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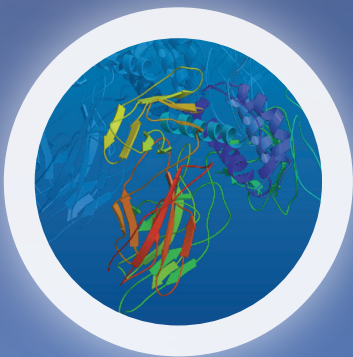


Gary Walsh



# Proteins

Biochemistry and  
Biotechnology



WILEY Blackwell

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Biochemistry and Biotechnology

Second Edition

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WILEY Blackwell

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*This book is dedicated to a most precious collection of proteins,  
my children Eithne, Shane and Alice.*







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# Preface

This textbook aims to provide a comprehensive and up-to-date overview of proteins, both in terms of their biochemistry and applications. The first edition was published over a decade ago and in the intervening period this field has continued to rapidly evolve. The new edition retains the overall structure of the original one. Chapters 1–4 are largely concerned with basic biochemical principles. In these chapters issues relating to proteomics, protein sources, structure, engineering, purification and characterization are addressed. The remaining 10 chapters largely focus on the production of proteins and their applications in medicine, analysis and industry.

Despite the similarity in overall structure, the new edition has been extensively revised and updated to reflect recent progress in the area. Relative to the earlier edition there is greater emphasis on protein biochemistry, engineering and proteomics. The production of proteins via fermentation and animal cell culture are considered in new sections, which better balance the subsequent consideration of protein purification. The protein application chapters have been updated to reflect recent trends and developments. Thus, for example, recent bioprocess developments such as the use of disposable bioreactors are considered, there is greater relative emphasis on recombinant production systems and engineered products, therapeutic antibodies now are

considered in a full dedicated chapter, and newer industrial applications such as the use of enzymes in biofuel generation are also included. The chapters considering protein applications have also been strengthened via the incorporation of numerous specific commercial product case studies.

The text caters mainly for advanced undergraduate and graduate students undertaking courses in applied biochemistry/biotechnology, but it should also be of value to students pursuing degrees in biochemistry, microbiology, or any branch of the biomedical sciences. Its scope also renders it of interest to those currently working in the biotechnology sector.

A sincere note of thanks is due to a number of people who have contributed to the successful completion of this project. Thank you to J.J. Tobin, Tewfik Soulimane and Jayne Murphy for useful scientific discussions and to Angela Boyce, Madlen Witt, Martin Wilkinson, Brigit Hogan and Jimmy Kelly for helping provide many of the photographs included. I am grateful too to John Wiley & Sons for their professionalism, efficiency and never-ending patience as I spectacularly over-ran my manuscript submission date.

Gary Walsh  
Limerick, June 2013



# About the companion website

This book is accompanied by a companion website:

[www.wiley.com/go/walsh/proteinsbiochemistry](http://www.wiley.com/go/walsh/proteinsbiochemistry)

The website includes:

- Powerpoints of all figures from the book for downloading
- PDFs of all tables from the book for downloading





# Chapter 1

## Proteins and proteomics

Throughout this book, I will consider various aspects of protein structure, function, engineering and application. Traditionally, protein science focused on isolating and studying one protein at a time. However, since the 1990s, advances in molecular biology, analytical technologies and computing has facilitated the study of many proteins simultaneously, which has led to an information explosion in this area. In this chapter such proteomic and related approaches are reviewed.

### 1.1 Proteins, an introduction

While we consider protein structure in detail in Chapter 2, for the purposes of this chapter it is necessary to provide a brief overview of the topic. Proteins are macromolecules consisting of one or more polypeptide chains (Table 1.1). Each polypeptide consists of a chain of amino acids linked together by peptide (amide) bonds. The exact amino acid sequence is determined by the gene coding for that specific polypeptide. When synthesized, a polypeptide chain folds up, assuming a specific three-dimensional shape (i.e. a specific

conformation) that is unique to the protein. The conformation adopted depends on the polypeptide's amino acid sequence, and this conformation is largely stabilized by multiple, weak interactions. Overall, a protein's structure can be described at up to four different levels.

- *Primary structure*: the specific amino acid sequence of its polypeptide chain(s), along with the exact positioning of any disulfide bonds present.
- *Secondary structure*: regular recurring arrangements of adjacent amino acid residues, often over relatively short contiguous sequences within the protein backbone. The common secondary structures are the  $\alpha$ -helix and  $\beta$ -strands.
- *Tertiary structure*: the three-dimensional arrangement of all the atoms which contribute to the polypeptide. In other words, the overall three-dimensional structure (conformation) of a polypeptide chain, which usually contains several stretches of secondary structure interrupted by less ordered regions such as bends/loops.
- *Quaternary structure*: the overall spatial arrangement of polypeptide subunits within a protein composed of two or more polypeptides.

**Table 1.1** Selected examples of proteins. The number of polypeptide chains and amino acid residues constituting the protein are listed, along with its molecular mass and biological function.

Protein	Polypeptide chains	Total no. of amino acids	Molecular mass (Da)	Biological function
Insulin (human)	2	51	5800	Complex, but includes regulation of blood glucose levels
Lysozyme (egg)	1	129	13,900	Enzyme capable of degrading peptidoglycan in bacterial cell walls
Interleukin-2 (human)	1	133	15,400	T-lymphocyte-derived polypeptide that regulates many aspects of immunity
Erythropoietin (human)	1	165	36,000	Hormone which stimulates red blood cell production
Chymotrypsin (bovine)	3	241	21,600	Digestive proteolytic enzyme
Subtilisin ( <i>Bacillus amyloliquefaciens</i> )	1	274	27,500	Bacterial proteolytic enzyme
Tumour necrosis factor (human TNF- $\alpha$ )	3	471	52,000	Mediator of inflammation and immunity
Haemoglobin (human)	4	574	64,500	Gas transport
Hexokinase (yeast)	2	800	102,000	Enzyme capable of phosphorylating selected monosaccharides
Glutamate dehydrogenase (bovine)	~40	~8300	~1,000,000	Enzyme that interconverts glutamate and $\alpha$ -ketoglutarate and $\text{NH}_4^+$

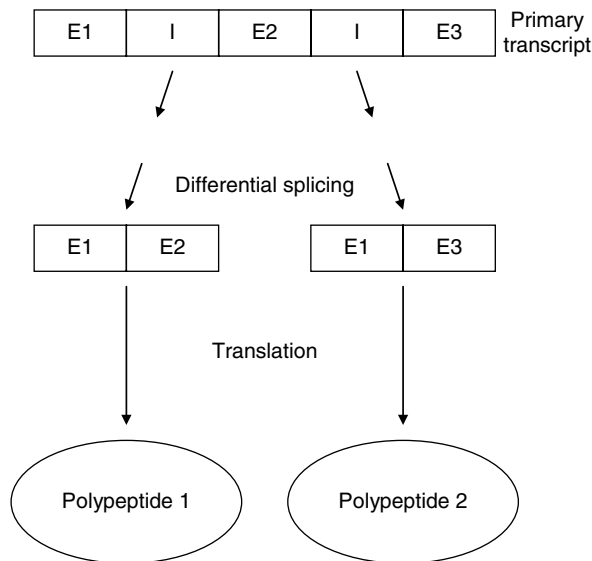
The majority of proteins derived from eukaryotes undergo covalent modification either during, or more commonly after, their ribosomal synthesis. This gives rise to the concept of co-translational and post-translational modifications, although both modifications are often referred to simply as post-translational modifications (PTMs), and such modifications can influence protein structure and/or function. Proteins are also sometimes classified as 'simple' or 'conjugated'. Simple proteins consist exclusively of polypeptide chain(s) with no additional chemical components being present or being required for biological activity. Conjugated proteins, in addition to their polypeptide components, contain one or more non-polypeptide constituents known as prosthetic groups. The most common prosthetic groups found in association with proteins include carbohydrates (glycoproteins), phosphate groups (phosphoproteins), vitamin derivatives (e.g. flavoproteins) and metal ions (metalloproteins).

## 1.2 Genes, genomics and proteomics

The term 'genome' refers to the entire complement of hereditary information present in an organism or virus. In the overwhelming majority of cases it is encoded in DNA, although some viruses use RNA as their genetic material. The term 'genomics' refers to the systematic study of the entire genome of an organism. Its core aims are to:

- sequence the entire DNA complement of the cell; and
- to physically map the genome arrangement (assign exact positions in the genome to the various genes and non-coding regions).

Prior to the 1990s, the sequencing and study of a single gene represented a significant task. However, improvements in sequencing technologies and the development



**Figure 1.1** Differential splicing of mRNA can yield different polypeptide products. Transcription of a gene sequence yields a 'primary transcript' RNA. This contains coding regions (exons) and non-coding regions (introns). A major feature of the subsequent processing of the primary transcript is 'splicing', the process by which introns are removed, leaving the exons in a contiguous sequence. Although most eukaryotic primary transcripts produce only one mature mRNA (and hence code for a single polypeptide), some can be differentially spliced, yielding two or more mature mRNAs. The latter can therefore code for two or more polypeptides. E, exon; I, intron.

of more highly automated hardware systems now renders DNA sequencing considerably faster, cheaper and more accurate. Cutting-edge sequencing systems now in development are claimed capable of sequencing small genomes in minutes, and a full human genome sequence in a matter of hours and for a cost of approximately \$1000. By early 2014, the genomes online database (GOLD; [www.genomesonline.org](http://www.genomesonline.org)), which monitors genome studies worldwide, documented some 36,000 ongoing/complete genome projects, and the rate of completion of such studies is growing exponentially. From the perspective of protein science, the most significant consequence of genome data is that it provides full sequence information pertinent to every protein the organism can produce.

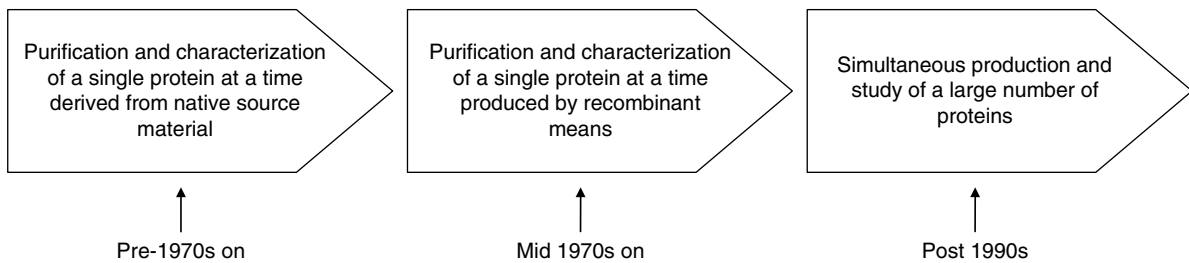
The term 'proteome' refers to the entire complement of proteins expressed by a specific cell/organism. It is more complex than the corresponding genome in that:

- at any given time a proportion of genes are not being expressed;
- of those genes that are expressed, some are expressed at higher levels than others;

- the proteome is dynamic rather than static because the exact subset of proteins expressed (and the level at which they are expressed) in any cell changes with time in response to a myriad of environmental and genetic influences;
- for eukaryotes, a single gene can effectively encode more than one polypeptide if its mRNA undergoes differential splicing (Figure 1.1);
- many eukaryotic proteins undergo PTM.

The last two points in particular generally signify that the number of proteins comprising a eukaryotic organism's proteome can far exceed the number of genes present in its genome. For example, the human genome comprises approximately 22,000 genes whereas the number of distinct protein structures present may exceed 1 million, with any one cell containing an estimated average of approximately 10,000 proteins.

Traditionally, proteins were identified and studied one at a time (Figure 1.2) (see Chapters 2, 3 and 4). This generally entailed purifying a single protein directly from a naturally producing cellular source,



**Figure 1.2** Evolution of the various approaches used to study proteins. Refer to text for details.

or from a recombinant source in which the gene/cDNA coding for the protein was being expressed. While this approach is still routinely used, a proteomic approach can potentially yield far more 'global' protein information far more quickly.

Proteomics refers to the large-scale systematic study of the proteome or, depending on the research question being asked, a defined subset of the proteome, such as all proteome proteins that are phosphorylated or all the proteome proteins that increase in concentration when a cell becomes cancerous. It is characterized by the integrated study of hundreds, more usually thousands or even tens of thousands of proteins. This in turn relies on high-throughput techniques/processes that facilitate the production, purification or characterization of multiple proteins rapidly and near simultaneously, usually by using automated/semi-automated and miniaturized processes/procedures. Standard techniques of molecular biology, for example, allow convenient global genome protein production (Figure 1.3) as well as facilitating the attachment of affinity tags to the proteins (as discussed later in this chapter and in Chapter 4), thereby enabling high-throughput purification efforts. Proteomics relies most of all on techniques that allow high-throughput analysis of the protein complement under investigation. Among the more central techniques in this regard are two-dimensional electrophoresis, high-pressure liquid chromatography (HPLC) and mass spectrometry (MS).

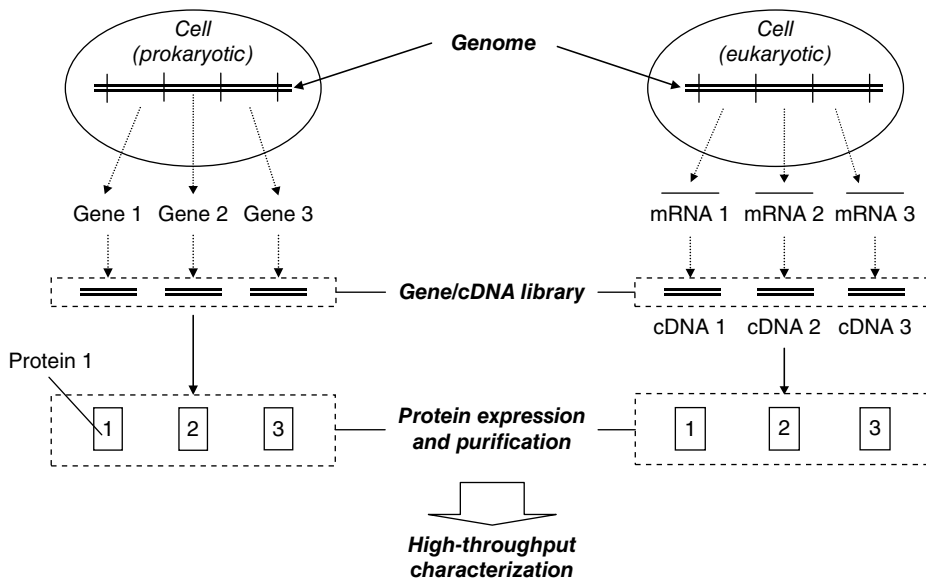
Before we consider the goals and applications of proteomics in more detail, it is worth reviewing these analytical techniques. In the context of proteomics, they are often applied in combination to characterize a target proteome, with electrophoretic and/or HPLC-based methods initially used to separate

individual constituent proteome proteins from each other, followed by MS-based analysis. These techniques can also be used for the detailed analysis of individual proteins characteristic of classical protein science studies or, for example, as part of a quality control process for commercial protein preparations such as biopharmaceuticals. Such applications will be considered further in later chapters.

### 1.2.1 Electrophoresis

Electrophoresis is an analytical technique that separates analytes from each other on the basis of charge. The technique involves initial application of the analyte mixture to be fractionated onto a supporting medium (e.g. filter paper or a gel) with subsequent activation of an electrical field. Each charged substance then moves towards the cathode or the anode at a rate of migration that depends on the ratio of charge to mass (i.e. the charge density) of the analyte as well as on any interactions with the support medium. As described in Chapter 2, proteins are charged species, with their exact charge density being dependent on their amino acid sequence.

The most common electrophoretic method applied to proteins is one-dimensional polyacrylamide gel electrophoresis (PAGE) run in the presence of the negatively charged detergent sodium dodecyl sulfate (SDS-PAGE), and is most often used to analyse protein purity (see Chapter 4). In the case of PAGE, migration occurs through a polyacrylamide gel, the average pore size of which is largely dependent on the concentration of polyacrylamide present. A sieving effect therefore also occurs during PAGE so that the rate of protein migration is influenced by its size/shape as well as charge density.



**Figure 1.3** Global proteomics approach. While target proteins may be obtained from native (i.e. naturally producing) source material, they are most commonly obtained by recombinant means via the construction of gene/cDNA libraries. In the case of a prokaryotic cell source, a collection of individual genes can be isolated and cloned by standard molecular biology techniques, forming a genomic library (consisting of just three genes in the simplified example portrayed here). Eukaryotic genes generally consist of coding sequences (exons) interrupted by non-coding sequences (introns), while processed mRNA transcripts derived from those genes reflect the coding sequence for the final polypeptide product only. Isolation of total cellular mRNA followed by incubation with a reverse transcriptase enzyme yields complementary double-stranded DNA (cDNA) sequences, directly encoding the polypeptide sequences of the complement of expressed genes, thereby generating a cDNA library. Again by using standard molecular biology techniques the gene/cDNA library products can be expressed, yielding the recombinant protein products. The proteins, in turn, can be purified and characterized via techniques considered in subsequent sections of this chapter, as well as in Chapters 4 and 5.

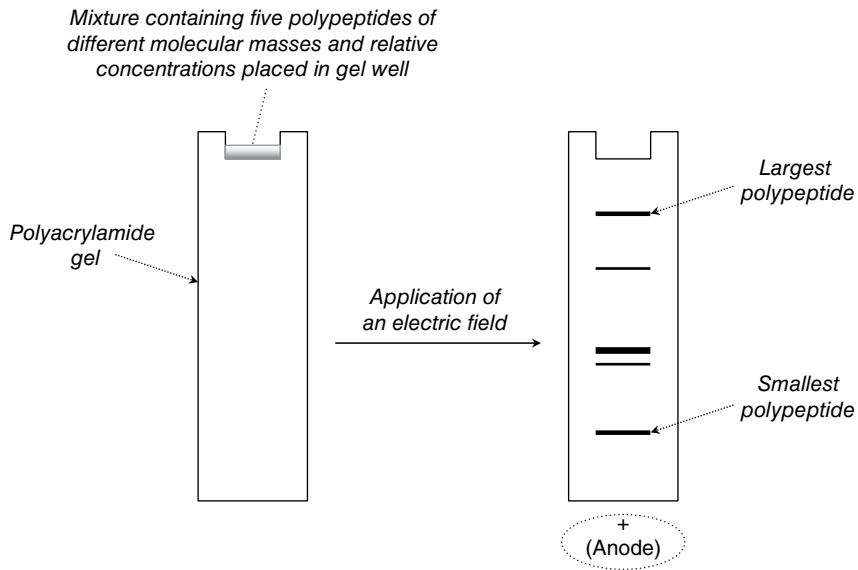
Incubation of the protein with SDS has two notable effects: (i) it denatures most proteins, giving them all approximately the same shape, and (ii) it binds directly to the protein at the constant rate of approximately one SDS molecule per two amino acid residues. In practice this confers essentially the same (negative) charge density to all proteins. Separation of proteins by SDS-PAGE therefore occurs by a sieving effect, with the smaller proteins moving fastest towards the anode (Figure 1.4).

#### 1.2.1.1 Isoelectric focusing

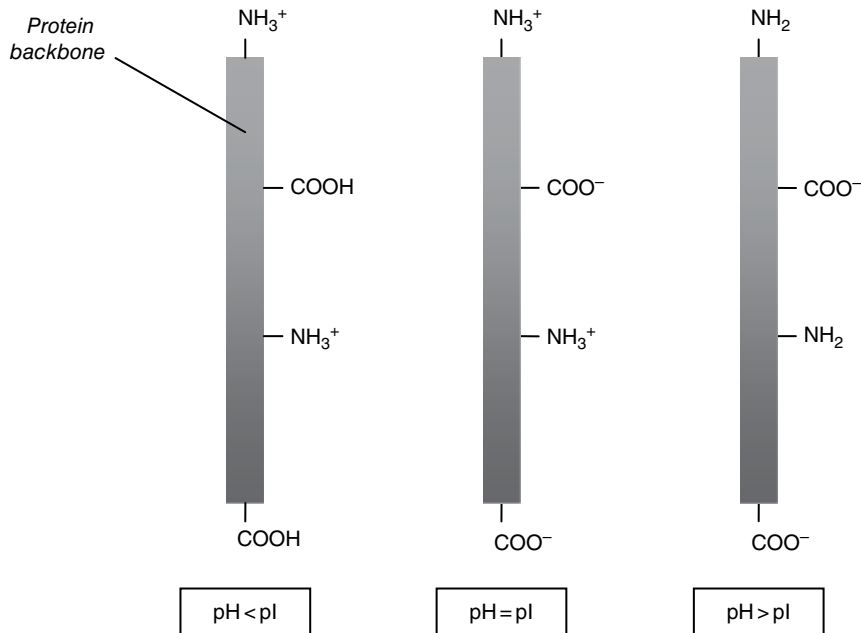
Isoelectric focusing is an additional form of electrophoresis. A modified gel is used which contains polyacrylamide to which a gradient of acidic and basic buffering groups are covalently attached.

As a result an immobilized pH gradient is formed along the length of the gel. The gel is normally supported on a plastic strip. The protein solution to be applied is normally first incubated with a combination of urea and a non-ionic detergent such as Triton or CHAPS and a reducing agent to break any disulfide linkages present. This ensures that all sample proteins are completely disaggregated and fully solubilized. On application of the protein sample, the proteins present migrate in the gel until they reach a point at which the pH equals their isoelectric point (pI) (Figure 1.5).

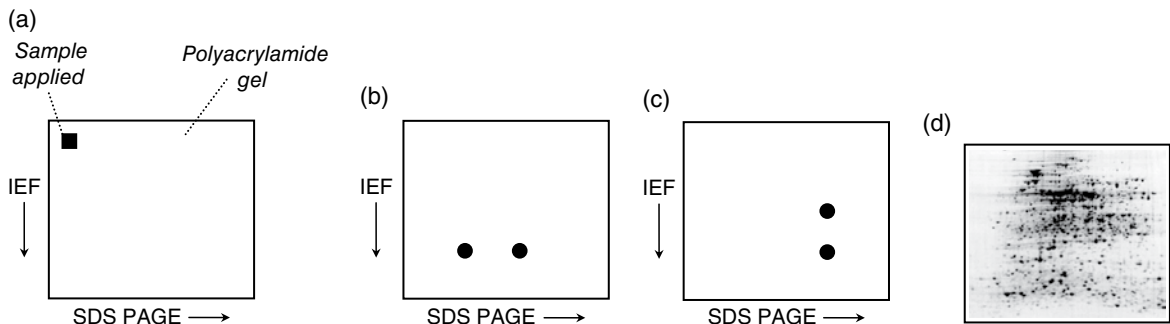
Neither SDS-PAGE nor isoelectric focusing, by themselves, can fully separate (resolve) very complex mixtures of proteins, such as would characterize an entire cell's proteome. Each separation mode can individually resolve about 100 protein



**Figure 1.4** Separation of proteins by SDS-PAGE. Protein samples are incubated with SDS (as well as reducing agents, which disrupt disulfide linkages). The electric field is applied across the gel after the protein samples to be analysed are loaded into the gel wells. The rate of protein migration towards the anode depends on protein size. After electrophoresis is complete individual protein bands may be visualized by staining with a protein-binding dye.



**Figure 1.5** Proteins are amphoteric molecules, displaying a positive, negative or zero overall net charge depending on the pH of the solution in which are they dissolved. Contributing to the overall charge of a protein are all the positive and negative charges of its amino acid side chains as well as the free amino and carboxyl groups present at its amino and carboxyl termini, respectively. The state of ionization of these groups is pH dependent. The pH at which the net number of positive charges equal the net number of negative charges (i.e. the protein has an overall net electric charge of zero, and hence will not move under the influence of an electric field) is known as its isoelectric point (pI).



**Figure 1.6** Principle of two-dimensional gel electrophoresis. The protein sample is applied to the polyacrylamide gel and first subjected to isoelectric focusing (IEF). After this is complete the protein bands are subjected to SDS-PAGE in the perpendicular direction (a). This combination has greater resolving power than either technique alone. (b) Resolution of two proteins with equal pI values but different molecular masses. (c) Resolution of two proteins of equal molecular mass but differing pI values. (d) Example of a two-dimensional gel in which a microbial proteome has been resolved.

bands, but when combined about 1000–2000 bands can be resolved. As such, combining them into so-called two-dimensional electrophoresis (Figure 1.6) can achieve far better resolution of a complex protein mixture, and hence this approach is often used to achieve initial separation of a protein set prior to additional proteomic analysis and individual protein identification/sequencing (usually via MS). In this context, two-dimensional electrophoresis has a number of strengths, including:

- high-resolution separation;
- straightforward technique;
- relatively inexpensive.

However, it also has a number of potential drawbacks, in particular:

- exact reproducibility of gel banding patterns often challenging to consistently achieve;
- not amenable to genuine high-throughput experiments.

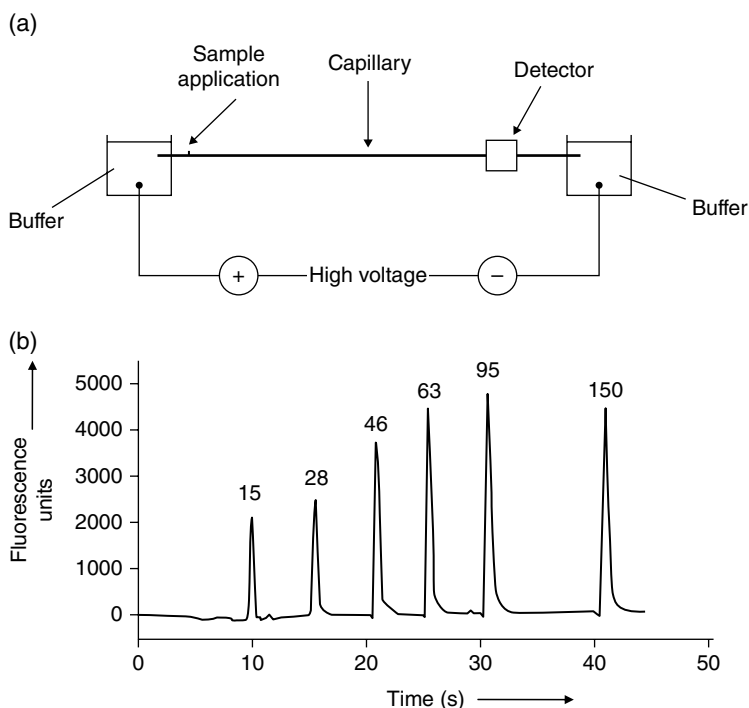
### 1.2.1.2 Capillary electrophoresis

Capillary electrophoresis (CE) is yet another electrophoretic format, and separates molecules on the basis of charge density. In this case, however, electrophoretic separation occurs not in a polyacrylamide gel but along a narrow-bore capillary tube

usually containing a conductive buffer (Figure 1.7). Typically, the capillary will have an internal diameter of 50–75  $\mu\text{m}$  and be up to, or greater than, 1 m in length. The dimensions of this system yield greatly increased surface area to volume ratios (when compared with polyacrylamide gels), hence greatly increasing the efficiency of heat dissipation from the system. This in turn allows operation at a higher current density, thus speeding up the rate of migration through the capillary. Sample analysis is usually completed within 15 minutes. In some ways CE is more similar to liquid chromatography (see section 1.2.2) than conventional electrophoresis. It exhibits very high resolving power, and its short analysis time and simple instrumentation is amenable to high-throughput analysis. CE is most typically used in proteomics to achieve separation of a peptide or a protein mix, with the separated species being fed into a mass spectrometer for analysis (CE-MS).

### 1.2.2 High-pressure liquid chromatography

Chromatography refers to the separation of individual constituents of a mixture via their differential partitioning between two phases: a solid stationary phase and a liquid mobile phase. In the context of protein chromatography, the stationary



**Figure 1.7** (a) Schematic representation of capillary electrophoresis. After sample application, a high voltage is applied and the proteins migrate under the influence of the resultant electric field. Visualization of proteins eluting is achieved using an in-line UV/visible, fluorescence or other appropriate detector. (b) Separation of individual constituents of a protein mixture, with the molecular mass of individual proteins (kDa) indicated above each peak.

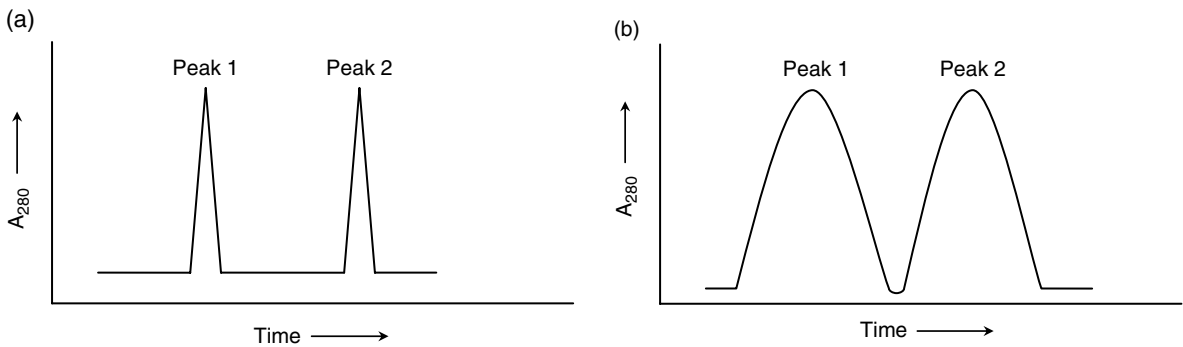
phase is usually chromatographic beads, packed into a cylindrical column, and the mobile phase is usually a buffer and chromatographic separation takes advantage of differences in protein characteristics such as size and shape, charge or hydrophobicity.

Chromatography can be used at a preparative or analytical level, and both applications are considered in detail in Chapters 4 and 5. Preparative chromatography in particular is usually performed under relatively low pressures, where flow rates through the column are generated by low-pressure pumps (low-pressure liquid chromatography or LPLC). Fractionation of a single sample on such chromatographic columns typically requires several hours to complete. Low flow rates are required because as the protein sample flows through the column, the proteins are brought into contact with the surface of the chromatographic beads by direct (convective) flow. The protein molecules then rely entirely on molecular diffusion to enter the porous

gel beads. This is a slow process, especially when compared with the direct transfer of proteins past the outside surface of the gel beads by liquid flow. If a flow rate significantly higher than the diffusional rate is used, protein band spreading (and hence loss of resolution) will result. This occurs because any protein molecules which have not entered the bead will flow downward through the column at a faster rate than the (identical) molecules which have entered into the bead particles. Such high flow rates will also result in a lowering of adsorption capacity as many molecules will not have the opportunity to diffuse into the beads as they pass through the column.

One approach that allows increased chromatographic flow rates without loss of resolution entails the use of microparticulate stationary-phase media of very narrow diameter. This effectively reduces the time required for molecules to diffuse in and out of the porous particles. Any reduction in particle





**Figure 1.8** HPLC-based chromatographic separation generally gives rise to better-resolved protein peaks (a) than do low pressure-based systems (b).

diameter dramatically increases the pressure required to maintain a given flow rate. Such high flow rates may be achieved by utilizing HPLC systems (also often known as high-performance liquid chromatographic systems). By employing such methods sample fractionation times may be reduced from hours to minutes, and when experimental conditions are optimized chromatographic peak width is generally reduced compared with low-pressure systems and hence resolution power is higher (Figure 1.8).

The successful application of HPLC was made possible largely by (i) the development of pump systems which can provide constant flow rates at high pressure and (ii) the identification of suitable pressure-resistant chromatographic media. Traditional soft gel media utilized in low-pressure applications are totally unsuited to high-pressure systems due to their compressibility. Traditionally, HPLC bead diameter was typically in the 3–5  $\mu\text{m}$  range (although beads with diameters up to 50  $\mu\text{m}$  can be used in some applications). More recent advances in bead chemistry have allowed the development of mechanically stronger, even smaller beads (diameter <2  $\mu\text{m}$ ). Coupled with refined high-pressure pump design, this has still further improved flow rate (speed) and resolution, and is sometimes termed ultra performance liquid chromatography (UPLC). The high resolving power of HPLC, together with fast running times, makes it a suitable proteomic technique for achieving protein separation from complex mixtures, with individual protein peaks usually being fed directly to mass spectrometers

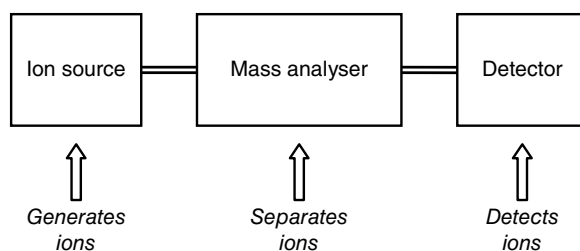
(LC-MS) for further analysis. If the protein sample being analysed is very complex, the use of so-called multidimensional LC prior to MS analysis may be required. This generally entails contiguous separation by two HPLC modalities (e.g. ion-exchange-based HPLC, followed by reverse-phase HPLC separation of various fractions eluting from the initial ion-exchange column).

### 1.2.3 Mass spectrometry

MS is the analytical technique most intimately associated with proteomics. MS separates a mixture of (vaporized and ionized) analytes on the basis of their mass to charge ratio. It can very accurately determine the molecular mass of analytes and its basic principle of operation is outlined in Figure 1.9.

MS has for many years been a central technique for determining the molecular mass of small molecules. Its routine application to protein work has only been made possible relatively recently, principally by the development of suitable ionization techniques that allow generation of gas-phase ionized proteins. It can determine the mass of proteins up to 500 kDa, with an accuracy of better than 0.01%.

MS now finds routine application in protein science, both in the context of high-throughput proteomic analysis and in the analysis of single proteins. Although applied in areas such as characterization and quality control of biopharmaceuticals



**Figure 1.9** Basic principle of mass spectrometry. The system is composed of three essential components: an ion source which generates gas phase-ionized analytes; a mass analyser, which sorts the ions by mass via the application of, for example, electric or magnetic fields; and a detector, which detects and quantifies the ions. Data from the detector thus provides the mass and abundance of each ion present. Refer to text for further detail.

(see Chapter 5), the focus in this chapter is on its use in proteomics. However, overall MS is used to:

- determine protein mass;
- generate partial or full amino acid sequence data for a protein;
- quantify the amount of protein present in a sample;
- detect and identify protein PTMs;
- detect protein modification such as oxidation, deamidation or proteolysis;
- provide some information on protein structural detail.

Ultimately, these applications rely on the fact that all the amino acids, or other constituent biomolecules of the protein (e.g. specific sugars in the case of glycoproteins), have known molecular masses, and that potential modifications to a protein's structure (e.g. a PTM or the oxidation of an amino acid) will have predictable effects on the protein's molecular mass.

### 1.2.3.1 Ionization methods

Various methods can be used to ionize analytes for the purposes of MS, including the following commonly used approaches.

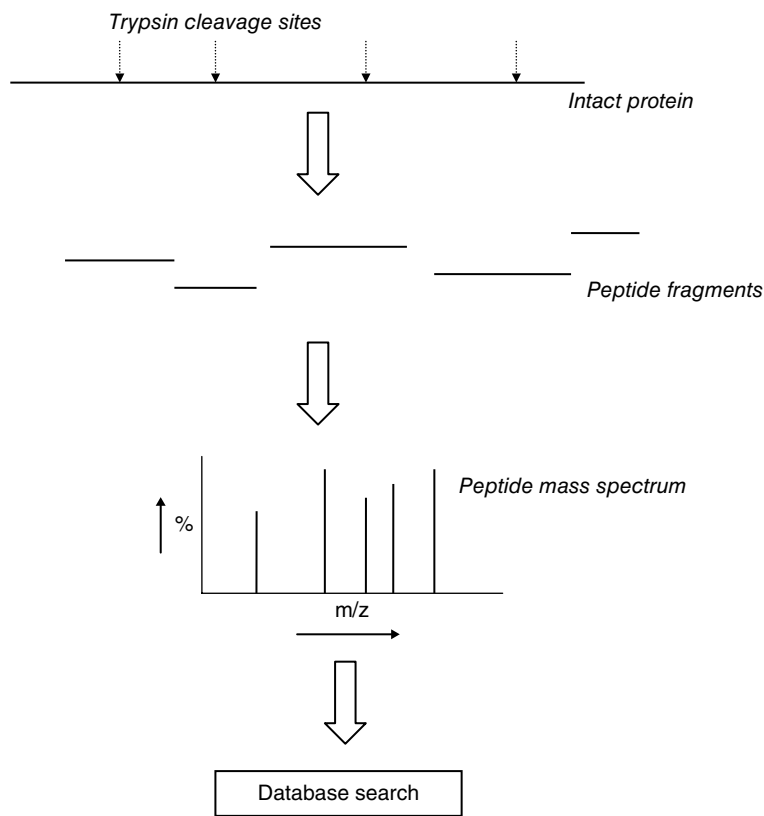
- *Electron ionization* (EI), which involves bombarding the analytes with electrons.
- *Chemical ionization* (CI), in which analytes are collided with a reactive gas.
- *Fast atom bombardment* (FAB), in which the analytes are bombarded with argon gas.
- *Electrospray ionization* (ESI), in which the analytes are sprayed into an electric field.

- *Matrix-assisted laser desorption ionization* (MALDI), in which the analytes are co-crystallized with a matrix substance (a UV-absorbing substance such as sinapinic acid), followed by exposure to an electric field and a pulsed laser beam. The matrix molecules absorb the laser photons, become excited and are transferred into the gas phase along with the neighbouring analyte molecules. A proportion of both matrix and analyte molecules become ionized by this process and the applied electric field accelerates the ions towards the analyser.

The exact ionization (and subsequent analyser mode; see Figure 1.9) chosen will depend on the research question posed. Ionization methods can be classified as 'soft' or 'hard'. Soft ionization methods such as ESI and MALDI can achieve ionization while leaving the protein intact (and thus are usually used if a protein's molecular mass is to be determined; this is known as 'top-down' MS). Hard ionization methods such as EI and FAB result in protein fragmentation as well as ionization, yielding a fragment fingerprint analysed by mass ('bottom-up' MS).

### 1.2.3.2 Protein molecular mass determination

'MALDI-TOF' MS is a popular approach for determining the molecular mass of an intact protein. As described above, the MALDI approach achieves ionization of the intact protein, which is then fed into a time of flight (TOF) analyser. As they enter the analyser tube all the protein ions have essentially the same kinetic energy and charge. Because of this, the time required for each protein ion to reach the



**Figure 1.10** Schematic representation of a common approach to protein identification via MS-based peptide mass fingerprinting. Refer to text for detail.

detector reflects its molecular mass, with smaller proteins travelling fastest. A sample size of as little as a few femtomoles ( $10^{-15}$  mol) of protein is all that is required for analysis.

Alternatively, ESI-MS can be used to determine the mass of an intact protein. It is also a soft ionization method, and even non-covalent protein complexes can remain intact (giving rise to the potential for some protein interaction analysis). It is often used with a quadrupole analyser (which contains four rod metal electrodes, which effectively serve as a mass filter). As ESI processes analytes in solution, the sample can be pumped into the mass analyser continuously and thus it can be connected directly to LC or CE instruments and used for high-throughput analysis. Because the sample must be co-crystallized (dry powder) for MALDI operation, MALDI cannot be used in continuous format with pre-separation LC/CE methods.

### 1.2.3.3 MS-based protein identification

While accurate determination of a protein's molecular mass is one application of MS, the approach finds more routine use in the identification of proteins and the determination of a partial/full amino acid sequence. Protein identification obviously forms a central element of proteomics, but these techniques can also be used to better characterize a single protein isolated via a classical protein science approach, or can be used as quality control checks on purified biopharmaceutical products in order to verify identity/sequence. The more common approaches for achieving these objectives are outlined below. As these approaches involve initially fragmenting the intact protein, followed by mass analysis of the peptide fragments, they are termed bottom-up MS analyses.

Peptide mass fingerprinting is an approach commonly used to identify proteins (Figure 1.10).

The intact protein sample is initially treated with either a proteolytic enzyme (e.g. trypsin) or a chemical (e.g. CNBr) which selectively cleaves specific peptide bonds along the protein's backbone, thereby generating a peptide mix. As each protein has its own unique amino acid sequence, each generates its own unique peptide map or fingerprint. The peptides generated are then further analysed by MS using soft ionization techniques (MALDI or ESI) that do not further fragment them. This generates a peptide mass spectrum. Identification of the protein is then undertaken by using specialist computer software that compares the experimentally determined peptide masses with theoretical digestion data for all the proteins whose amino acid sequence is known and has been deposited in sequence databases (see section 1.3).

A variant approach that is more 'information rich' and which can often generate complete/near complete amino acid sequence information of the protein under investigation is that of tandem MS (MS/MS) analysis. The basic approach, as the name suggests, involves interrogation of the protein using two mass analysers in sequence, in other words in tandem, separated only by a collision cell. In the case of MS/MS, the protein to be sequenced is first chemically or enzymatically fragmented. The fragments are separated along the first analyser tube. One peptide ion fragment is selected at a time and fed (alone) into the collision tube, where it collides with inert gas molecules (He or Ar). This promotes further fragmentation into a range of complementary peptides that are separated on the basis of mass in the second tube. Computerized analysis of the mass of each fragment generated in the second tube can yield nearly complete/complete sequence data.

## 1.3 Bioinformatics

A central characteristic of genomics and proteomics is the vast amount of biological data, such as gene and protein sequences, that it generates. This provides two challenges: (i) how to store all this information and (ii) how to analyse, interrogate and use this data in order to understand its actual biological significance, apply it to research questions

and generate new knowledge. Bioinformatics represents the scientific discipline that addresses these challenges. It is a multidisciplinary field that concerns itself with storing, retrieving and analysing biological data and draws expertise mainly from biology, mathematics and computer science. Bioinformatics is thus underpinned by two main activities: (i) the establishment of computer databases in which raw biological information (e.g. genome and protein sequences) are deposited and stored, and (ii) the development and operation of computer programs that allow users to interrogate, analyse and derive new understanding/information.

While there are many specialist databases available worldwide (some of which we will encounter in subsequent chapters, e.g. enzyme-based databases outlined in Table 11.6), there are three main global, publically accessible databases that serve as repositories for DNA sequence data. Each deposited sequence is given a unique, internationally recognized accession number and these repositories share information deposited on a daily basis, so all contain virtually the same data. The three databanks are GenBank, the European Molecular Biology Laboratory (EMBL) database and the DNA database of Japan. These databases are hosted by the National Center for Biotechnology Information (NCBI) in the USA, the European Bioinformatics institute (EBI) and the (Japanese) National Institute of Genetics (Table 1.2). Nucleotide sequence information can be used to generate protein sequence information, as does direct protein sequencing efforts. Protein sequence databanks are therefore also maintained by these host bioinformatic institutes.

In addition to maintaining sequence databases themselves, the host organizations generally maintain (and often develop) bioinformatic computer software programs/tools which facilitate data analysis and/or cooperate with additional organizations that maintain databases and/or develop bioinformatic analytical tools used to derive biological knowledge from primary sequence information. As a result numerous bioinformatic resources are available for public use (usually via dedicated websites), hosted by various organizations and capable of providing/generating often overlapping sets of bioinformatic information. Generally, such protein-focused bioinformatic

**Table 1.2** The three main global sequence databases, their host organizations and web addresses. Refer to text for details.

Database name	Host	Web address
GenBank	The (USA) National Center for Biotechnology Information (NCBI)	www.ncbi.nlm.nih.gov/genbank
The EMBL database	The European Bioinformatics Institute (EBI)	www.ebi.ac.uk/embl
The DNA Database of Japan	The (Japanese) National Institute of Genetics (NIG)	www.ddbj.nig.ac.jp

web-based resources can be grouped in terms of their use as follows.

- *Sequence databases*: house primary DNA/protein sequence information (see, for example, Table 1.2).
- *Protein family databases/resources*: classify proteins into families based on sequence similarities. This can, for example, help elucidate potential functional and structural characteristics of a specific protein, as well as establishing likely evolutionary relationships.
- *Protein structural databases/resources*: organize and store experimentally determined protein three-dimensional structures or generate putative structural models of a protein based on sequence similarities to proteins whose structure has been determined experimentally.
- *Protein function databases/resources*: maintain information about protein function, most commonly relating to metabolic pathways and protein interactions.
- *Proteomics databases*: store proteomic MS and two-dimensional electrophoretic data.

Some bioinformatic resources can be applicable to more than one of the categories above and the number of databases established, as well as which databases will most conveniently answer a particular research question posed, can be somewhat confusing. However, some of the main international bioinformatic organizations maintain 'gateway resource portals' on their homepages, which serve as single entry points into multiple specific databases/resources and/or allow a simultaneous search of such multiple databases/resources with a specific search term (such as a protein's name). For example, the Swiss Institute of Bioinformatics maintains a bioinformatic resource portal called ExPASy (Box 1.1), while the NCBI maintain a portal called Entrez.

### Box 1.1 ExPASy

ExPASy ([www.expasy.org](http://www.expasy.org)) is the Swiss Institute of Bioinformatics resource portal that serves as a single search system/entry point for a whole range of bioinformatic databases and software tools. The databases and tools are categorized under a number of headings, including proteomics, genomics, structural bioinformatics, systems biology and population genetics. Specifically under the proteomics category, over 30 databases and some 250 tools are listed. Examples of both databases and tools, as well as the type of information provided/generated by these, are listed below and these resources generally focus on:

- protein sequences, similarity and identification;
- protein characterization and function;
- protein families;
- protein structure;
- protein-protein interaction;
- post-translational modifications;
- mass spectrometry and two-dimensional electrophoretic data.

The collection of databases and resources are collectively searchable using a key word or an accession number. Thus, for example, a key word search of the site (limited to the proteomics category) using the term 'cellulase' reveals almost 16,000 hits, some 14,000 of which are derived from the UniProtKB resource (see below). Each entry in UniProtKB provides information on a specific cellulase, including its source, size

and sequence as well as a list of literature references.

### Examples of proteomic-focused databases and tools which are accessible/searchable via ExPASy

#### Databases

UniProtKB: functional information on proteins

STRING: protein–protein interactions

Swiss Model repository: protein structure homology models

PROSITE: protein domains and families

Enzyme: enzyme nomenclature

GlycoSuiteDB: glycan database

#### Tools

APSSP: advanced protein secondary structure prediction

BLAST: sequence similarity searches

ClustalW: multiple sequence alignment

FindMod: protein PTM prediction

InterProScan: family domain database search

Mascot: protein identification for MS data

Peptide cutter: protein cleavage site prediction

PredictProtein: prediction of protein physico-chemical properties

RasMol: molecular graphics visualization

T-Coffee: sequence and structure multiple alignment

TargetP: subcellular localization prediction

Swiss model workspace: structure homology modelling

We will encounter some of the better-known protein-focused bioinformatic databases/tools in some subsequent chapters.

## 1.4 Proteomics: goals and applications

While a central goal of proteomics is to separate and identify/record individual proteins constituting a cell or organism's proteome (or a subset of the proteome), proteomics also incorporates additional goals of protein analysis.

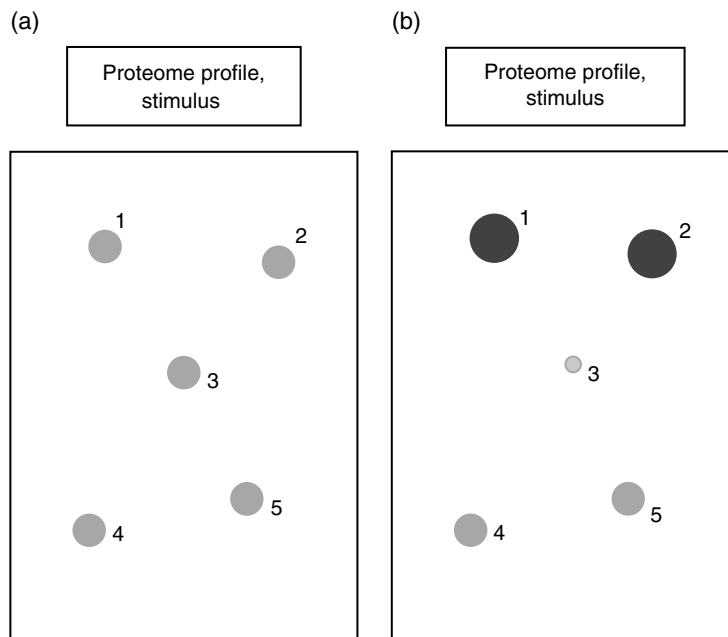
- *Expression proteomics*: allows analysis of the expression of individual proteins in the proteome, and how these change in response to stimuli such as genetic or environmental factors.
- *Functional proteomics*: aims (ultimately) to assign a biological function to each protein in the proteome.
- *Structural proteomics*: aims to gain as much information as possible relating to the three-dimensional structure of proteome proteins.

It is important to note that there is overlap between these areas, for example changes in protein expression levels in response to a specific stimulus can provide valuable information about a protein's likely function, while structural information can also provide insight into protein function.

These areas of proteomic analysis are operationalized by the application of a wide range of analytical ('wet chemistry') techniques. Some such techniques, including electrophoretic, chromatographic and MS-based analyses, have already been introduced while others, such as yeast two-hybrid systems and protein microarrays, are described in sections 1.4.2.1 and 1.4.2.2. It is also important to emphasize that such direct analytical approaches can be complemented by bioinformatic-based approaches. Thus, for example, computer programs exist which facilitate the assignment of a putative function to a protein based on amino acid sequence comparisons to proteins of known function. Similarly, bioinformatic tools exist which facilitate prediction of a protein's likely three-dimensional structure based on amino acid sequence comparisons to those found in proteins of known (experimentally determined) three-dimensional structure. Some such bioinformatics programs will be considered in the next chapter.

### 1.4.1 Expression proteomics

Various classical techniques (e.g. immunoassays, see Chapter 10) may be used to detect and quantify the concentration of a specific protein in a biological sample. Quantitative or expression proteomics focuses on the simultaneous detection and quantification of many different proteins in a proteomic sample or, more usually, the simultaneous detection and quantification



**Figure 1.11** Diagrammatic representation of the quantitative proteomic approach as illustrated by two-dimensional gel electrophoretic-based analysis. In this simplified illustrative example, the ‘proteome’ consists of just five proteins derived from a biological source material under investigation (e.g. a specific cell type exposed to two different stimuli). It is clear that, relative to stimulus (a), stimulus (b) results in an increase in the concentration of proteins 1 and 2, a decrease in the concentration of protein 3, while making no difference to the concentration of proteins 4 and 5. In reality, proteomic samples analysed would generally contain hundreds or thousands of different proteins.

of differences in concentrations of many different proteins in two or more different proteomic samples that have been exposed to different stimuli. Electrophoretic, chromatographic and MS-based techniques may all be applied to such analyses (Figure 1.11).

Detecting and identifying changes in the expression levels of specific proteins/groups of proteins in response to a specific stimulus can of course provide clues as to protein function. At a basic research level therefore this approach can for example be used to identify groups of proteins likely involved in specific cellular processes. From an applied perspective, studying the changes in proteome expression profiles of clinical samples can provide potentially useful medical information, and such ‘clinical proteomics’ now forms a valuable part of medical-based research and development. For example, the approach can be used to potentially identify biomarkers for specific diseases/conditions and identify potential new drugs.

A biomarker is a specific measurable characteristic of a biological system whose quantity correlates in

some way with a biological process. In the context of clinical science, most biomarkers are biomolecules whose levels in biological samples (e.g. blood, urine or tissue samples) are correlated with some disease or condition. Biomarker detection and measurement over time can therefore reflect the occurrence of a disease/condition, how it is progressing with time and perhaps how it is responding to therapy. Many established biomarkers are proteins (e.g. the gonadotrophic hormone hCG serves as a biomarker for pregnancy; Chapter 10). The high throughput and rapid nature of proteomics provides a very powerful tool for the identification of potential new disease biomarkers. Once identified and validated, standard classical diagnostic assays (e.g. immunoassays) for the biomarker can be developed and used in clinical chemistry laboratories (see Chapter 10). Moreover, comparative proteomic analysis of, for example, a cancer cell versus an untransformed cell of the same type could lead to the identification of cellular proteins fuelling the cancer phenotype. Such proteins could therefore represent targets for future anticancer drugs.

### 1.4.2 Functional proteomics

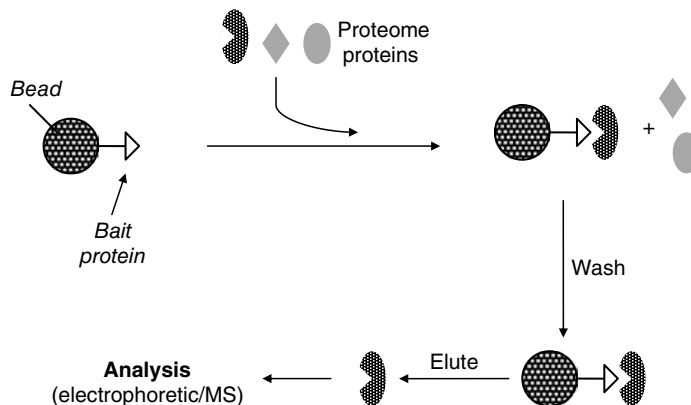
Genome sequencing studies have generated enormous amounts of protein sequence information. However, the function of the majority of such proteins remains to be elucidated, and assigning such functionality represents a major challenge. For example, the function of the majority of protein sequences identified by the Human Genome Project remains unknown and, even in the case of very well-studied organisms (e.g. *Escherichia coli*) function remains unassigned for a significant minority of proteins. Various genomic/bioinformatic/proteomic approaches may be pursued in an effort to assign function.

At a purely bioinformatic level, and as already mentioned, computer programs exist which help assign a putative function to a protein based on amino acid sequence comparisons to proteins of known function (see Chapter 2). At a genomic level, for example, knockout studies can be employed. Such studies entail the disruption of a specific gene with subsequent analysis of the effect on the organism.

At a proteomic level, analysing changes in the expression levels of specific proteins/groups of proteins in response to a specific stimulus can, as mentioned previously, provide clues as to protein

function. However, the core laboratory-based approaches adopted in functional proteomics attempt to identify protein–protein interactions (the ‘interactome’), usually by using a protein of interest as a ‘bait molecule’ to fish out proteins capable of interacting with it from a proteome of interest (‘prey molecules’). The resultant ‘prey’ proteins recovered are likely to be functionally related to the bait protein. Careful experimental design and execution is required to ensure that any proteins recovered are interacting with the bait protein in a biospecific manner. If non-specific binding occurs, the assumption that the proteins are functionally related will of course be inaccurate.

Various experimental approaches may be pursued in order to identify protein interactions. One approach involves incubating the bait protein with the proteome of interest to allow the formation of interactions with prey protein partners. Antibodies raised against the bait protein are then added, which precipitate the bait–prey complex out of solution; the precipitate can then be fractionated by SDS-PAGE, with subsequent analysis of the protein components present via MS. An alternative approach entails immobilizing the bait protein on a chromatographic bead, followed by incubation with the proteome of interest (Figure 1.12).



**Figure 1.12** Approach to interactome studies using a bait protein immobilized on a chromatographic bead. Beads can be incubated with the target proteome (which in this simplified example contains only three proteins). Only proteins interacting with the bait molecule in a biospecific manner will be retained on the column. After washing away the additional (non-binding) proteins, the captured (prey) protein(s) can be eluted from the bead and analysed in order to establish prey protein identity.



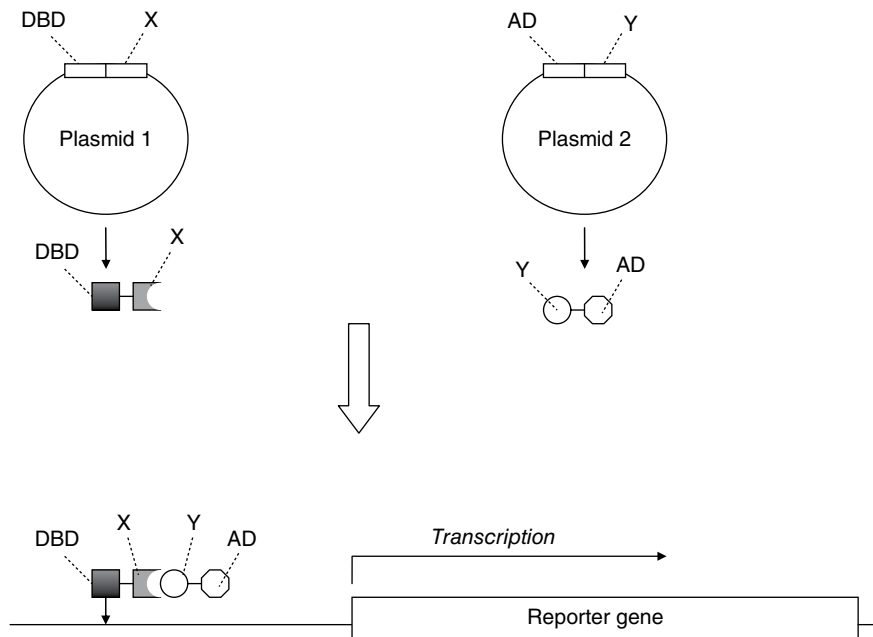
Among the most prominent interactome techniques are the yeast two-hybrid (Y2H) system and protein microarrays. Before we consider these approaches it is important to recognize that the goals of functional proteomics are broader than simply assigning function to an individual protein. These goals also incorporate identification of the subcellular location in which the protein functions, determination of the composition and function of macromolecular complexes, and promotion of a broader understanding at a molecular level of cellular mechanisms/processes in which proteins participate and how some processes are interlinked.

#### 1.4.2.1 Yeast two-hybrid system

The Y2H system is a molecular biology technique developed to investigate protein–protein interaction. The technique is based on the fact that gene expression

requires the presence of a transcription activator (a protein that binds DNA, thereby stimulating transcription of a nearby gene, usually by facilitating/enhancing RNA polymerase binding). Transcription activators typically consist of two domains: a DNA-binding domain (DBD), which docks the protein at a specific DNA sequence, and an activator domain (AD), which actually facilitates transcription of the target gene(s) downstream of the DBD domain.

Using this system, as overviewed in Figure 1.13, the bait protein of interest is expressed as a fusion protein which incorporates the transcription factor's DBD domain. A possible interacting protein (prey protein) is expressed as a fusion product incorporating the transcription activator's AD domain. If bait–prey interaction does indeed occur, the transcription factor's DBD and AD domains are effectively reunited in the resultant protein complex (Figure 1.13). This in turn triggers expression of the downstream reporter gene.



**Figure 1.13** The basis on which the yeast two-hybrid (Y2H) system detects protein–protein interactions. Plasmid 1 (in yeast 1) contains a fusion construct housing a nucleotide sequence coding for a transcription activator DNA-binding domain (DBD) fused to a nucleotide sequence coding for the bait protein (X). Plasmid 2 (in yeast 2) contains a fusion construct housing a nucleotide sequence coding for a transcription activator domain (AD) fused to a nucleotide sequence coding for a possible prey protein (Y). The yeast are allowed to mate (or are transformed), bringing both plasmids into the one cell. If the bait and prey proteins (X–Y) actually do interact, they bring the transcription factor DBD and AD domains together in the one complex, which in turn specifically activates the downstream reporter gene. Refer to text for exact detail.

Reporter gene expression leads to some observable change in cellular phenotype, facilitating straightforward detection. Among the most common reporter genes is the *lacZ* gene, coding for  $\beta$ -galactosidase, which turns expressing yeast colonies blue by degrading the chromomeric substrate X-gal. Additional reporter genes include the *HIS3* gene (encodes a dehydratase enzyme essential in the biosynthesis of histidine and which therefore allows expressing cells to grow on a media devoid of histidine) and the *luc* gene (encodes a luciferase enzyme which can oxidize luciferin to produce green light). Sometimes a combination of reporter genes is used.

The Y2H system is amenable to high-throughput screening, making it particularly useful from a proteomic perspective. For example, a large 'prey' cDNA library (encoding the sequence of all proteins in the proteome of interest) can be generated and subsequently screened against any specific bait protein of interest.

#### 1.4.2.2 Protein microarrays

Protein-protein interaction can also be investigated in high-throughput mode (i.e. simultaneous analysis of many different proteins derived from a proteome/proteome subset of interest) using a protein microarray (protein chip) approach. This approach entails:

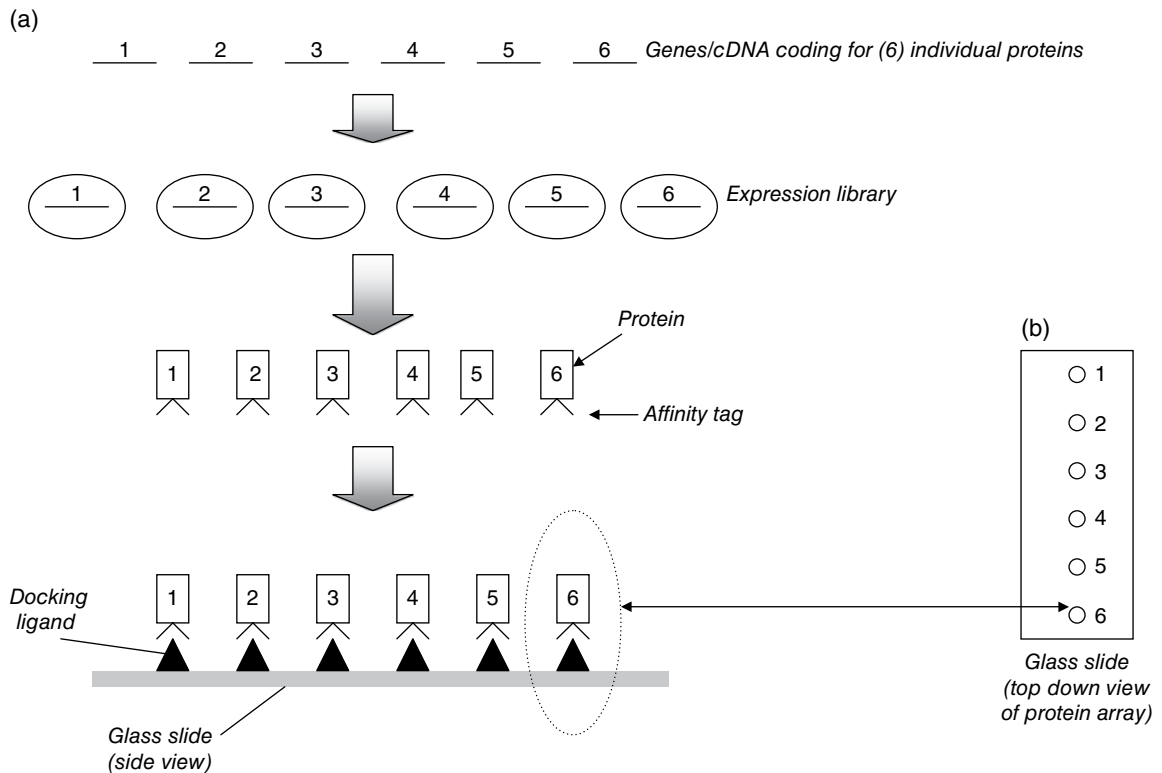
- initial immobilization of the collection of proteins with which you wish to probe samples of interest for interacting proteins, thereby generating the actual protein array;
- exposure of the protein array to the sample you wish to analyse;
- subsequent analysis of the array to detect and identify any binding partners/interactions.

The collection of proteins immobilized will be dictated by the research question posed, but one common broad approach would be to source these proteins from a library of an organism's genome via recombinant production (Figure 1.3). By using this approach it is also possible to incorporate an affinity tag at one or other end of all the proteins produced, which can subsequently facilitate both affinity-based

protein purification and affinity-based immobilization of the purified proteins (Figure 1.14a). Affinity tags will be discussed in Chapter 4 but, briefly, one such common tag is a short sequence of histidine residues (usually six, i.e. His-6) attached at the end of the protein. The His tag binds to divalent metals such as nickel ( $\text{Ni}^{2+}$ ), which can therefore act as a capture ligand. In the context of protein purification, a chromatographic column containing  $\text{Ni}^{2+}$  capture ligand can selectively purify the tagged protein, while  $\text{Ni}^{2+}$  immobilized on an appropriate surface can act as an affinity anchor for individual proteins of the protein array.

Once the gene/cDNA library coding for the tagged proteins that will constitute the array is constructed (each protein-encoding gene/cDNA being present in a single engineered recombinant cell), each recombinant protein can be expressed, purified and immobilized onto a solid surface, often made from glass or nitrocellulose (Figure 1.14a), thus producing the protein array. The different protein samples are typically applied using robotic microspotting equipment (arrayers). Individual spots will contain hundreds to thousands of individual (identical) copies of one particular protein. The pitch (i.e. distance between any two spots) can be a little as  $300\ \mu\text{m}$ , facilitating the printing of up to 20,000 individual protein spots on a single glass microscope slide (Figure 1.14b).

The use of affinity tags provides a convenient means of protein immobilization on the array support surface. Moreover, the tag itself acts as a spacer arm, keeping the protein at a (short) distance from the support surface and ensuring that all the protein molecules are oriented in an identical direction. This usually maximizes the ability of interacting proteins to, in turn, bind to the array proteins during interaction analysis. However, an alternative immobilization approach involves the direct covalent linkage of the proteins to the solid support. This can be conveniently undertaken by using supports containing chemically reactive groups (e.g. aldehydes or activated esters) which are capable of forming direct covalent linkages with functional groups commonly found on proteins (e.g. amino, carboxyl or thiol groups). The covalent nature of such links prevents protein leakage



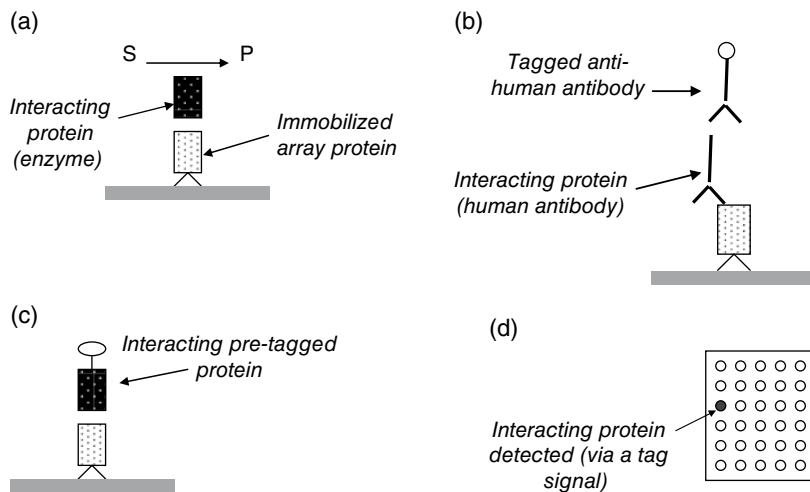
**Figure 1.14** Generation of a protein array. (a) The genes/cDNAs coding for the proteome of interest (only six proteins in this simplified example) are expressed in a recombinant microbial library (i.e. individual genes/cDNAs are inserted into individual microbial cells which, when grown individually, will produce the recombinant protein product). This molecular biology approach also allows the attachment of affinity tags at the end of each protein, facilitating affinity-based protein purification subsequent to protein expression. The tags also allow the docking (attachment) of the proteins to a solid support (e.g. a glass slide) if a docking ligand for the affinity tag is first immobilized on that support (b). Refer to text for further details.

(desorption) from the array and the approach can be undertaken to immobilize proteins devoid of affinity tags (e.g. non-recombinant proteins). However, proteins are immobilized in direct contact with the support surface and at random orientations, which can potentially negatively affect protein–protein interaction when the array is in use.

Detection of the interaction between proteins from the sample being analysed with array proteins may be achieved in different ways (Figure 1.15). In some instances an array may be designed to detect a specific protein type such as an enzyme or an antibody. Under such circumstances, interaction detection may rely on some inherent characteristic of the molecule captured by the array. If the array were designed to capture a specific enzyme (Figure 1.15a),

the enzyme captured from samples analysed could be detected using a chromogenic substrate (a molecule the enzyme is able to catalytically transform into a coloured product). Likewise, if the array were designed to detect specific antibody molecules in for example human blood, a second antibody which specifically binds to human antibodies and to which a fluorescent tag has been attached could be used (Figure 1.15 b).

However, a more widespread approach is to first pretreat the samples to be analysed such that a tag (usually a fluorescent molecule) is attached to all analyte molecules in the sample. After such samples are incubated with the array (and the array is subsequently rinsed in order to remove any unbound tag present), bound molecules can be detected via a



**Figure 1.15** Some approaches that facilitate the detection of protein interactions. (a) The use of a substrate molecule which generates a coloured/fluorescent product if the array is designed to interact with a specific enzyme. (b) The use of a labelled (tagged) antibody capable of binding to human antibodies if the array is designed to interact with human antibodies. (c) Interaction detection via the use of a sample in which all analytes are pre-tagged. (d) A (simplified) array image in which molecules from a sample analysed have bound to one specific array protein. Refer to text for further detail.

fluorescent signal (Figure 1.15c). Signal can be visualized using a microarray laser scanner. This generates an image of the microarray spots in which those participating in interactions generate a fluorescent or other signal (Figure 1.15d).

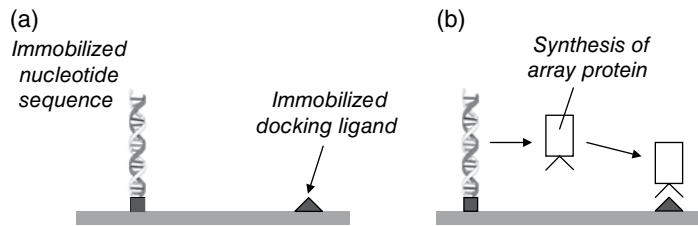
Once the occurrence of protein interactions has been established, the next step in protein array experiments is normally aimed at identifying the interacting proteins. This is most often achieved by subjecting the proteins interacting with the array to MS analysis in order to establish identity/sequence. Protein microarrays may also be used to identify protein–non-protein interactions (e.g. protein–DNA or protein–carbohydrate interactions) by pursuing the same approach as in the case of protein–protein analysis.

Array technology may be used for applied as well as academic purposes. For example, antibody-based arrays have been developed to simultaneously detect various cytokines or other molecules of diagnostic/prognostic value present in clinical samples.

While the high-throughput miniaturized nature of protein array technology renders it an attractive analytical technique, the approach is not without its limitations. For example, the occurrence of

non-specific binding reactions lead to false-positive results. Moreover, many if not most proteins are relatively labile molecules and array construction/storage prior to use can trigger protein modification and/or denaturation. This can prevent normal interactions (generating false-negative results) or can lead to artefactual interactions, leading to false-positive results.

Another limitation of array technology is the difficulty in obtaining sufficiently pure protein to construct large arrays. While the generation of libraries expressing perhaps thousands of different proteins (Figure 1.14) can be relatively straightforward, subsequent purification of each recombinant protein, even when using tag-based affinity purification systems, is usually more labour-intensive and complex (see Chapter 4). For example, affinity-based purification columns must often be followed by a second chromatographic step in order to fully purify the target protein. One approach which could potentially overcome this limitation is the development of so-called self-assembling protein microarrays. In this approach individual protein-encoding genes/cDNA (which are also engineered to contain an affinity tag at one end) are first immobilized on the



**Figure 1.16** Self-assembling protein arrays. The nucleotide sequences (e.g. cDNA) coding for individual tagged proteins are immobilized on the array solid support surface, as are tag docking ligands. Only a single illustrative sample is shown here (a). A commercial cell-free expression system is then incubated on the array surface. Cell-free expression systems contain a cocktail of the components necessary to transcribe and translate a coding sequence (RNA polymerase, ribosomes, tRNA and ribonucleotides), thus allowing protein synthesis to occur *in vitro*. The result therefore is synthesis of the tagged array proteins, which then spontaneously immobilize on the solid support surface via binding to the docking ligands (b).

microarray support surface (Figure 1.16). These are then expressed *in situ* (i.e. directly on the support surface) using a cell-free expression system. The tagged proteins, once synthesized, then bind to a tag docking ligand, which has also been pre-immobilized onto the microarray support surface. This approach would bypass the need to purify individual array proteins. Furthermore, by undertaking the cell-free protein expression step immediately prior to array application, storage stability-related concerns may no longer be an issue. However, a potential drawback is that some proteins may not fold properly in this *in vitro* environment nor will this approach support protein PTM.

### 1.4.3 Structural proteomics

After their synthesis, proteins fold into a specific three-dimensional shape (specific conformation) and protein function normally depends on it retaining that conformation. The ultimate goal of structural proteomics is to provide a complete three-dimensional description of each protein constituting a proteome.

A detailed description of protein architecture and associated methods of determining three-dimensional structure is given in Chapter 2. However, briefly, X-ray crystallography is the technique most commonly used to resolve the three-dimensional structure of proteins. Nuclear magnetic resonance (NMR) can also be used to

determine the three-dimensional structure of some, mainly smaller, proteins. Traditionally, attempts to study three-dimensional structure was undertaken on a protein-by-protein basis. The protein under study was first purified, either from a naturally producing biological source material, or from a recombinant system producing the protein. Structural analysis then ensued. The structural proteomic approach essentially pursues the same approach, but attempts to study a number of target proteome proteins at the same time. Thus, a structural proteomic starting point is often characterized by generation of a recombinant expression library expressing the target group of proteins. The recombinant proteins invariably include an affinity tag, which facilitates subsequent protein purification (see also Chapter 3). Once purified (which usually incorporates tag removal using a proteolytic enzyme), the proteins are subject to structural analysis.

The molecular biology element described above potentially allows the simultaneous/near simultaneous production and follow-on affinity purification of many proteins (i.e. has potential high-throughput characteristics). However, complications can arise including:

- the occurrence of low-level recombinant protein expression (making it difficult to source sufficient sample protein to conveniently work with);
- incomplete/no protein folding (i.e. the recombinant protein accumulates in a non-functional unfolded form, useless to structural studies);

- the extent of purity achieved by a single-step tag affinity-based purification system (highly purified protein is required).

In such instances considerable variation in experimental protocols may be required in order to optimize protein production and purification. For some proteins, an appropriate level of optimization may simply not be achieved.

Follow-on structural elucidation experiments often prove even less amenable to high-throughput automated analysis. For both X-ray crystallography and NMR spectroscopy, considerable protein-specific optimization of sample preparation is required. In the case of X ray crystallography, proteins must first be successfully crystallized, a process that again requires considerable protein-specific optimization and which ultimately may not prove successful. Moreover, the actual process of gathering and interpreting structural detail is often quite time-consuming. Overall, therefore, structural proteomics has some way to go before it becomes a genuinely automated high-throughput process.

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# Chapter 2

## Protein structure and engineering

The basics of protein structure was reviewed at the beginning of Chapter 1 in order to facilitate the subsequent consideration of proteomics. In this chapter I consider protein structure, and how it can be modified via protein engineering, in far more detail.

### 2.1 Primary structure

Polypeptides are linear unbranched polymers, usually containing up to 20 different monomers (i.e. amino acids) linked together in a precise predefined sequence. The primary structure of a polypeptide refers to its amino acid sequence, along with the exact positioning of any disulfide bonds present. The 20 commonly occurring amino acids are listed in Table 2.1, along with their three-letter and one-letter abbreviations. The structures of these amino acids are presented in Figure 2.1. Of these amino acids, 19 contain a central ( $\alpha$ ) carbon atom, to which is attached a hydrogen atom (H), an amino group ( $\text{NH}_2$ ), a carboxyl group ( $\text{COOH}$ ), and an additional side-chain (R) group, which differs from amino acid to amino acid. The amino acid proline is unusual in that its R group forms a direct covalent

bond with the nitrogen atom of what is the free amino group in other amino acids.

As will be evident from the next section, peptide bond formation between adjacent amino acid residues involves the establishment of covalent linkages between the amino and carboxyl groups attached to their central ( $\alpha$ ) carbon atoms. Hence the free functional (i.e. chemically reactive) groups in polypeptides are almost entirely present as part of the constituent amino acids' R groups. In addition to determining the chemical reactivity of a polypeptide, these R groups also play a significant role in dictating the final conformation adopted by a polypeptide. Stabilizing/repulsive forces between different R groups (as well as between R groups and the surrounding aqueous media) largely dictate what final shape the polypeptide adopts.

The R groups of the non-polar aliphatic amino acids (Gly, Ala, Val, Leu, Ile and Pro) are devoid of chemically reactive functional groups. These R groups are noteworthy in that, when present in a polypeptide's backbone, they tend to interact with each other non-covalently (via hydrophobic interactions). These interactions have a significant stabilizing influence on protein conformation.

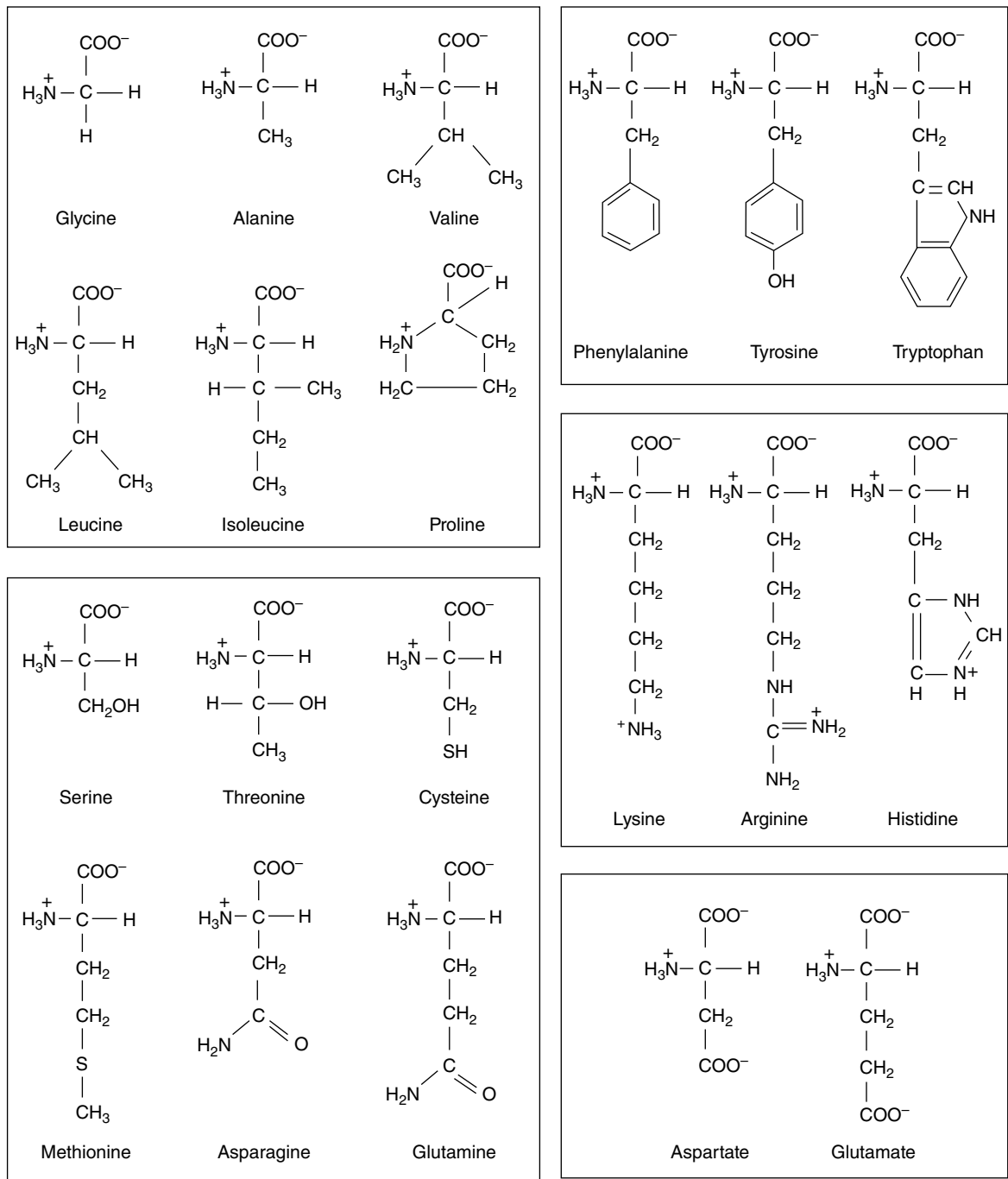
**Table 2.1** The 20 commonly occurring amino acids. They may be subdivided into five groups on the basis of side-chain structure. Their three- and one-letter abbreviations are also listed (one-letter abbreviations are generally used only when compiling extended sequence data, mainly to minimize writing space and effort). In addition to their individual molecular masses, the per cent occurrence of each amino acid in an 'average' protein is also presented. This data was generated from sequence analysis of over 1000 different proteins.

R group classification	Amino acid	Abbreviated name (3 letter)	Abbreviated name (1 letter)	Molecular mass (Da)	Per cent occurrence in 'average' protein
Non-polar, aliphatic	Glycine	Gly	G	75	7.2
	Alanine	Ala	A	89	8.3
	Valine	Val	V	117	6.6
	Leucine	Leu	L	131	9.0
	Isoleucine	Ile	I	131	5.2
	Proline	Pro	P	115	5.1
Aromatic	Tyrosine	Tyr	Y	181	3.2
	Phenylalanine	Phe	F	165	3.9
	Tryptophan	Trp	W	204	1.3
Polar but uncharged	Cysteine	Cys	C	121	1.7
	Serine	Ser	S	105	6.0
	Methionine	Met	M	149	2.4
	Threonine	Thr	T	119	5.8
	Asparagine	Asn	N	132	4.4
	Glutamine	Gln	Q	146	4.0
Positively charged	Arginine	Arg	R	174	5.7
	Lysine	Lys	K	146	5.7
	Histidine	His	H	155	2.2
Negatively charged	Aspartic acid	Asp	D	133	5.3
	Glutamic acid	Glu	E	147	6.2

Glycine is noteworthy in that its R group is a hydrogen atom. This means that the  $\alpha$ -carbon of glycine is not asymmetric (i.e. is not a chiral centre; to be a chiral centre the carbon would have to have four different chemical groups attached to it, in this case two of its four attached groups are identical). As a consequence glycine does not occur in multiple stereoisomeric forms, unlike the remaining amino acids which occur as either D or L isomers. Only L-amino acids are naturally found in polypeptides.

The side chains of the aromatic amino acids (Phe, Tyr and Trp) are not particularly reactive chemically, but they all absorb ultraviolet (UV) light. Tyr and Trp in particular absorb strongly at 280 nm, allowing detection and quantification of proteins in solution by measuring the absorbance at this wavelength (see Chapter 4).

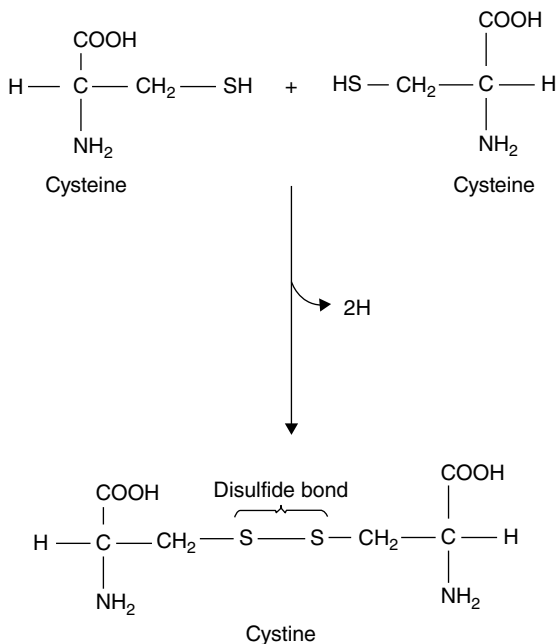
Of the six polar but uncharged amino acids, two (cysteine and methionine) are unusual in that they contain a sulfur atom. The side chain of methionine is non-polar and relatively unreactive, although the sulfur atom is susceptible to oxidation (Chapter 4). In contrast, the thiol ( $-\text{C}-\text{SH}$ ) portion of cysteine's R group is the most reactive functional group of any amino acid side chain. *In vivo* this group can form complexes with various metal ions and is readily oxidized, forming cystine residues in which two cysteine's are covalently linked (Figure 2.2). The formation of intrachain cystines (i.e. a disulfide linkage between two cysteine residues within the same polypeptide backbone) helps stabilize the three-dimensional structure of such polypeptides. Interchain disulfide linkages can also form, in which cysteines from two different polypeptides participate.



**Figure 2.1** The chemical structure of the 20 amino acids commonly found in proteins.

This is a very effective way of covalently linking adjacent polypeptides. Disulfide bonds are considered to be a post-translational modification (PTM; see section 2.9).

Of the four remaining polar but uncharged amino acids, the R groups of two (serine and threonine) contain hydroxyl (OH) groups while the R groups of asparagine and glutamine contain amide (CONH<sub>2</sub>)



**Figure 2.2** The formation of cystine via disulfide bond formation between two cysteines.

groups. None are particularly reactive chemically, although on exposure to high temperatures or extremes of pH the latter two can deaminate, yielding aspartic acid and glutamic acid respectively (Chapter 4).

Aspartic and glutamic acid are themselves negatively charged under physiological conditions. This allows them to chelate certain metal ions, and also to markedly influence the conformation adopted by polypeptide chains in which they are found.

Lysine, arginine and histidine are positively charged amino acids. The arginine R group consists of a hydrophobic chain of four  $\text{CH}_2$  groups (Figure 2.1), capped with an amino ( $\text{NH}_2$ ) group, which is ionized ( $\text{NH}_3^+$ ) under most physiological conditions. However, within most polypeptides there is normally a fraction of unionized lysines, and these (unlike their ionized counterparts) are quite chemically reactive. Such lysine side chains can be chemically converted into various analogues. The arginine side chain is also quite bulky, consisting of three  $\text{CH}_2$  groups, an amino group ( $-\text{NH}_2$ ) and a ionized guanido group ( $=\text{NH}_2^+$ ). The 'imidazole' side chain of histidine can be described chemically as a tertiary amine ( $\text{R}_3-\text{N}$ ), and thus it can act as a

strong nucleophilic catalyst (the nitrogen atom houses a lone pair of electrons, making it a 'nucleus lover' or nucleophile. It can donate its electron pair to an 'electron lover' or electrophile). As such, the histidine side chain often constitutes an essential part of some enzyme active sites (e.g. the protease subtilisin Carlsberg; see Chapter 12).

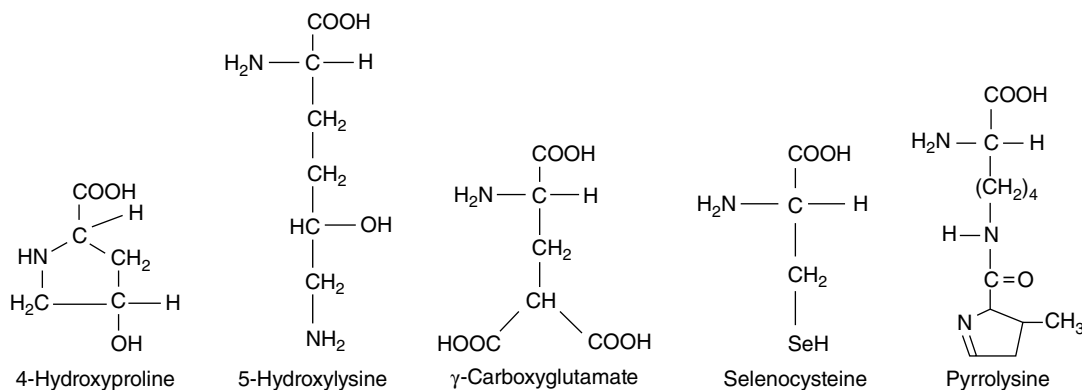
In addition to the 20 common amino acids, some modified amino acids are also found in several proteins. In most instances these modified amino acids are formed by PTM reactions, as discussed later in this chapter. However, two amino acids (selenocysteine and pyrrolysine; Figure 2.3) exist as a preformed amino acid in their own right and are hence sometimes called the 21st and 22nd proteinogenic amino acids.

Selenium in the form of selenocysteine (Sec or U) is an essential component of a small number of enzymes in some species (including glutathione peroxidase, thioredoxin reductases and some hydrogenases). The nucleotide sequence of the genes coding for such enzymes contains a UGA codon, which codes for selenocysteine. In non-selenocysteine proteins, UGA normally functions as a termination codon. The reading of UGA as selenocysteine rather than the more usual stop codon is apparently dependent on the presence of a so-called *cis*-acting selenocysteine insertion sequence element.

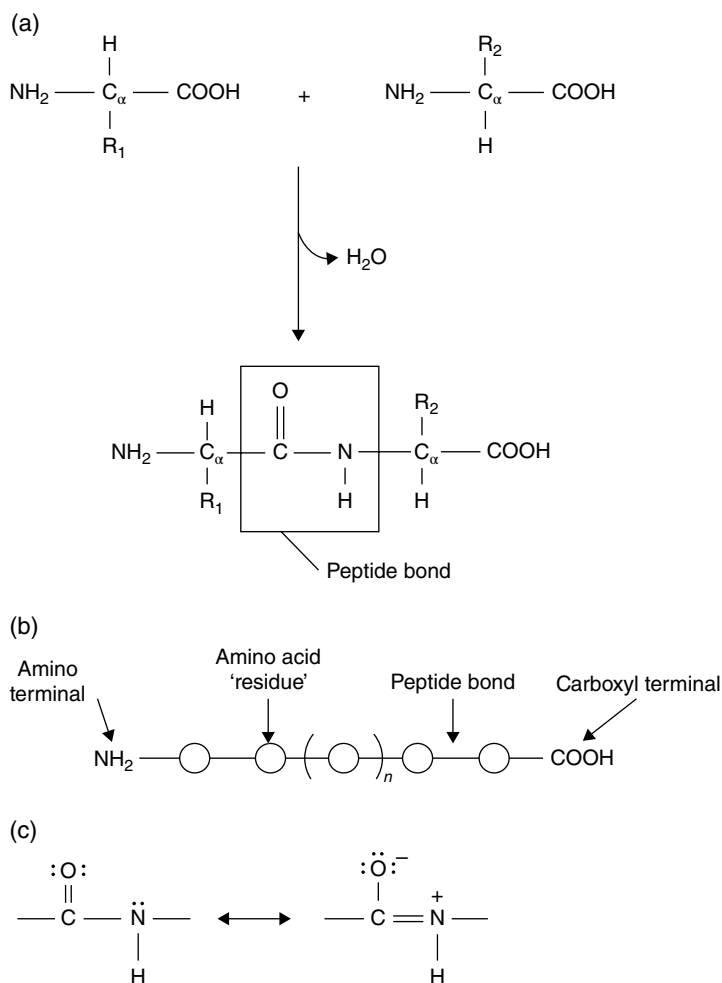
Pyrrolysine (Pyl or O) displays a side chain similar to lysine, with the presence of an added pyrroline ring at the end of the lysine side chain. Similarly to Sec, Pyl is encoded by a codon which normally functions as a stop signal (UAG), with Pyl insertion likely requiring a pyrrolysine insertion sequence element. Its presence appears to be restricted to a small number of methanogenic, mainly archael, microorganisms, where it appears to reside within the active site of several methyltransferase enzymes, playing a direct catalytic role therein.

### 2.1.1 The peptide bond

Successive amino acids are joined together during protein synthesis via a 'peptide' (i.e. amide) bond (Figure 2.4). This is a condensation reaction, as



**Figure 2.3** Structure of some modified amino acids.



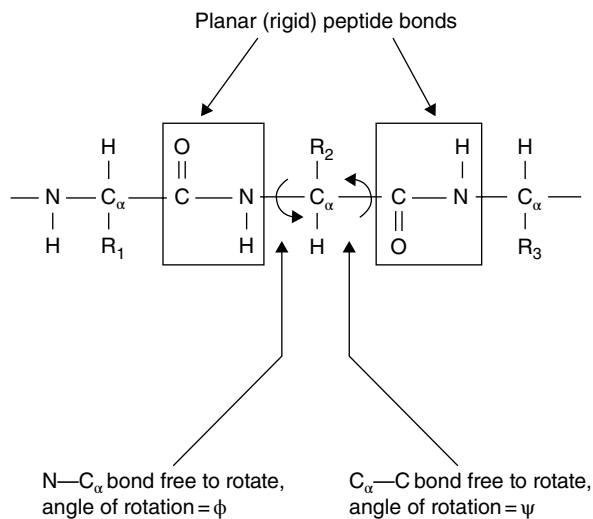
**Figure 2.4** (a) Peptide bond formation. (b) Polypeptides consist of a linear chain of amino acids successively linked via peptide bonds. (c) The peptide bond displays partial double-bonded character.

a water molecule is eliminated during bond formation. Each amino acid in the resultant polypeptide is termed a 'residue', and the polypeptide chain will display a free amino ( $\text{NH}_2$ ) group at one end and a free carboxyl ( $\text{COOH}$ ) group at the other end. These are termed the amino and carboxyl termini, respectively.

The peptide bond has a rigid planar structure and is in the region of  $1.33 \text{ \AA}$  ( $0.133 \text{ nm}$ ) in length. Its rigid nature is a reflection of the fact that the amide nitrogen lone pair of electrons are delocalized across the bond (i.e. the bond structure is a halfway house between the two forms illustrated in Figure 2.4c). In most instances, peptide groups assume a *trans* configuration (Figure 2.4a). This minimizes steric interference between the R groups of successive amino acid residues.

While the peptide bond is rigid, the other two bond types found in the polypeptide backbone, the  $\text{N}-\text{C}_\alpha$  bond and the  $\text{C}_\alpha-\text{C}$  bond (Figure 2.5), are free to rotate. The polypeptide backbone can thus be viewed as a series of planar 'plates' which can rotate relative to one another. The angle of rotation around the  $\text{N}-\text{C}_\alpha$  bond is termed  $\phi$  (phi) while that around the  $\text{C}_\alpha-\text{C}$  bond is termed  $\psi$  (psi). These angles are also known as rotation angles, dihedral angles or torsion angles. By convention, these angles are defined as being  $180^\circ$  when the polypeptide chain is in its fully extended *trans* form. In principle, each bond can rotate to any value between  $-180^\circ$  and  $+180^\circ$ . However, the degrees of rotation actually observed are restricted due to the occurrence of steric hindrance between atoms of the polypeptide backbone and those of amino acid side chains.

For each amino acid residue in a polypeptide backbone, the actual  $\phi$  and  $\psi$  angles that are physically possible can be calculated, and these angle pairs are often plotted against each other in a diagram termed a Ramachandran plot. Sterically allowable angles fall within relatively narrow bands in most instances. A greater than average degree of  $\phi/\psi$  rotational freedom is observed around glycine residues, due to the latter's small R group, hence steric hindrance is minimized. On the other hand, bond angle freedom around proline residues is quite restricted due to this amino acid's unusual structure (Figure 2.1). The  $\phi$  and  $\psi$  angles allowable around each  $\text{C}_\alpha$  in a polypeptide backbone obviously exerts



**Figure 2.5** Fragment of polypeptide chain backbone illustrating rigid peptide bonds and the intervening  $\text{N}-\text{C}_\alpha$  and  $\text{C}_\alpha-\text{C}$  backbone linkages, which are free to rotate.

a major influence upon the final three-dimensional shape assumed by the polypeptide.

### 2.1.2 Amino acid sequence determination

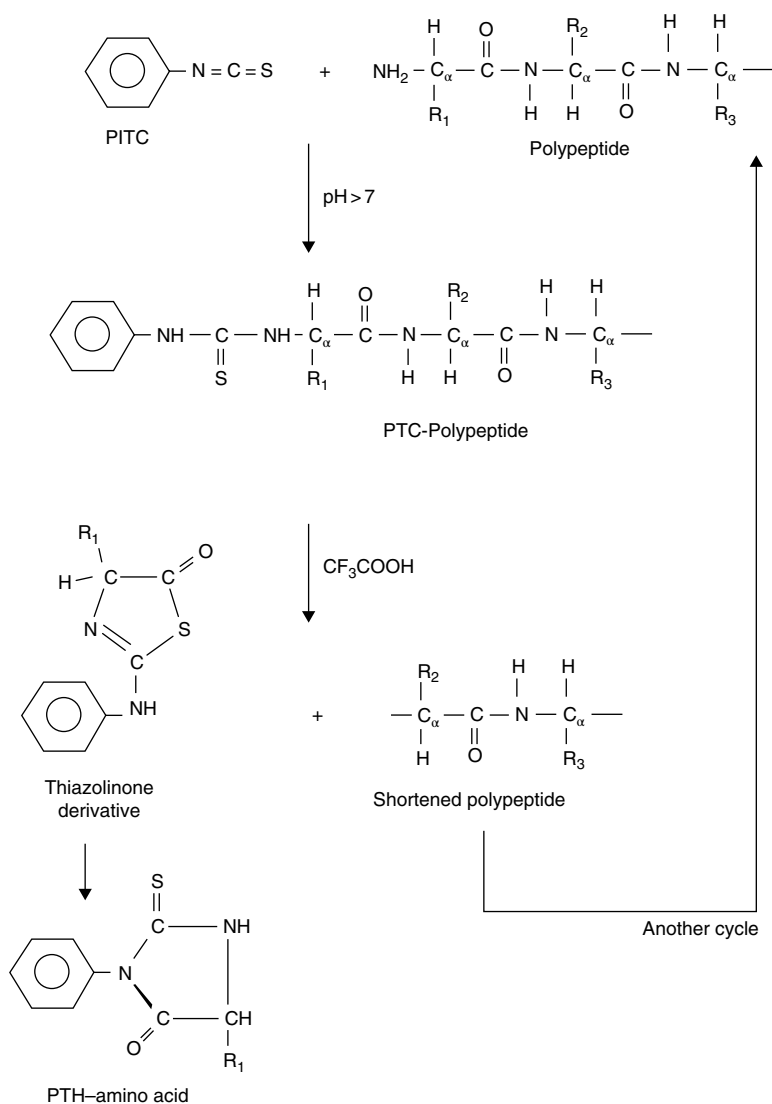
Traditionally, the amino acid sequence of an isolated polypeptide was determined directly via chemical (Edman) sequencing (Box 2.1). Comparatively more recently mass spectrometry (MS)-based approaches have come to the fore, as described in Chapter 1. MS-based approaches display several potential advantages over the Edman sequencing approach.

- MS-based approaches are faster and more convenient than Edman degradation.
- Unlike the Edman approach, MS-based approaches are amenable to high-throughput analyses and therefore generally more useful for proteomics.
- MS-based approaches are more sensitive: the Edman technique, though sensitive, usually requires  $1-10 \text{ pmol}$  ( $1-10 \times 10^{-12} \text{ mol}$ ) of protein sample, whereas MS requires only a few femtomoles ( $10^{-15} \text{ mol}$ ) of protein, making MS between 10 and 1000 times more sensitive (see Chapter 1).
- MS-based approaches can provide sequence information from blocked/modified peptides.

**Box 2.1 Direct chemical sequencing via the Edman method**

The Edman degradation method represents the classical (but now far less commonly used) approach for directly determination of the amino acid sequence of a polypeptide. The approach entails sequential labelling, removal and identification of amino acid residues, beginning at the N-terminal end of the polypeptide (see diagram). The polypeptide is first incubated with the reagent phenylisothiocyanate (PITC) which, at high pH values, reacts with the polypeptide N-terminal amino group yielding a phenylthio-

carbamyl (PTC) derivative. Subsequent addition of anhydrous trifluoroacetic acid cleaves the PTC derivative, yielding a thiazolinone derivative (consisting of the PITC-N-terminal amino acid derivative) and a polypeptide chain lacking its N-terminal amino acid. The thiazolinone derivative is then solvent extracted and converted into the more stable phenylthiohydantoin (PTH) derivative by acidification of the media. The PTH derivative is compared chromatographically to PTH-amino acid standards in order to identify



the amino acid moiety. The shortened polypeptide is then subjected to a second round of the cycle in order to identify amino acid number two of the original polypeptide. Further rounds of the cycle allow determination of the entire sequence.

Prior to commencement of Edman sequencing the individual polypeptides of multi-polypeptide proteins must be separated, and each independently sequenced. In addition, any disulfide linkages must be broken, usually by incubation of the polypeptide with a suitable reducing agent.

Theoretically, the Edman method should facilitate sequence analysis of proteins of any size. However, with each repeat of the sequencing cycle, a small number of unreacted N-terminal amino acid residues will remain. Such errors are obviously cumulative, and the background 'noise' increases steadily until a point is reached where the amino acid residues being processed can no longer be assigned with absolute certainty. Larger polypeptides must first be fragmented into shorter peptides. The fragments are then separated and independently sequenced. Fragmentation may be achieved chemically or enzymatically and a range of chemical reagents and proteolytic enzymes capable of hydrolysing particular backbone peptide

bonds are available. The peptide fragments are then separated, either by one- or two-dimensional electrophoresis, or more commonly by HPLC.

In order to determine the correct order in which the (now sequenced) peptide fragments occurred in the intact polypeptide it is necessary to independently fragment a fresh sample of the polypeptide, using an alternative fragmentation reagent. The new set of fragments generated are then separated and sequenced. Identification of overlapping sequences between the original and new set of fragments allows determination of the full sequence data.

Sequencing can also be used to determine the location of disulfide linkages within primary sequence data. In this case, a fresh sample of protein is fragmented without prior reduction (i.e. breaking) of any disulfide linkages. The presence of a disulfide bond results in the generation of a peptide mix that will contain a large polypeptide 'fragment' actually consisting of two fragments linked by the disulfide bond. On its isolation the 'fragment' is reduced, and the two fragments released are independently sequenced. This pinpoints the cysteine molecules participating in the disulfide linkage.

The last point in particular has always been a complicating factor when applying the Edman approach to eukaryotic-derived proteins. Up to 80% of such proteins display chemically altered N-terminal amino acid residues, which do not react with the Edman PITC reagent (Box 2.1). The most common N-terminal chemical alteration observed is acetylation (see section 2.9.4), but blocking may also be the result of glycosylation and formylation for example.

Today, however, the vast majority of protein sequences are obtained/predicted indirectly via nucleotide sequence data generated from genome sequencing projects (Chapter 1), which now means that amino acid sequence data for several tens of millions of different proteins are available and may be accessed and interrogated through databases

such as the Uniprot database ([www.uniprot.org](http://www.uniprot.org); Box 2.2).

Despite the central importance of the genomic approach, direct sequencing methods remain important/essential for a number of applications. For example, direct sequencing (full-length or at least partial sequencing of the first 10–20 amino acids at the N-terminus of a protein) can be used to:

- design polymerase chain reaction (PCR) primers to assist in the ultimate cloning of the gene coding for the protein if the protein has been purified directly from, for example, a source for which no genome sequence data is available;
- serve as a quality control tool to directly verify the identity/sequence of protein products such as biopharmaceuticals.



**Box 2.2 The UniProt databases**

UniProt (Universal Protein Resource) is a comprehensive web-based resource ([www.uniprot.org](http://www.uniprot.org)) housing information on proteins, particularly protein sequence and function. It is a collaboration between three bioinformatic-based institutes: the European Bioinformatics Institute, the Swiss Institute of Bioinformatics, and the Protein Information Resource institute. At its heart is the UniProt knowledgebase (UniProtKB), a protein database that can be interrogated via (among other methods) a key word query search, and which provides protein name, sequence information, taxonomic data, available functional data and literature information for each protein entry therein. Virtually all the protein sequences provided by UniProtKB are derived from the translation of coding sequences submitted to public nucleic acid databases (EMBL, GenBank and DDBJ; see Chapter 1, in particular Table 1.2).

For example, a UniProtKB search using the term 'human erythropoietin' in the search query box reveals a list of erythropoietins (EPOs) and related proteins from various species, with human EPO residing at the top of the entry list (entry no. P01588). In addition to its full amino acid sequence, the human EPO entry houses additional information on the protein's structure and function (e.g. molecular processing, post-translational modifications, natural variants, secondary structural detail, functional information, pharmaceutical uses, references and links to additional pertinent databases). The full 193 amino acid sequence provided is shown below (single letter format; see Table 2.1). The first 27 residues represent a signal peptide, subsequently removed to produce the 166 amino acid mature product (see Chapter 8).

```
      10      20      30      40      50      60
MGVHECPAWL  WLLLSLLSLP  LGLPVLGAPP  RLICDSRVLE  RYLLEAKEAE  NITTGCAEHC

      70      80      90     100     110     120
SLNENITVPD  TKVNFYAWKR  MEVGQQAVEV  WQGLALLSEA  VLRGQALLVN  SSQPWEPLQL

     130     140     150     160     170     180
HVDKAVSGLR  SLTTLRLALG  AQKEAISPPD  AASAAPLRTI  TADTFRKLFR  VYSNFLRGKL

     190
KLYTGEACRT  GDR
```

### 2.1.3 Bioinformatic analysis of sequence data

The elucidation of the amino acid sequence of any protein is in itself a legitimate goal of protein science. However, much additional information can be leveraged from the vast and growing quantity of protein sequence information now available via bioinformatic analysis. As outlined in overview in Chapter 1, a major goal, and indeed achievement, of bioinformatics has been the development of computer programs/software tools which can interrogate and analyse raw protein sequence information in order to generate additional information. As also outlined in Chapter 1, many such tools can be conveniently accessed via bioinformatic resource portals such as ExPASy (see Box 1.1).

In this context, a word of caution is also warranted. The quality of information derived from the application of any bioinformatic program is only as good as the principles and assumptions on which the program is based. For example (as we will see later), the precise ‘rules’ by which a protein’s primary structure dictates that protein’s three-dimensional structure remain to be fully elucidated. As such, a theoretical three-dimensional model of a protein generated on the basis of amino acid sequence information by a bioinformatic tool may or may not be accurate and is best validated by direct structural analysis.

Various and often multiple different bioinformatic programs/tools are available that interrogate protein sequence information/databases in order to:

- identify proteins containing similar amino acid sequences (i.e. run similarity searches) and assess how closely related two (or more) proteins are, or if there is a high probability that they undertake similar functions (see next section);
- calculate a theoretical molecular mass, isoelectric point (see Chapter 4) or other physicochemical property of a protein;
- predict elements of a protein’s higher-order structure (secondary and tertiary structure, or for example protein domains, as discussed in section 2.2.2);

- predict if a protein is likely to undergo PTMs (see section 2.9), and at what point(s) along the protein backbone this is likely to occur;
- predict where in the cell the protein is likely to function (or if it is likely exported from the cell).

While we return to such tools as appropriate later in the chapter, we consider those used to identify sequence similarities in the following section.

#### 2.1.3.1 Sequence similarity and sequence alignment analyses

A basic question posed for any protein sequence, particularly if newly identified (e.g. from a genome sequencing project), is if it is related to any previously identified protein sequences. A high degree of sequence similarity usually infers a common ancestry and possibly a similar function. A commonly used sequence similarity search tool is BLAST (Basic Local Alignment Search Tool), accessible via the NCBI webpage (see Table 1.2) or via other bioinformatic resource pages such as UniProt (Box 2.2). A BLAST search compares a ‘query’ sequence to a library or database of sequences, generating a list of similarity ‘hits’ usually ordered by per cent sequence identity (Table 2.2).

A more in-depth sequence analysis may be undertaken by using such software to generate an amino acid sequence alignment between two or more sequences, i.e. a pairwise or a multiple sequence alignment, respectively. A pairwise alignment is presented in Figure 2.6.

A number of terms are routinely encountered when undertaking sequence similarity comparisons, including homology, identity and similarity. Homology (homologous proteins) implies that proteins share a common evolutionary ancestry. Note that the term ‘homology’ is an absolute one; a term such as ‘per cent homology’ is incorrect. Identity and similarity quantitatively describe the extent to which two sequences are related. Per cent identity refers to the percentage of residues in two (or more) aligned sequences which are identical. Per cent similarity includes consideration of both identical and similar matches in aligned sequences.

**Table 2.2** Top matches obtained from a BLAST search using the human erythropoietin (EPO) amino acid sequence as a query sequence against the 42 million sequence entries present in the UniProtKB database (Box 2.2). A total of 121 hits were obtained, the top 26 of which are presented here. Unsurprisingly, the highest matches were to the human EPO sequence entries already present in the database. Many of the additional hits are EPO sequences from other species. An outline of how similarity is graded is presented in the main text.

Accession	Entry name	OQuery hit193	OMatch hit (sqrt scale)2453i	Name (organism)
Query	2013072970QOV94AU2	=====	==	
G9JKG7	G9JKG7_HUMAN	=====	==	Erythropoietin ( <i>Homo sapiens</i> )
P01588	EPO_HUMAN	=====	==	Erythropoietin ( <i>Homo sapiens</i> )
H2QV42	H2QV42_PANTR	=====	==	Uncharacterized protein ( <i>Pan troglodytes</i> )
G3RS27	G3RS27_GORGO	=====	==	Uncharacterized protein ( <i>Gorilla gorilla gorilla</i> )
B7ZKK5	B7ZKK5_HUMAN	=====	==	EPO protein ( <i>Homo sapiens</i> )
G1RMP4	G1RMP4_NOMLE	=====	==	Uncharacterized protein ( <i>Nomascus leucogenys</i> )
G3RPR5	G3RPR5_GORGO	=====	==	Uncharacterized protein ( <i>Gorilla gorilla gorilla</i> )
P07865	EPO_MACFA	=====	==	Erythropoietin ( <i>Macaca fascicularis</i> )
Q28513	EPO_MACMU	=====	==	Erythropoietin ( <i>Macaca mulatta</i> )
G7POD4	G7POD4_MACFA	=====	==	Putative uncharacterized protein ( <i>Macaca fascicularis</i> )
F6WN92	F6WN92_MACMU	=====	==	Erythropoietin ( <i>Macaca mulatta</i> )
F7DTH0	F7DTH0_CALJA	=====	==	Uncharacterized protein ( <i>Callithrix jacchus</i> )
Q867B1	EPO_HORSE	=====	==	Erythropoietin ( <i>Equus caballus</i> )
17AKF2	17AKF2_FELCA	=====	==	Erythropoietin ( <i>Felis catus</i> )
13MLF9	13MLF9_SPETR	=====	==	Uncharacterized protein ( <i>Spermophilus tridecemlineatus</i> )
F7DQY8	F7DQY8_HORSE	=====	==	Erythropoietin ( <i>Equus caballus</i> )
P33708	EPO_FELCA	=====	==	Erythropoietin ( <i>Felis catus</i> )
D2HX05	D2HX05_AILME	=====	==	Putative uncharacterized protein ( <i>Ailuropoda melanoleuca</i> )
G1M830	G1M830_AILME	=====	==	Uncharacterized protein ( <i>Ailuropoda melanoleuca</i> )
G3UDT5	G3UDT5_LOXAF	=====	==	Uncharacterized protein ( <i>Loxodonta africana</i> )
K4Q170	K4Q170_CANFA	=====	==	Erythropoietin ( <i>Canis familiaris</i> )
M3YWD4	M3YWD4_MUSPF	=====	==	Uncharacterized protein ( <i>Mustela putorius furo</i> )
H0Y1U0	H0Y1U0_OTOGA	=====	==	Uncharacterized protein ( <i>Otolemur garnettii</i> )
L5K6F9	L5K6F9_PTEAL	=====	==	Erythropoietin ( <i>Pteropus alecto</i> )
F1PPB9	F1PPB9_CANFA	=====	==	Erythropoietin ( <i>Canis familiaris</i> )
J9NYY7	J9NYY7_CANFA	=====	==	Erythropoietin ( <i>Canis familiaris</i> )

```

1 -----MGVHECPAWLWLLLSLLSLPLGLPVLGAPPRLICDSRVLERYLLEAK 47
1 MCEPAPPPTQSAWHSFPECPA-LFLLLSLLLPGLPVLGAPPRLICDSRVLERYILEAR 59
      . . **** *:***** *****:*****:****:
      . . **** *:***** *****:*****:****:

48 EAENITTGCAEHCSLNENITVPDPTKVNIFYAWKRMEVGGQAVEVWQGLALLSEAVLRGQAL 107
60 EAENVTMGCAQGCSENFENITVPDPTKVNIFYTWKRMDVGGQALEVWQGLALLSEAILRGQAL 119
      ****:*  ***:  **: .*****:*****:*****:*****:*****:*****:
      ****:*  ***:  **: .*****:*****:*****:*****:*****:*****:

108 EVNSSQPWEPLQLHVDKAVSGLRSLTLLRRLALGAQKEAISPDAASAAPLRTTTADTFRK 167
120 LANASQPSETPQLHVDKAVSSLRSLTLLRRLALGAQKEAMSLPEEASPAPLRTFTVDTLCK 179
      *.:** *  *****.*****:*****:*****:*****:*****:*.*** *
      *.:** *  *****.*****:*****:*****:*****:*****:*.*** *

168 LFRVYSNFLRGKCLKLYTGEACRTGDR 193 P01588 EPO_HUMAN
180 LFRVYSNFLRGKCLKLYTGEACRRGDR 205 J9NYY7 J9NYY7_CANFA
      ****:*****.***** ****

```

**Figure 2.6** A pairwise sequence alignment between the amino acid sequence of human erythropoietin (EPO, top line of each twin sequence) and canine EPO (bottom line of each twin sequence) (a). The sequence alignment was undertaken via the UniProt website. Asterisks are automatically placed underneath sequence positions housing identical amino acid residues while double or single dots (i.e. a colon or a period) appear underneath residue positions which display strongly or weakly similar properties, respectively. Thus, human and canine EPOs contain identical residues at 155 positions (i.e. they display approximately 75% identity) and similar residues at a further 24 positions. The software also facilitates the generation of additional information such as the positioning of amino acid residues with particular properties.

Sequence analysis alone is often insufficient to unequivocally demonstrate that two or more proteins are homologous. For example, two proteins can turn out to be homologous without displaying high-level per cent identity. This situation can arise, for example, if mainly conservative substitutions occur over the evolutionary period (i.e. substitutions by amino acids displaying similar properties, such as aspartic acid to glutamic acid, both of which display negatively charged side chains). Although this results in a divergence of sequence, it may not alter the functional capacity or three-dimensional structure of the protein to nearly the same degree. In general, three-dimensional structural detail of homologous proteins diverge much more slowly than do their corresponding amino acid sequence identity.

## 2.2 Higher-level structure

Thus far I have concentrated on the primary structure (amino acid sequence) of a polypeptide. Higher-level protein structure can be described at various levels, i.e. secondary, tertiary and quaternary.

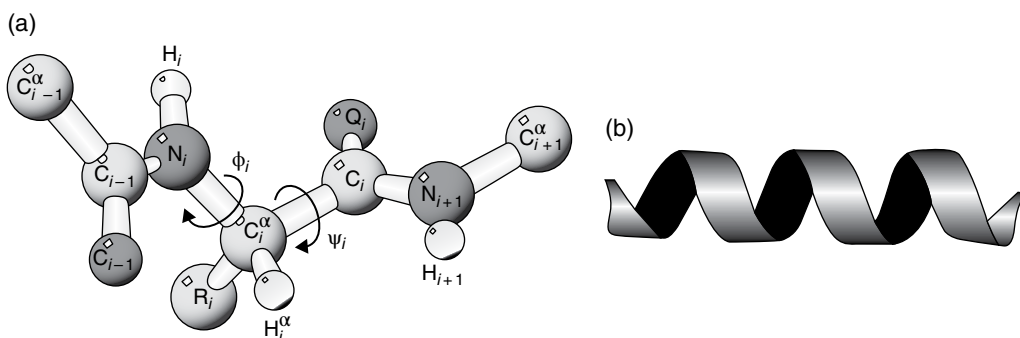
- Secondary structure can be described as the local spatial conformation of a polypeptide's backbone,

excluding the constituent amino acids' side chains. The major elements of secondary structure are the  $\alpha$ -helix and  $\beta$  strands, as described below.

- Tertiary structure refers to the three-dimensional arrangement of all the atoms which contribute to the polypeptide.
- Quaternary structure refers to the overall spatial arrangement of polypeptide subunits within a protein composed of two or more polypeptides.

### 2.2.1 Secondary structure

By studying the backbone of most proteins, stretches of amino acids which adopt a regular, recurring shape usually become evident. The most commonly observed secondary structural elements are termed the  $\alpha$ -helix and  $\beta$  strands. Fibrous proteins often display but one type of secondary structure, stretching fully from one end of the polypeptide to the other. The entire backbone of the fibrous protein  $\alpha$ -keratin, for example, adopts an  $\alpha$ -helical shape, while the entire backbone of another fibrous protein, fibroin, assumes the  $\beta$  conformation. In contrast, the backbone of globular proteins generally exhibits several stretches of regular secondary structure ( $\alpha$ -helix and/or  $\beta$  strands), separated by stretches largely devoid of



**Figure 2.7** Ball and stick and ribbon representation of an  $\alpha$ -helix. Reproduced from *Current Protocols in Protein Science* by kind permission of the publisher, John Wiley & Sons, Ltd.

regular, recurring conformation. The  $\alpha$ -helix and  $\beta$  sheets are commonly formed because they maximize formation of stabilizing intramolecular hydrogen bonds and minimize steric repulsion between adjacent side-chain groups, while also being compatible with the rigid planar nature of the peptide bonds (i.e. remain compatible with allowable dihedral angles on a Ramachandran plot).

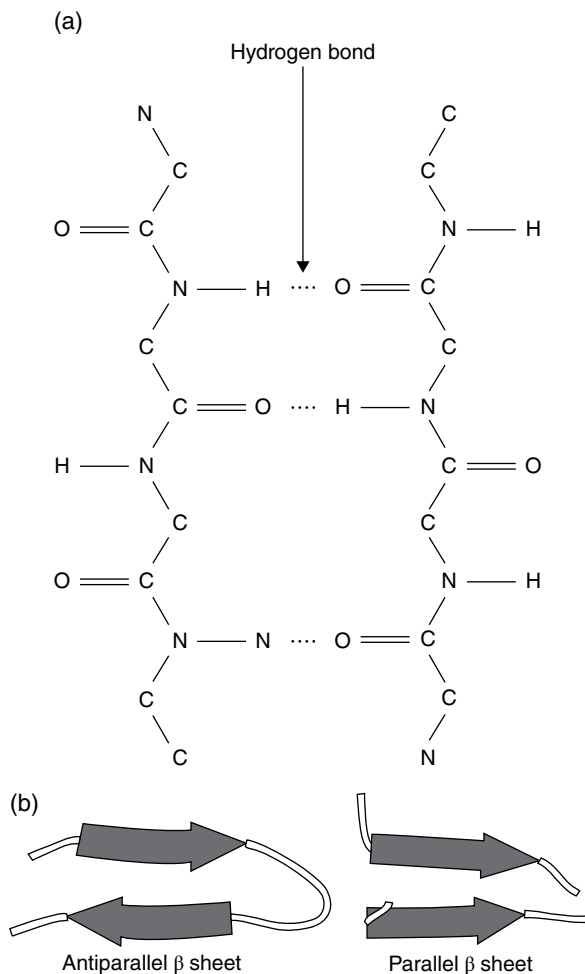
The  $\alpha$ -helix is a right-handed one, containing 3.6 amino acid residues in a full turn (Figure 2.7). This approximates to a length of 0.56 nm along the long axis of the helix. The participating amino acid side chains protrude outward from the helical backbone. Amino acids most conducive with  $\alpha$ -helix formation include alanine, leucine, methionine and glutamate. Proline, as well as the occurrence in close proximity of multiple residues with either bulky side groups or side groups of the same charge, tend to disrupt  $\alpha$ -helical formation. The helical structure is stabilized by hydrogen bonding, with every backbone C=O group forming a hydrogen bond with the N-H group four residues ahead of it in the helix. Stretches of  $\alpha$ -helix found in globular polypeptides can vary in length from a single helical turn to greater than 10 consecutive helical turns. The average length is about three turns.

$\beta$  Strands represent the other major recurring structural element of proteins.  $\beta$  Strands usually are 5–10 amino acid residues in length, with the residues adopting an almost fully extended zigzag conformation. Single  $\beta$  strands are rarely, if ever, found alone. Instead, two or more of these strands align themselves together to form a  $\beta$  sheet. The  $\beta$  sheet is

a common structural element stabilized by maximum hydrogen bonding (Figure 2.8). The individual  $\beta$  strands participating in  $\beta$ -sheet formation may all be present in the same polypeptide, or may be present in two polypeptides held in close juxtaposition.  $\beta$  Sheets are described as being either parallel, antiparallel or mixed. A parallel sheet is formed when all the participating  $\beta$  stretches are running in the same direction (e.g. from the amino terminus to the carboxy terminus, Figure 2.8). An antiparallel sheet is formed when successive strands have alternating directions (N-terminus to C-terminus followed by C-terminus to N-terminus, etc.). A  $\beta$  sheet containing both parallel and antiparallel strands is termed a mixed sheet.

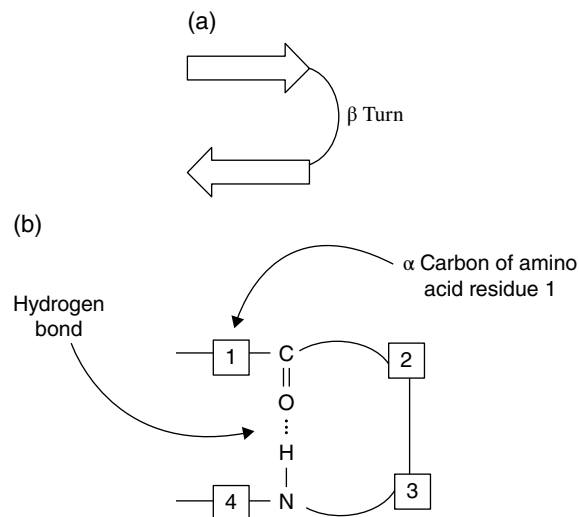
The presence of common secondary structures (the  $\alpha$ -helix and  $\beta$  conformation) in a protein can be detected and quantified (i.e. the percentage of the protein's backbone adopting each confirmation) using a form of spectroscopy known as circular dichroism. This entails exposing the protein to circularly polarized light in the 190–250 nm wavelength region. The structural symmetry characterized by different secondary structure types will result in differential and characteristic absorption patterns of the left-handed versus right-handed polarized light, which can be plotted as an absorption spectrum.

In terms of secondary structure, most proteins consist of several segments of  $\alpha$ -helix and/or  $\beta$  strands separated from each other by various loop regions. These regions can vary in length and shape, and allow the overall polypeptide to fold into a compact tertiary structure. In general, loops are present



**Figure 2.8** The  $\beta$  sheet. (a) Two segments of  $\beta$  strands (antiparallel) forming a  $\beta$  sheet via hydrogen bonding. The  $\beta$  strand is drawn schematically as a thick arrow. By convention the arrowhead points in the direction of the polypeptide's C-terminus. (b) Schematic of a two-strand  $\beta$  sheet in parallel and antiparallel modes.

on the surface of polypeptides. They are rich in polar/charged amino acid residues that, along with the N—H and C=O groups of their associated peptide bonds, hydrogen bond with the surrounding water molecules. Loop regions are usually quite flexible and the longer such regions can be susceptible to proteolytic cleavage. In addition to their obvious role in connecting stretches of regular secondary elements, loop regions themselves often participate or contribute directly to the polypeptide's



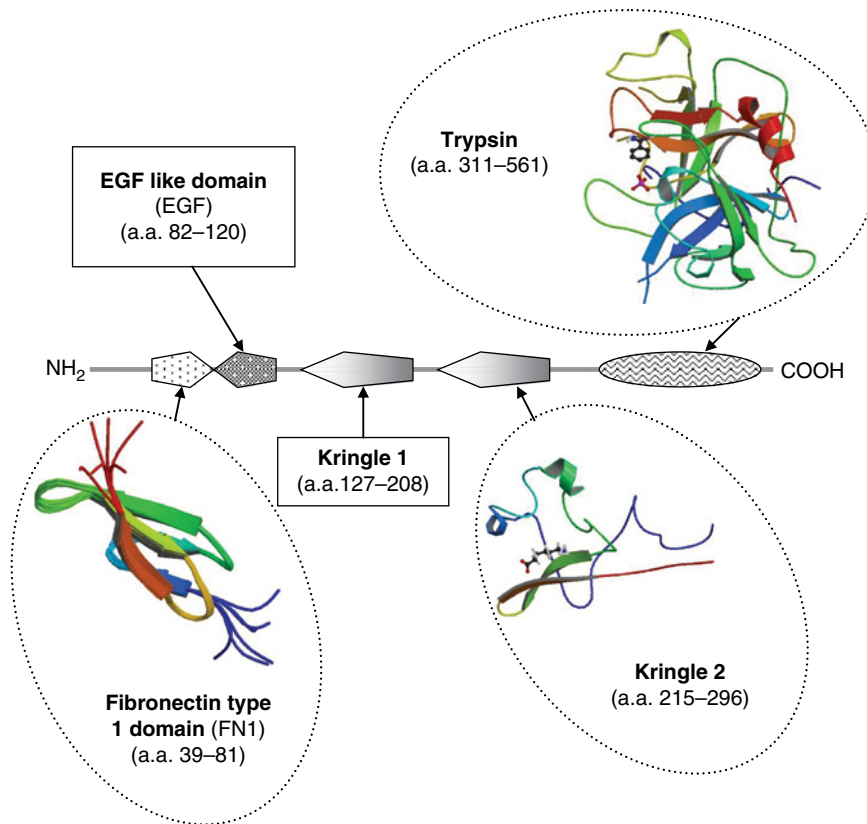
**Figure 2.9** (a) The  $\beta$  bend or  $\beta$  turn is often found between two stretches of antiparallel  $\beta$  strands. (b) It is stabilized in part by hydrogen bonding between the C=O bond and the NH groups of the peptide bonds at the neck of the turn.

biological function. The antigen-binding region of antibodies, for example, are largely constructed from six loop regions. Such loops also often form the active site of enzymes.

One loop structure, termed a  $\beta$  turn or  $\beta$  bend, is a characteristic feature of many polypeptides. The  $\beta$  bend achieves a  $180^\circ$  alteration in backbone direction over the course of four amino acid residues, and is most often found between two stretches of antiparallel  $\beta$  strands (Figure 2.9). Glycine and proline residues are often found at  $\beta$  turns. Glycine minimizes steric hindrance, due to its small side chain. Proline, by virtue of its unusual structure, naturally introduces a kink or bend in the polypeptide backbone. The loop is stabilized in part by the formation of a hydrogen bond between the C=O of the first residue and the N—H of the fourth residue (Fig. 2.9).

## 2.2.2 Tertiary structure

As previously mentioned, a polypeptide's tertiary structure refers to its exact three-dimensional structure, relating the relative positioning in space of all the polypeptide's constituent atoms to each



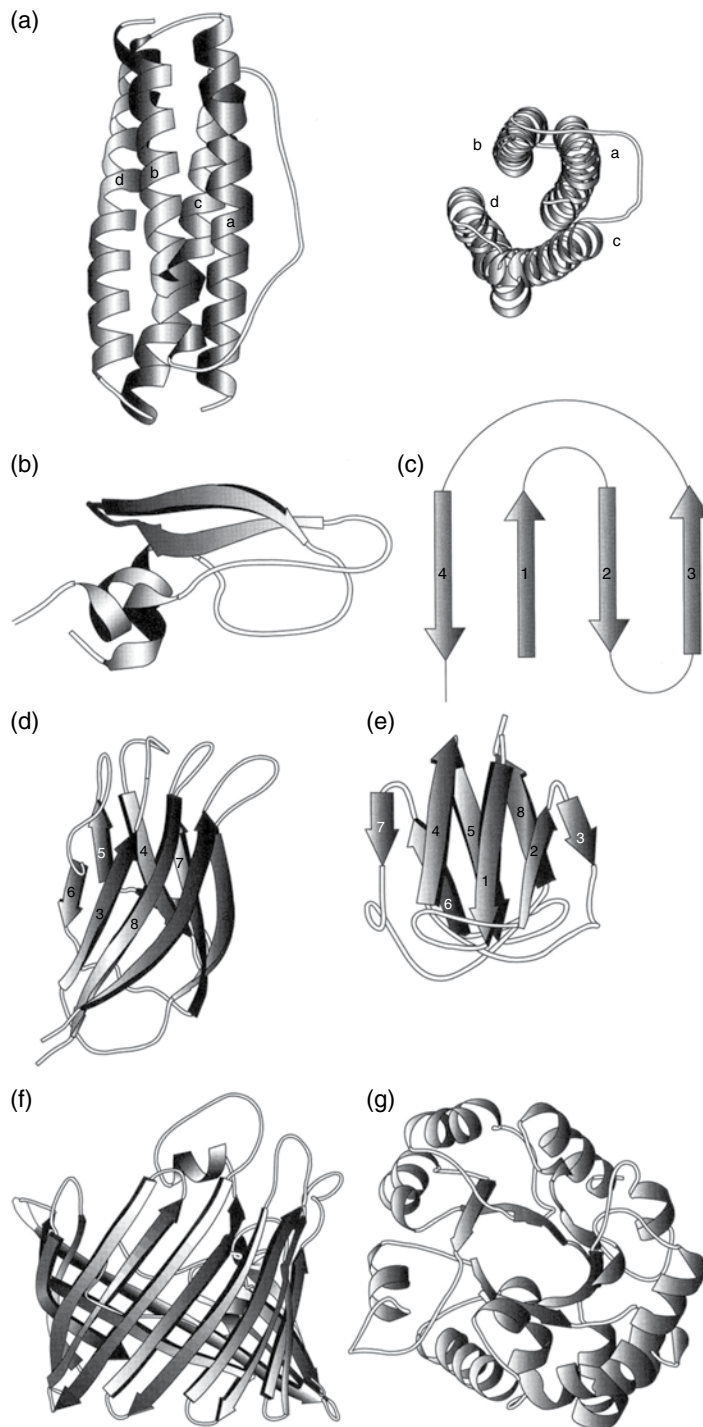
**Figure 2.10** Schematic representation of the domain structure of human tissue plasminogen activator, a 526 amino acid protein. A number of these domains have been independently produced via recombinant DNA technology and their three-dimensional structure resolved. These are also included (protein databank entries 1TPN, 1PK2 and 1RTF; [www.pdb.org](http://www.pdb.org)).

other. The tertiary structure of small polypeptides (approximately 200 amino acid residues or less) usually forms a single discrete structural unit. However, when the three-dimensional structure of many larger polypeptides is examined, the presence of two or more structural subunits within the polypeptide becomes apparent. These are termed domains. Domains are therefore (usually) tightly folded subregions of a single polypeptide, connected to each other by more flexible or extended regions. As well as being structurally distinct, domains often serve as independent units of function. For example, both domains of the protein troponin C serve to bind calcium ions, and cell surface receptors usually contain one or more extracellular domains (some or all of which participate in ligand binding), a transmembrane domain (hydrophobic in nature

and serving to stabilize the protein in the membrane) and one or more intracellular domains that play an effector function (e.g. generation of second messengers).

The domain structure of tissue plasminogen activator (tPA) is presented in Figure 2.10. tPA is a clot-degrading protein used medically to treat heart attacks and strokes. The function of its various domains, as well as the generation of domain-deleted engineered variants for medical use, is considered in Chapter 6. More information on this protein may be obtained from the UniProt KB database (entry P00750).

When closely studied, it becomes apparent that domains themselves are usually composed of 'building blocks' known as structural motifs. Structural motifs (sometimes called supersecondary structures) are



**Figure 2.11** Some structural motifs commonly associated with (globular) polypeptides: (a) a four-helix bundle (b) a hairpin structure (c) a  $\beta$  sheet with a Greek key topology (d) a jelly roll motif (e) a  $\beta$  sandwich (f) a  $\beta$  barrel (g) an  $\alpha/\beta$  barrel. Refer to text for further details. Reproduced from *Current Protocols in Protein Science* by kind permission of the publisher, John Wiley & Sons, Ltd.



composed of a few stretches of secondary structure (e.g. stretches of  $\alpha$ -helix or  $\beta$  strands), linked via loops and all arranged in a specific three-dimensional conformation. Any given single structural motif is often found in a wide variety of polypeptides, which may have either related or unrelated biological functions. Some of the more commonly observed structural motifs include the helical bundle, the  $\beta$  hairpin, the Greek key motif, the jelly roll, the  $\beta$  sandwich and  $\beta$  barrels (Figure 2.11).

The term 'helical bundles' refers to the structural motif consisting of several stretches of  $\alpha$ -helix separated by short bends/loops. The  $\alpha$ -helical elements are usually (though not always) almost fully parallel or antiparallel to each other. The axis of the helical bundle is sometimes twisted, giving the entire bundle a twisted appearance. Helical bundles may also have different numbers of constituent helical stretches. Several haematopoietic cytokines, for example, display a four-helical bundle, while cytochrome C oxidase displays a 22-helical bundle.

The  $\beta$ -hairpin motif (also termed a  $\beta$  ribbon or  $\beta$ - $\beta$  unit) is a simple structural motif consisting of two stretches of  $\beta$  secondary structure connected by a loop. The axis of the resultant  $\beta$  sheet is usually twisted (Fig. 2.11). Hairpin motifs are found in a wide range of polypeptide types. Also common in many polypeptides is the Greek key motif, which is composed of four adjacent antiparallel  $\beta$  strands folded into this characteristic structure (Fig. 2.11). A jelly roll motif is essentially composed of two closely associated Greek key motifs (Fig. 2.11). In this instance,  $\beta$  strands 1, 2, 7 and 8 form the first Greek key motif, while strands 3, 4, 5 and 6 comprise the other. Overall, this forms a nearly fully closed barrel shape.

As the name suggests, a  $\beta$  sandwich structural motif consists of two  $\beta$  sheets packed face to face against each other. Variations of this sandwich structure are also found in some polypeptides. The  $\alpha\beta$  sandwich, for example, consists of a layer of  $\beta$  sheet packed tightly between two  $\alpha$ -helical stretches. This type of motif is often associated with nucleotide-binding proteins.

$\beta$  Barrels are yet another motif type found in a wide variety of polypeptides. These are assemblages of stretches of  $\beta$  strands, separated by loops and

folded into a barrel-like structure. Depending on the polypeptide, the 'barrel' can consist of anything from 5 to 16 individual  $\beta$  strands. A variation of this structure is the  $\alpha/\beta$  barrel which, as the name suggests, is a barrel-like motif composed of alternating  $\alpha$  and  $\beta$  stretches.

Another term often used in the context of protein structure is the protein fold. Some use the term 'fold' and 'structural motif' interchangeably. However, the term 'fold' is more usually reserved for more complex folding patterns which can include the more structurally complex motifs and/or whole protein domains. Tighter definitions of terms including motifs, folds and domains would be a welcome development.

## 2.3 Protein classification on the basis of structure

Polypeptides are most often characterized on the basis of their biological activity/function (e.g. catalytic proteins, transport proteins). An alternative categorization of proteins into groupings or families may be made on the basis of polypeptide sequence similarities, which imply similar structural and/or functional attributes. However, it is now clear that there exists a far greater degree of sequence diversity as opposed to structural diversity in the protein world. There appears to be no more than 1000–1500 different protein folds in existence, which form the building blocks of the tens of millions of proteins in existence. It follows that various different sequences, which in themselves display little or no sequence similarity, can in fact yield very similar higher-order structural elements in proteins. One consequence of this is that sequence-based approaches such as multiple alignments will not identify all proteins displaying homology/functional similarity.

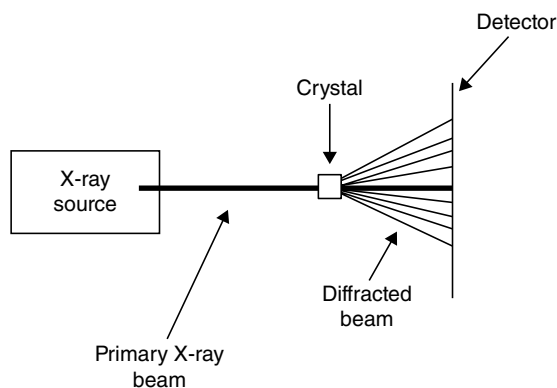
However, there are various protein structural classification databases now in existence, of which the Structural Classification of Proteins (SCOP) database and the CATH structural database are the two best known. Such classifications can also help elucidate an evolutionary relationship between

proteins. Proteins displaying significant similarity in primary sequence and tertiary structure and/or function are classified as belonging to the same protein family. Family members generally display a strong evolutionary relationship. Members of two or more protein families, although displaying little direct sequence similarity, may share considerable higher-order structural and functional similarities. Such families are grouped into superfamilies, and are likely to share an evolutionary relationship, albeit a distant one.

### 2.3.1 Higher structure determination

X-ray diffraction and nuclear magnetic resonance (NMR) are the techniques most widely used to obtain high-resolution protein structural information. Although recent advances in both the analytical equipment available and associated computing power renders these techniques more suited to polypeptide/protein structural analysis, the procedures and pre-requirements necessary for their successful application mean that the full three-dimensional structure of only a relatively modest number of proteins has thus far been elucidated, as compared to protein sequences available.

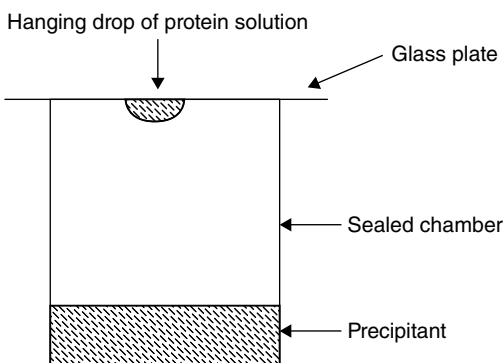
Any structure can be visualized only if electromagnetic radiation of a wavelength comparable to its dimensions is used. In the case of proteins, the appropriate size is of the order of ångströms ( $10^{-10}$  m, 0.1 nm). The wavelength of X-rays approximates to this, hence the use of X-ray diffraction technology. X-ray diffraction entails bombarding a sample of the protein in crystalline form with a beam of X-rays (Figure 2.12). Most of these X-rays pass straight through the crystal but some are diffracted by the electrons of the atoms in the crystal. The resultant diffraction pattern, recorded on a detector, is a reflection of the three-dimensional structure of the protein molecules present in the crystal. Individual atoms are normally distinguishable with a resolution of 1–1.5 Å (0.1–0.15 nm), which provides atomic level structural resolution.



**Figure 2.12** Overview of the principles of X-ray diffraction. Refer to text for details.

The initial prerequisite for protein X-ray diffraction is the generation of protein crystals. Although many lower-molecular-mass substances crystallize relatively easily, this is not the case for the vast majority of globular proteins, which are extremely large and display irregular surfaces. Even if induced to crystallize, protein crystals will contain solvent-filled channels/pores between individual protein molecules. The solvent (usually water) generally occupies 30–80% of crystal volume. Only a small proportion of the surface of individual proteins interact with each other and, as a result, the crystals are soft and relatively easily destroyed.

A number of approaches may be adopted in order to grow protein crystals. Generally, the protein must be very pure in order to crystallize successfully. A crystal forms when protein molecules are precipitated very slowly from supersaturated solutions, and this is usually achieved by vapour diffusion or by dialysis. Vapour diffusion, which is most commonly employed, entails slow concentration of protein molecules in the presence of a suitable precipitant such as polyethylene glycol (PEG) (Figure 2.13). Crystallization will also be affected by factors such as solution pH, the presence of specific buffer salts, metal ions, low-molecular-mass organic molecules, etc. The optimal crystallization conditions must usually be determined by direct experimentation. Crystals suitable for X-ray diffraction must typically have a



**Figure 2.13** Growth of protein crystals by the vapour diffusion (hanging drop) method. A small (20- $\mu$ L) drop of a concentrated purified protein solution containing a suitable precipitant (e.g. polyethylene glycol or ammonium sulfate) is placed on a glass surface. This is subsequently inverted and sealed (e.g. with vacuum grease) to the top of a chamber containing a reservoir of the precipitant. The apparatus is then incubated at a temperature of the order of 22°C, resulting in slow evaporation of water from the protein-containing hanging drop. A supersaturated solution is slowly generated, which is conducive to crystal growth.

minimum diameter of 50  $\mu$ m. Such crystals may contain up to  $10^{16}$  protein molecules.

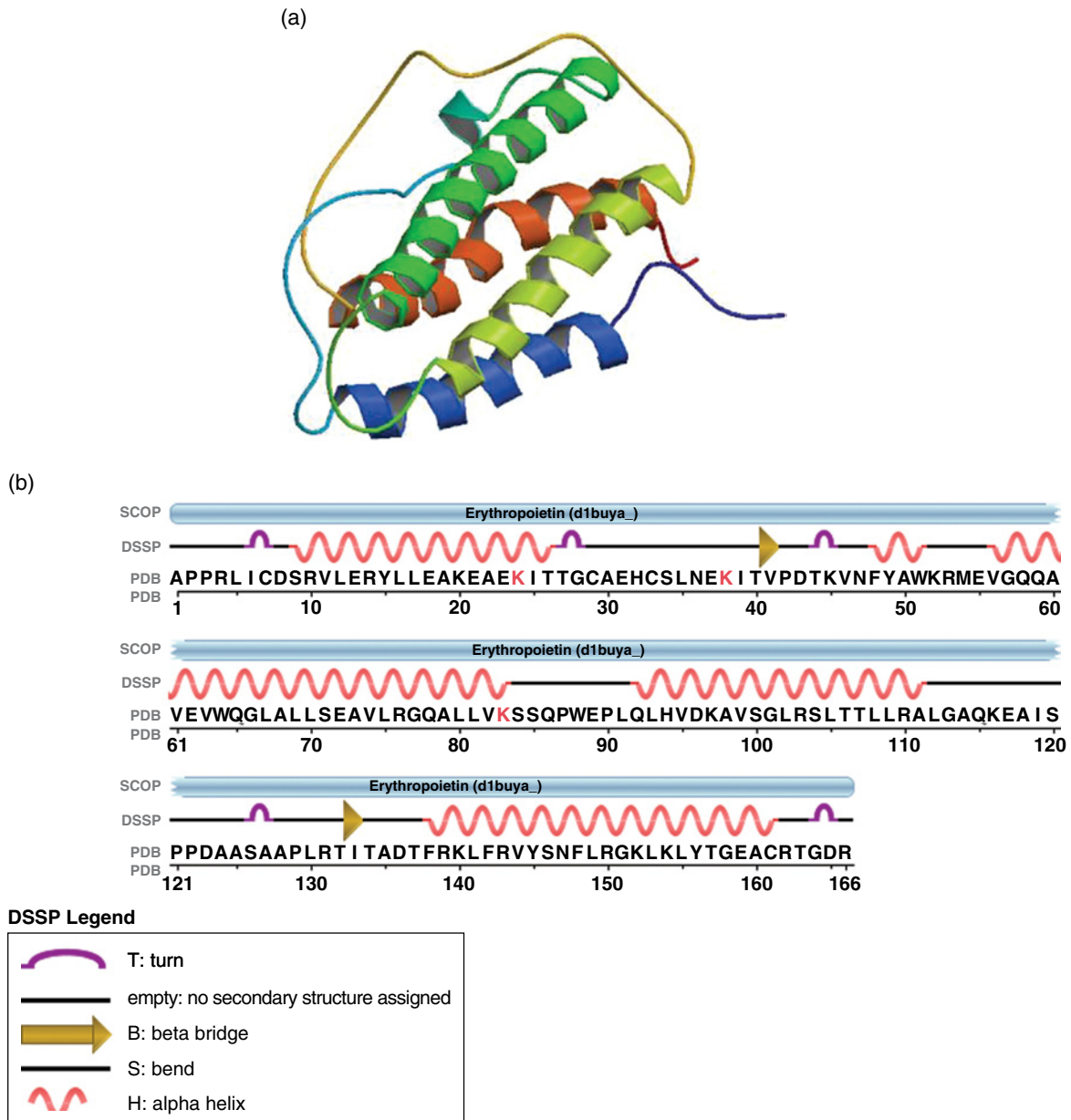
X-rays are generated when a metal plate is bombarded by accelerating electrons. This is normally achieved in high-voltage tubes, but more powerful X-ray beams may be generated in synchrotron storage rings, using electrons travelling close to the speed of light. An X-ray beam is allowed to escape from its source through a narrow window and it then passes through the protein crystal (Figure 2.12). The diffracted spots are recorded on an image plate which is then scanned, and the diffraction pattern is stored on computer. Information from the diffraction pattern may then be analysed by a mathematical expression termed a Fourier transform. This yields information regarding the relative positioning of the atoms present in the protein.

A major bottleneck in the determination of protein structure by X-ray diffraction relates to difficulties encountered in inducing many proteins to

crystallize. NMR may be used to determine the structure of proteins in free solution. However, the complexity of this technique, and particularly the data generated, often limits the use of this approach to relatively small proteins. However, recent technical advances now render practicable the analysis of proteins of 40–50 kDa or more. The solution-based nature of NMR means that the technique generates a range of closely related conformational structures, which largely reflects the fact that protein conformation can flex or ‘breathe’ in solution

NMR analysis involves applying a strong magnetic field to a sample of the protein of interest. Electromagnetic radiation in the radio-frequency range is then applied. NMR analysis is based on the fact that a number of atomic nuclei display a magnetic moment. These include  $^1\text{H}$ ,  $^{13}\text{C}$ ,  $^{15}\text{N}$  and  $^{31}\text{P}$ . This arises because nuclei behave as if they are spinning about an axis. As the above nuclei are positively charged, such spinning nuclei act like tiny magnets and will therefore interact with an applied magnetic field. If a protein is placed in a strong magnetic field, the spin on such nuclei aligns along this field. This alignment can be converted to an excited state if radiofrequency energy of the appropriate frequency is applied. Subsequently, the nuclei revert to their unexcited state, in the process emitting radiofrequency radiation, which may be detected and measured. The exact frequency emitted by any given nucleus is influenced by its molecular environment, and these shifts in frequency emitted can be used to provide three-dimensional structural information about the protein.

Experimentally determined protein three-dimensional structural information is stored in various databases as sets of atomic coordinates. The principal such database is the protein databank (PDB; [www.pdb.org](http://www.pdb.org)), which currently houses almost 100,000 resolved protein structures. However, the database does display considerable redundancy in that many proteins are represented by multiple entries (e.g. different isolated domain entries or the same protein/protein domain bound to different ligand molecules). PDB also houses/contains links to



**Figure 2.14** Three-dimensional structure of human erythropoietin (EPO) as deposited in the PDB (entry 1BUY). As well as the actual three-dimensional structure (a), entries contain additional information about proteins, including details of primary and secondary structure (b).

various molecular visualization software programs. The three-dimensional structure of human erythropoietin (EPO), as deposited in the PDB, is depicted in Figure 2.14. The site also contains additional structural information

relating to deposited structures, including sequence and secondary structural information (Figure 2.14b). Many databases such as SCOP and CATH base their structural classification schemes on PDB data.

## 2.4 Protein structural stability

After biosynthesis, a polypeptide folds into its native conformation (overall tertiary structure) that is structurally stable and functionally active. The conformation adopted ultimately depends on the polypeptide's amino acid sequence and arises as a result of:

- folding constraints placed on the polypeptide by the types, positioning and extent of secondary structural elements present therein, which tend to form first;
- interactions between amino acid residues, some of which may be far apart from each other in terms of amino acid sequence, but which are brought into close proximity by protein folding.

The major stabilizing forces of a polypeptide's overall conformation are:

- hydrophobic interactions;
- electrostatic attractions;
- covalent linkages.

Hydrophobic interactions are the single most important stabilizing influence on protein native structure. The 'hydrophobic effect' refers to the tendency of non-polar substances to minimize contact with a polar solvent such as water. Non-polar amino acid residues constitute a significant proportion of the primary sequence of virtually all polypeptides. These polypeptides will fold in such a way as to maximize the number of such non-polar residue side chains buried in the polypeptide's interior, away from the surrounding aqueous environment. This situation is most energetically favorable.

Stabilizing electrostatic interactions include van der Waals' forces (which are relatively weak), hydrogen bonds and ionic interactions. Although nowhere near as strong as covalent linkages (Table 2.3), the large number of such interactions existing within a polypeptide renders them collectively quite strong.

While polypeptides display extensive networks of intramolecular hydrogen bonds, such bonds do not contribute very significantly to overall conformational stability. This is because atoms that hydrogen bond with each other in a folded polypeptide can form energetically equivalent hydrogen bonds with water molecules if the polypeptide is in the unfolded state. Ionic attractions between (oppositely) charged amino acid side chains also contribute modestly to overall protein conformational stability. Such linkages are termed 'salt bridges' and, as one would expect, they are located primarily on the polypeptide surface.

Disulfide bonds represent the major type of covalent bond and can help stabilize a polypeptide's native three-dimensional structure. Intracellular proteins, although generally harbouring multiple cysteine residues, rarely form disulfide linkages due to the reducing environment which prevails within the cell. Extracellular proteins in contrast are usually exposed to a more oxidizing environment, conducive to disulfide bond formation. In many cases the reduction (i.e. breaking) of disulfide linkages has little effect on the native conformation of polypeptides. However, in other cases (particularly disulfide-rich proteins) disruption of this covalent linkage does render the protein less conformationally stable. In these cases the disulfide linkages likely serve to 'lock' functional/structurally important elements of domain/tertiary structure in place.

For a typical globular polypeptide the total of all the bonding energies associated with stabilizing interaction (Table 2.3) sums to several thousand kilojoules per mole. Thermodynamic analysis, however, shows that the free energy difference between a typical 200-residue polypeptide in its folded

**Table 2.3** Approximate bond energies associated with various (non-covalent) electrostatic interactions, as compared with a carbon-carbon single bond.

Bond type	Bond strength (kJ/mol)
Van der Waals' forces	10
Hydrogen bond	20
Ionic interactions	86
Carbon-carbon bond	350

versus denatured form is only of the order of 80–100 kJ/mol. This equates to just a few hydrogen bond equivalents. In the unfolded state, intrachain non-covalent (stabilizing) interactions are not maximized, but some new such interactions are formed which can ‘stabilize’ the denatured state. These include the aforementioned extensive hydrogen bonding between appropriate amino acid groups and surrounding water molecules. Moreover, the second law of thermodynamics states that it is more energetically favourable for a molecule to exist in a random order as opposed to a highly ordered state (the concept of entropy). The fact that proteins are only marginally more stable in their folded form is functionally significant. Structurally this renders proteins somewhat flexible, enabling them to more readily undergo various conformational changes central to their biological activity.

The description of protein structure as presented thus far may lead to the conclusion that proteins are static, rigid structures. This is not the case. A protein’s constituent atoms are constantly in motion and groups ranging from individual amino acid side chains to entire domains can be displaced via random motion by up to about 0.2 nm. A protein’s conformation therefore displays a limited degree of flexibility and such movement is termed ‘breathing’.

Breathing can sometimes be functionally significant by, for example, allowing small molecules to diffuse in or out of the protein’s interior. In addition to breathing some proteins may undergo more marked (usually reversible) conformational changes. Such changes are usually functionally significant. Most often they are induced by biospecific ligand interactions (e.g. binding of a substrate to an enzyme or antigen binding to an antibody). In this context, I will also discuss a special type of protein whose structure is intrinsically disordered in section 2.7.

The factors influencing the intrinsic stability of native polypeptide conformation have largely been elucidated via the study of proteins which function under relatively mild environmental conditions. More recently the study of proteins derived from extremophiles (organisms living under extreme environmental conditions, see Chapter 11) has further

extended our understanding of conformational stability. The three-dimensional structure of a number of homologous proteins derived from various psychrophiles, mesophiles, thermophiles and hyperthermophiles (Chapter 11) have now been determined. This facilitates the identification of changes in structural features that help render the protein stable under its particular native physiological conditions. Thermodynamic analysis reveals that the principle of marginal stability between the native versus denatured state extends to proteins isolated from such extreme environments.

One might expect that proteins isolated from thermophiles/hyperthermophiles would exhibit an increased level of intramolecular stabilizing interactions, in order to compensate for the destabilizing influence of elevated temperature. Conversely, it could be predicted that in order to remain at the appropriate degree of conformational flexibility, proteins from psychrophiles would display decreased levels of such stabilizing interactions. In broad terms these principles have been borne out, although the methods by which this is achieved differs for different proteins and the overall picture is by no means complete. Increased thermal stability is generally related to one or more of the following structural adaptations:

- an increase in the number of intramolecular polypeptide hydrogen bonds;
- an increase in the number of salt bridges;
- increased polypeptide compactness (improved packing of the hydrophobic core);
- extended helical regions.

Conversely, enhanced stability/functional flexibility of proteins derived from psychrophiles appears to be achieved by one or more of the following adaptations:

- fewer salt links;
- reduced aromatic interactions within the hydrophobic core (reduction in hydrophobicity);
- increased hydrogen bonding between the protein surface and the surrounding solvent;
- occurrence of extended surface loops.

## 2.5 Higher-order structure prediction

Given the complexities of resolving three-dimensional structure, it is not surprising that scientists are continually attempting to develop methods by which higher-order structure can be predicted from amino acid sequence data.

### 2.5.1 Secondary structure prediction

Over 20 different methods of secondary structure prediction have been reported (Table 2.4). Traditionally these approaches fall into two main categories.

1. Empirical statistical methods based on data generated from studying proteins of known three-dimensional structure and correlation of primary amino acid sequence of such proteins with structural features.
2. Methods based on physicochemical criteria such as fold compactness (i.e. the generation of a folded form displaying a tightly packed hydrophobic core and a polar surface).

The Chou and Fasman method represents one of the most popular of these traditional predictive methods. These scientists studied structural details of a number of proteins whose three-dimensional structure had been experimentally determined by X-ray

**Table 2.4** Some secondary structure predictive methods.

Method	Basis of prediction
Chou and Fasman	Empirical statistical method
Garnier, Osguthorpe and Robson (GOR) method	Empirical statistical method
EMBL profile neural network (PHD) method	Empirical statistical method
Protein sequence analysis (PSA) method	Empirical statistical method
Lim method	Physicochemical criteria

crystallography. This allowed them to construct 'conformational preference data' for each amino acid residue, which essentially relates the compatibility of the amino acid to  $\alpha$ -helical or  $\beta$  stretches (Table 2.5). By applying the preference data to the amino acid sequence of a polypeptide, putative  $\alpha$ -helical or  $\beta$ -strand regions can be assigned.

The analysis carried out by Chou and Fasman also allowed the following observations to be made.

- An  $\alpha$ -helical stretch is usually initiated by a six-residue sequence containing at least four H $\alpha$  or h $\alpha$  residues (Table 2.5).

**Table 2.5** Conformational preferences and assignments of amino acid residues with regard to stretches of  $\alpha$ -helix and  $\beta$  structure.

$\alpha$ -helix			$\beta$ strand		
Residue	P $\alpha$	Assignment	Residue	P $\beta$	Assignment
Glu	1.44	H $\alpha$	Val	1.64	H $\beta$
Ala	1.39	H $\alpha$	Ile	1.57	H $\beta$
Met	1.32	H $\alpha$	Thr	1.33	h $\beta$
Leu	1.30	H $\alpha$	Tyr	1.31	h $\beta$
Lys	1.21	h $\alpha$	Trp	1.24	h $\beta$
His	1.12	h $\alpha$	Phe	1.23	h $\beta$
Gln	1.12	h $\alpha$	Leu	1.17	h $\beta$
Phe	1.11	h $\alpha$	Cys	1.07	h $\beta$
Asp	1.06	h $\alpha$	Met	1.01	I $\beta$
Trp	1.03	I $\alpha$	Gln	1.00	I $\beta$
Arg	1.00	I $\alpha$	Ser	0.94	i $\beta$
Ile	0.99	i $\alpha$	Arg	0.94	i $\beta$
Val	0.97	i $\alpha$	Gly	0.87	i $\beta$
Cys	0.95	i $\alpha$	His	0.83	i $\beta$
Thr	0.78	i $\alpha$	Ala	0.79	i $\beta$
Asn	0.78	i $\alpha$	Lys	0.73	b $\beta$
Tyr	0.73	b $\alpha$	Asp	0.66	b $\beta$
Ser	0.72	b $\alpha$	Asn	0.66	b $\beta$
Gly	0.63	B $\alpha$	Pro	0.62	B $\beta$
Pro	0.55	B $\alpha$	Glu	0.51	B $\beta$

P $\alpha$ , propensity to form  $\alpha$ -helical regions; P $\beta$ , propensity to form  $\beta$  stretches; H $\alpha$ , strong helix former; h $\alpha$ , helix former; I $\alpha$ , weak helix former; i $\alpha$ , indifferent; b $\alpha$ , helix breaker; B $\alpha$ , strong helix breaker. Similar designations are used in the case of  $\beta$  formers, with 'b' replacing ' $\alpha$ '.

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- Proline residues, if present, are located at the amino terminus of the helix.
- Any group of four successive residues present in an  $\alpha$ -helix will have an average  $P\alpha$  value greater than 1.0 (Table 2.5).
- A  $\beta$  stretch is usually initiated by a five-residue sequence containing at least three  $H\beta$  or  $h\beta$  residues.
- Any group of four successive residues present in a  $\beta$  stretch will have an average  $P\beta$  value greater than 1.0.

Most such traditional predictive methods are at best 50–70% accurate. An indication of the accuracy may be obtained by comparing the predicted and actual percentage of various secondary structures in a range of proteins whose structures have actually been resolved. Some of the more recently developed programs also take into consideration multiple sequence alignment data but even the most modern programs usually achieve at best 70–75% accuracy. A range of such programs (e.g. APSSP, CFSSP, GOR, J Pred, Prof and SOPMA) are available via the ExPASy home page (see Box 1.1) and can be accessed by following the links pathway: ExPASy home page > proteomics > protein structure.

### 2.5.2 Tertiary structure prediction

Accurate prediction of a protein's three-dimensional structure is a still more complex problem. However, the fact that the architecture of all proteins is largely based on a limited number of building blocks (protein folds) helps in the development of such predictive tools. Moreover, as the number of proteins whose three-dimensional structure is resolved increases, associated bioinformatic analysis will continue to build a better picture of the range of amino acid sequences that can ultimately support the formation of specific protein folds.

Currently, three different approaches may be adopted in an attempt to predict the three-dimensional structure of a polypeptide from primary sequence data:

- comparative modelling;
- fold recognition approaches;
- *ab initio* structural prediction.

Homology modelling (comparative modelling) is applied when the target protein shares substantial sequence similarity to proteins whose three-dimensional structure has already been experimentally established. In this approach initial homology searches are undertaken using tools such as BLAST. Resolved structural details of homologous proteins can then be identified using structural databases such as PDB and CATH, allowing identification of conserved structural regions, as well as more variable regions. These provide a structural template with which the query sequence can be aligned, allowing a model of the target protein to be built. The accuracy of the predicted structure is closely related to the percentage amino acid identity shared by the query protein and its template. If sequence identity stands at 50% or greater, the predicted structure is usually quite accurate. Accuracy declines with decreasing percentage identity, particularly if it falls below about 30%.

Fold recognition approaches (also called threading) are based on the fact that proteins can share characteristic folds even if they are not homologous. Essentially the process entails 'threading' the target sequence (or subsets thereof) onto different known folds, while using software tools to evaluate likely compatibility of the sequence to the fold in question.

*Ab initio* (*de novo*) structure prediction is, understandably, the most high-risk approach to structure prediction and is applied in cases where the target sequence lacks detectable homology to any protein of known structure. One common approach to *ab initio* prediction entails comparing short (nine amino acid) sequence fragments of the target protein to resolved protein structures.

A range of protein structural prediction tools (e.g. CPHmodels, ESYPred3D, HHpred and Phyre2) are available via the ExPASy home page (see Box 1.1), and can be accessed by following the links pathway: ExPASy home page > proteomics > protein structure.

## 2.6 Protein folding

The above discussion focuses on the factors stabilizing the fully folded protein structure. In this section I focus on the folding event itself. Thermodynamically, a protein folds from a higher energy unfolded state to



a lower energy folded state. This process is usually a rapid one, often lasting from under 1 second to several seconds. The speed of folding indicates that this process occurs via a directed pathway rather than via random conformational searches until stumbling on the most stable structural arrangement.

The exact detail of protein folding is complex and incompletely understood. For example, protein folding pathways are likely to differ, in some aspects at least, depending on the complexity of the protein (e.g. single domain vs. multidomain vs. multi-subunit proteins and if the protein is subject to post-translational modifications), as well the synthesizing cell type (prokaryotic vs. eukaryotic) and specific cellular microenvironmental conditions, for example pH, solute concentrations, protein crowding, and whether folding occurs in the cytoplasm or (in eukaryotes) at least partly in the endoplasmic reticulum.

For most proteins the folding pathway appears to proceed via the initial rapid formation of the more compact, partially folded 'molten globule' followed by completion of the folding pathway at a slower pace (Figure 2.15). The molten globule exhibits most secondary structural elements of the native protein, but only a limited degree of ultimate tertiary structure. Its formation, which requires only several milliseconds, is termed a hydrophobic collapse. As the name suggests, this process is primarily driven by the favourable energy change achieved by bringing hydrophobic amino acid residues into contact with one another and away from surrounding water molecules. Hydrophobic collapse in turn drives secondary structure formation; the internalization of hydrophobic residues also internalizes the polar N—H and C=O groups of their associated peptide

bonds. This prevents the latter groups from forming stabilizing hydrogen bonds with surrounding water molecules. However, this energetically unfavourable situation can be counteracted if these groups are allowed to hydrogen bond with each other in a situation maximized by the formation of  $\alpha$ -helical or  $\beta$  stretches.

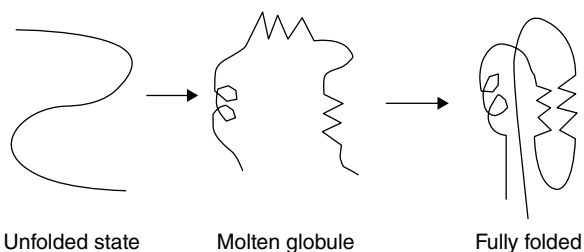
In the subsequent and final phase of protein folding, elements of tertiary structure form. Initially, these are most likely motifs/subdomains, which in turn lead to complete folding. In the case of large multidomain proteins, domains near the protein's N-terminus, being synthesized first, may be folded in part or in full before the entire protein is synthesized. While some proteins likely follow a single folding pathway others appear to have multiple pathways, which all ultimately lead to complete protein folding.

Many proteins can fold spontaneously *in vitro*, although some appear to fold more slowly/less accurately than they do *in vivo*. Although the primary sequence ultimately dictates tertiary structure, several obstacles to correct folding exist, including:

- aggregation of partially folded intermediates via intermolecular hydrophobic interactions;
- isomerization of proline residues;
- formation of disulfide linkages between incorrect pairs of cysteine residues.

Cells appear to have evolved various mechanisms by which such obstacles can be minimized or overcome.

Molecular chaperones are a class of protein which help polypeptides to fold into their correct native three-dimensional shape by preventing or correcting the occurrence of improper hydrophobic associations. Chaperones tend to slow down the folding process. They were first termed 'heat-shock proteins' (Hsp) as their intracellular concentration increases at elevated temperatures. Two major classes of chaperones are now known to exist: Hsp70 proteins and chaperonins. Both contain hydrophobic regions, which can interact with unfolded/misfolded polypeptides, helping them to fold correctly in an ATP-dependent process.



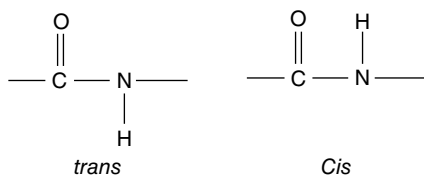
**Figure 2.15** Overview of the protein folding pathway. Refer to text for details.

The chaperonins of *Escherichia coli* have been studied in most detail. These consist of a large complex constructed from two protein types: the 60-kDa Hsp60 protein (known as GroEL) and the 10-kDa Hsp10 protein (known as GroES). The intact structure consists of 14 subunits of GroEL, which form a long hollow cylinder approximately 15 nm long and 14 nm in diameter; seven subunits of GroES interact and bind to one end of the cylinder, closing off the cavity at that end. The internal walls of the cavity display several regions rich in hydrophobic residues.

The intact chaperone will interact directly with unfolded/misfolded regions of polypeptide chains, but not with properly folded regions. The recognition mechanism is not fully understood but hydrophobic interactions between the presumably large exposed hydrophobic patches in unfolded/improperly folded regions and those in the internal surface of the chaperonin cavity likely play a central role. The exact molecular detail of how proper folding is subsequently assisted remains to be elucidated.

Peptidyl prolyl isomerases and protein disulfide isomerases also assist the efficient and proper folding of many proteins *in vivo*. The peptide bonds of polypeptide chains are generally in the *trans* conformation, as previously described. The *cis* conformation (Figure 2.16), although possible, is far less thermodynamically stable and hence rarely occurs in native polypeptides. In some polypeptides, however, a peptide bond in which the N—H group is derived from a proline residue does sometimes exist in the *cis* form. This often occurs at tight bends, and in such instances is required for structural flexibility. This *cis* form is stabilized by surrounding elements of three-dimensional structure. Prior to folding these stabilizing elements therefore do not exist.

In the unfolded state, an equilibrium between *cis* and *trans* forms of peptide bonds exist, but due to the latter's stability the equilibrium greatly favours the



**Figure 2.16** *Cis* versus *trans* forms of a peptide bond.

*trans* form. Unaided *trans*–*cis* isomerization of target proline residues is thus a slow process and can be rate limiting to folding. Peptidyl prolyl isomerases are found in most cell types and can enhance the rate of isomerization of such proline-based peptide bonds by rates of over  $10^6$ . These enzymes can therefore eliminate this rate-limiting step to folding *in vivo*.

The folding of proteins which contain disulfide linkages can pose special problems for the cell – that of ensuring the correct pairing of cysteine residues in such linkages. This process is assisted by protein disulfide isomerases. These enzymes themselves have free thiol (SH) groups derived from constituent cysteine residues. These groups can form transient disulfide linkages with cysteine residues present in the target protein. As such they can assist in the intramolecular rearrangements of incorrectly formed disulfide linkages.

## 2.7 Intrinsically disordered proteins

Conventional wisdom dictates that a well-defined three-dimensional structure is a prerequisite to protein function. It is not uncommon for proteins to display short relatively flexible sequences along the polypeptide backbone but it has become increasingly obvious over the past two decades that some proteins display complete or near-complete intrinsic structural disorder, while a significant number of proteins display surprisingly long stretches (>30 amino acid residues) that are intrinsically disordered. This can be experimentally investigated using NMR (which evaluates protein structure in solution, providing not a single defined structure but a range of structural variants). Bioinformatic analysis also reveals that intrinsically disordered (i.e. unstructured) regions have characteristic sequence signatures, including the presence of:

- low sequence complexity;
- low content of bulky hydrophobic amino acid residues;
- a high content of polar and charged amino acid residues.

These characteristics help explain the unstructured nature of the protein, as a primary step in protein folding appears to be hydrophobic collapse (see section 2.6). Moreover, many such disordered regions display a high degree of conservation, which has led to the development of several bioinformatic tools that can predict likely intrinsically disordered proteins or regions within proteins. The ExPASy bioinformatics portal houses links to several such tools. Moreover, a database housing details of proteins displaying unstructured regions in part or in entirety houses some 700 entries ([www.disprot.org](http://www.disprot.org)).

Sequence conservation also implies functional significance and many proteins involved in eukaryotic signal transduction and associated with cancer have an increased propensity for intrinsic disorder. Specific cellular processes in which intrinsically disordered proteins participate include the regulation of transcription, signal transduction, protein phosphorylation and the regulation of self-assembly of multiple protein complexes. Moreover, binding of these proteins to one or more ligands can induce their adoption of a specific conformation. The functional significance and molecular mechanism of action of these intriguing proteins is only beginning to be understood.

## 2.8 Protein engineering

Protein engineering describes the intentional alteration of a protein's amino acid sequence, usually with the aim of achieving either:

- a better understanding of the relationship between a protein's primary and higher-level structure, or its structure and function; or
- the development of a protein variant which, relative to the wild-type protein, displays some enhanced property in the context of its commercial use.

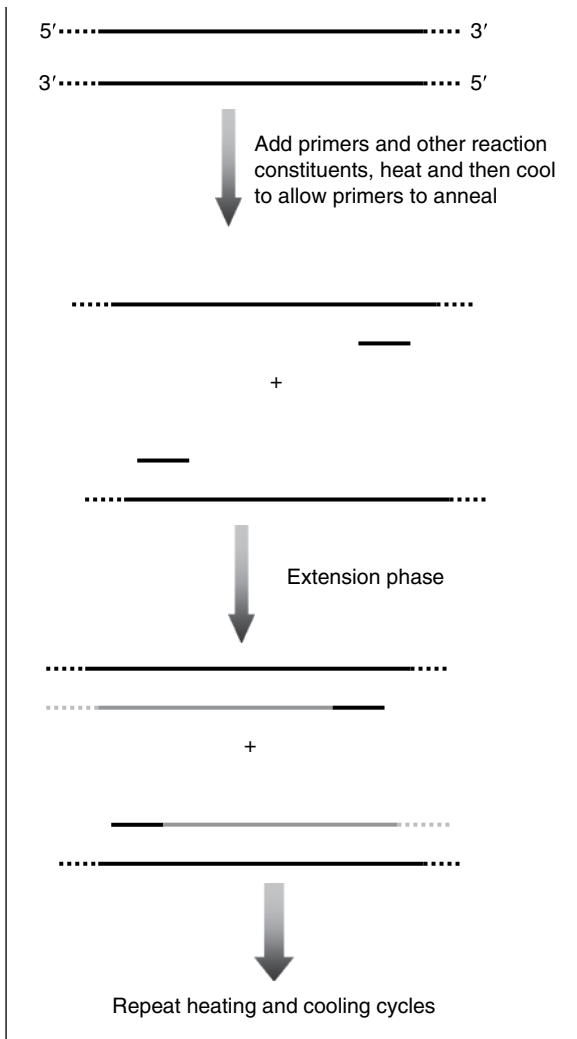
Protein engineering is facilitated by bioinformatics and molecular biology, in particular PCR (which is used to clone a specific nucleotide sequence such as one coding for a specific protein; Box 2.3). Many

such engineered proteins are now used commercially (Table 2.6). Protein engineering may be pursued via site-directed mutagenesis or directed evolution, or a combination of both.

### Box 2.3 PCR: an overview

The polymerase chain reaction (PCR) is a technique that allows the copying (amplification) of a selected DNA sequence, such as a gene or cDNA which codes for a specific protein. The process is initiated by the separation of the double-stranded DNA into its two constituent strands. This is achieved by heating the sample (usually to a temperature of 94°C). Also present in the reaction mixture are (i) two chemically synthesized oligonucleotide primers ('oligos') whose sequences are complementary to the sequences flanking the gene of interest; (ii) the enzyme DNA polymerase, which can extend the primers to synthesize a new DNA strand of complementary sequence to the single-stranded DNA template; and (iii) all the nucleoside precursors required for synthesis of the growing DNA strand (i.e. the deoxynucleoside triphosphates or dNTPs).

Once strand separation has been achieved, the reaction temperature is reduced in order to allow primers to anneal to complementary sequences on each strand and allow the DNA polymerase to extend the primers. This extension phase is normally carried out at 72–74°C. The DNA polymerase used is sourced from thermophilic microorganisms such as *Thermus aquaticus*, and is therefore heat stable and not inactivated by PCR operational temperatures (see also Chapter 13). This completes the first cycle of the PCR process and it has resulted in a doubling of the amount of target DNA present in the reaction mixture. The cycle is then repeated and with each repeat comes a doubling of the amount of target DNA. After 25–30 cycle repeats, several hundred million copies of the target DNA have been generated.



### 2.8.1 Site-directed mutagenesis

Site-directed mutagenesis facilitates the introduction of specific predefined sequence alterations into a protein's backbone (Box 2.4). Such alterations can include the insertion, deletion or replacement of either a single or multiple amino acid residues.

The approach can be useful in identifying if a particular residue (or indeed sequence) is important in the context of some aspect of protein structure and/or function. This can be achieved by, for example, removing the target amino acid

altogether or (more commonly) replacing it with a different amino acid residue. Alanine (alanine scanning) is often used in this context as its side chain is non-bulky, non-charged, chemically unreactive and is compatible with the formation of various structural motifs. Such engineering can be undertaken 'blind' via systematic replacement of all (or a defined subset) of backbone residues, or can be more targeted (e.g. the replacement of a specific amino acid believed to be within an enzyme's active site in order to more effectively map the active site).

If the function (or a functional consequence such as increased immunogenicity) of a particular amino acid/amino acid sequence is already known (or suspected via for example multiple alignment studies), then site-directed mutagenesis can provide an effective approach to alter or tailor that characteristic. Thus, for example, many modern detergent enzymes have been rendered oxidation resistant via the replacement of oxidation-sensitive methionine residues on their surface (Table 2.6 and see Chapters 11 and 12).

### 2.8.2 Directed evolution

Directed evolution entails introducing random mutations into the gene coding for the protein of interest, thus generating a large library of gene variants. This process is known as diversification. The library of variants is expressed and screened to identify variants displaying enhanced target characteristics (e.g. increased functional efficacy, increased stability). The gene coding for the desired variant is then cloned and sequenced in order to identify the exact amino acid alterations present. This variant gene can then be subject to further rounds of such directed evolution. Diversification is usually achieved in practice using a technique termed error-prone PCR.

With standard PCR applications (Box 2.3) it is desirable to use a DNA polymerase (see also Chapter 13) and PCR reaction conditions that ensure high-fidelity DNA replication. For error-prone PCR applications the opposite is desirable.

**Table 2.6** Representative engineered proteins which are now used commercially. These and other examples are considered in later chapters of this book.

Protein	Use	Engineering detail	Chapter
Tissue plasminogen activator (tPA)	Thrombolytic agent	Various engineered products developed with altered amino acid sequences or with whole domains deleted in order to make clot degradation more efficient or lengthen serum half-life	6
Antibodies	Various, including cancer treatment	Various engineered products developed, including mouse-derived antibodies in which large segments have been replaced by human antibody domains (in order to reduce immunogenicity in humans), or the development of antigen-binding antibody fragments (which could for example penetrate tumours more effectively)	7
Fusion proteins	Various, including treating rheumatoid arthritis and cancer	Generation of novel hybrid proteins by combining one or more domains from two different proteins together. The fusion product 'Enbrel' for example consists of the extracellular domain of the tumour necrosis factor (TNF) receptor (allowing it bind TNF), fused to antibody constant (Fc) domains (which increases its serum half-life)	7
Engineered insulins	Diabetes	The replacement/alteration of amino acids in the insulin backbone in order to make the engineered product either faster-acting or slower-acting than un-engineered insulin	8
Various detergent proteases and amylases	Added to detergents to enhance cleaning	Removal/replacement of oxidation-sensitive amino acid residues, allowing the enzymes to retain activity in the presence of oxidants usually also present in detergents	12
DNA polymerase	PCR reactions	Enhances enzyme's affinity for DNA	13

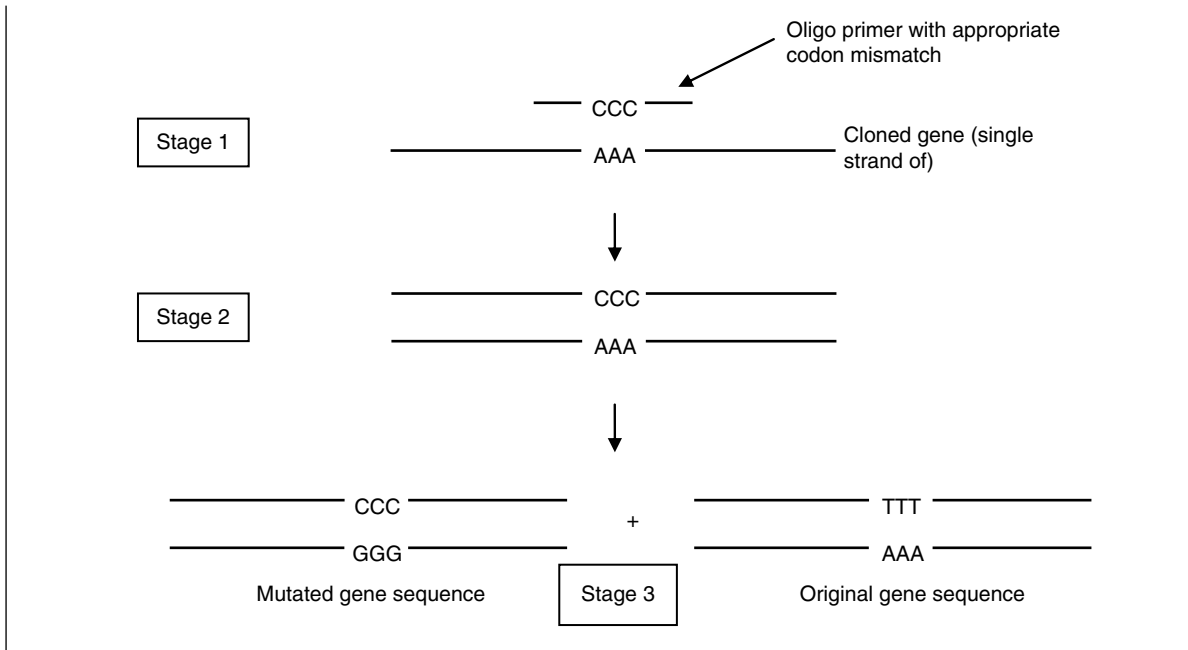
**Box 2.4** Alteration of amino acid sequence by site-directed mutagenesis

A variety of molecular approaches may be used to introduce a substitution, short insertion or short deletion into a protein's amino acid sequence via site-directed mutagenesis. Most are loosely based on the technique of PCR (reviewed in Box 2.3).

One approach involves initial cloning of the gene/cDNA coding for the wild-type (unaltered) protein. Next, an appropriate oligonucleotide ('oligo') is designed and chemically synthesized. The two end segments of the oligo are complementary in sequence to either side of the section of the cloned gene one wishes to alter. Its central portion, however, displays an altered nucleotide sequence corresponding to the alteration required. For example, in the case

illustrated diagrammatically below, a single amino acid substitution is desired – a change from lysine (corresponding to AAA in the gene sequence) to proline (corresponding to GGG on the coding strand). On annealing, the deliberately mismatched bases will 'loop out', but the sequences flanking each side will anneal fully via complementary base pairing.

The addition of DNA polymerase and dNTPs facilitates DNA synthesis (stage 2 in the diagram). Both strands can be replicated (stage 3 in the diagram). The mutant gene can then be recovered and cloned into an appropriate expression vector, allowing the production of large amounts of the desired mutant protein.



Thus a DNA polymerase such as Taq polymerase, which lacks proofreading capability (the ability to autocorrect insertional mistakes) is used. Moreover, PCR reaction conditions are controlled in order to reduce replication fidelity still further. This often entails the replacement of the polymerase's natural cofactor ( $Mg^{2+}$ ) with  $Mn^{2+}$  and including suboptimal relative concentrations of dNTPs in the reaction mixture (Box 2.3). Optimized error-prone protocols typically introduce error rates in the region of one to three base-pair substitutions per kilobase of DNA.

Error-prone PCR tends to introduce random point mutations in a protein's backbone. DNA shuffling represents a complementary technique which can potentially facilitate the recombination of several individual beneficial mutations from different mutants into a single protein. The process entails fragmentation of several mutant genes, generally into lengths in the region of 50 base pairs. The fragment mix is then subject to PCR, but in the absence of a primer. Overlapping fragments will anneal with each other, allowing eventual extension to full gene sequence size. The recombined genes can be cloned, expressed and screened for improved target characteristics.

## 2.9 Protein post-translational modification

Thus far in this chapter I have focused exclusively on the amino acid backbone of proteins. However, many polypeptides undergo covalent modification, either during or after their ribosomal synthesis, giving rise to the concept of co-translational and post-translational modification (generally simply called post-translational modification). PTMs are characteristic particularly of eukaryotic proteins and are generally introduced by specific enzymes or enzyme pathways. Many occur at the site of a specific characteristic protein sequence (signature sequence) within the protein backbone. This has allowed the development of bioinformatic tools capable of identifying potential PTM sites along a protein's backbone via sequence analysis. Moreover, many such PTMs will introduce a predefined mass difference into the affected polypeptide. Additional bioinformatic tools have thus been developed that can interrogate peptide mass fingerprinting (MS) data for mass differences between actual peptide fragments experimentally generated from a protein and theoretical peptides in databases in an effort to

identify the occurrence of specific PTMs. Indeed MS-based analysis has become a central tool in PTM analysis. Many PTM-focused bioinformatic tools are available via the ExPASy home page and can be accessed by following the links pathway: ExPASy home page > proteomics > post-translational modification.

Several hundred PTMs have been recorded, and these modifications invariably influence some aspect of a protein's structure or function. However, many such PTMs occur only rarely and their biology remains to be fully elucidated. Some of the more common PTMs are listed in Table 2.7. Some such PTMs are mainly/exclusively associated with intracellular proteins (e.g. phosphorylation and acetylation) while others are invariably associated only with extracellular proteins (e.g. disulfide linkages and glycosylation).

From an applied perspective, the majority of proteins approved for therapeutic use (biopharmaceuticals) are subject to one or often a combination of PTMs, and the PTM component can profoundly affect their therapeutic properties (see Chapters 6–9). As outlined in Chapter 3, the vast majority of such proteins are now produced by recombinant means, and the necessity for such proteins to exhibit

PTMs compatible with human biology often dictates which recombinant expression systems are suitable or unsuitable for protein production.

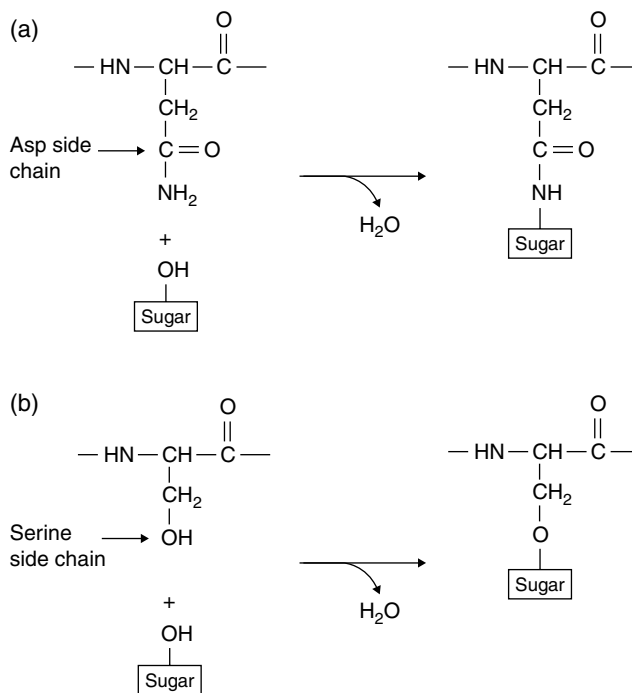
### 2.9.1 Glycosylation

Glycosylation (the attachment of carbohydrates) is one of the most common forms of PTM associated with eukaryotic proteins, particularly extracellular and cell-surface proteins. Limited protein glycosylation can also be undertaken by some bacteria. Two types of glycosylation generally occur: N-linked and O-linked. In the case of N-linked glycosylation, the sugar chain (the oligosaccharide) is attached to the protein via the nitrogen atom of an asparagine (Asn) residue, while in O-linked systems the sugar chain is attached to the oxygen atom of hydroxyl groups, usually those of serine or threonine residues (Figure 2.17).

Monosaccharides most commonly found in sugar side chain(s) include mannose, galactose, glucose, fucose, *N*-acetylgalactosamine, *N*-acetylglucosamine, xylose and sialic acid. Sialic acids are derivatives of the nine-carbon monosaccharide neuraminic acid, the most common one being *N*-acetylneuraminic

**Table 2.7** The more common forms of post-translational modifications that polypeptides may undergo. Refer to text for additional details.

Modification	Comment
Glycosylation	For some proteins glycosylation can increase solubility, influence biological half-life and/or biological activity
Proteolytic processing	Various proteins become biologically active only on their proteolytic cleavage (e.g. some blood factors)
Phosphorylation	Influences/regulates biological activity of various regulatory proteins including polypeptide hormones
Acetylation	Modulation of target protein activity
Acylation	May help some polypeptides interact with/anchor in biological membranes
Amidation	Influences biological activity/stability of some polypeptides
Sulfation	Influences biological activity of some neuropeptides and the proteolytic processing of some polypeptides
Hydroxylation	Important to the structural assembly of certain proteins
$\gamma$ -Carboxyglutamate formation	Important in allowing some blood proteins to bind calcium
ADP-ribosylation	Regulates biological activity of various proteins
Disulfide bond formation	Helps stabilize conformation of some proteins



**Figure 2.17** (a) N-linked versus (b) O-linked glycosylation. 'Sugar' represents an oligosaccharide chain, an example of which is provided in Figure 2.18.

acid. These monosaccharides can be joined together in various sequences and by a variety of glycosidic linkages. The carbohydrate chemistry of glycoproteins is therefore quite complex. The structure of two such oligosaccharide chains is presented in Figure 2.18.

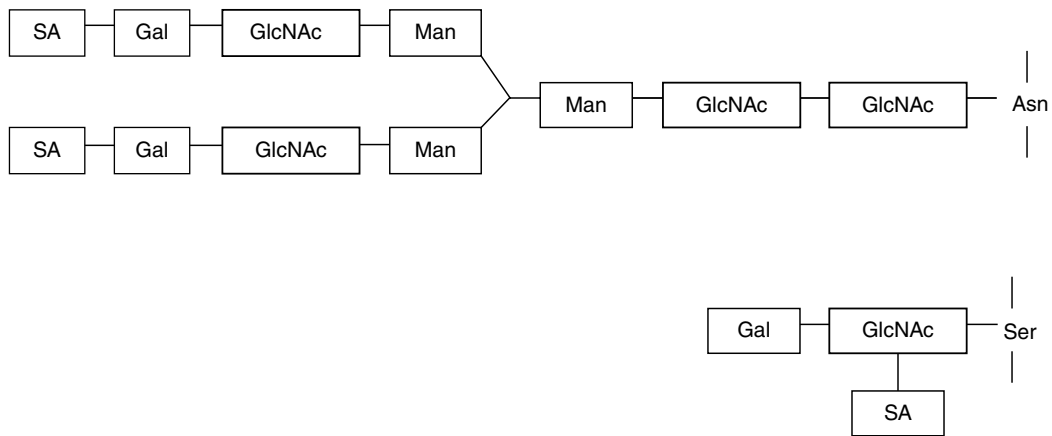
N-linked glycosylation is sequence specific, involving the transfer of a presynthesized oligosaccharide chain to an Asn residue found in a characteristic sequence Asn-X-Ser, Asn-X-Thr or Asn-X-Cys, where X represents any amino acid residue with the exception of proline. An additional glycosylation determinant must also apply, as not all potential N-linked sites are glycosylated in some proteins. The determinants of O-linked glycosylation are less well understood. Characteristic sequence recognition is not apparent in most cases and three-dimensional structural features may be more important in such instances.

Glycosylation is the most complex PTM associated with native human proteins, and it is estimated that 1–2% of the human genome encodes proteins contributing to glycosylation capacity.

Glycosylation can have a number of functional effects on proteins.

- It aids correct protein folding/assembly, for example the glycocomponent of gonadotrophic hormones (Chapter 8) has been implicated in folding and subunit assembly.
- It can aid in the targeting and trafficking of a newly synthesized protein to its final destination, for example the removal of two or more of EPO's three N-linked glycosylation sites (see Chapter 8) results in a protein variant which is very poorly secreted from the cell.
- It can play a role in ligand binding, for example an antibody's glycocomponent can play a role in triggering its so-called effector functions (see Chapter 7).
- The glycocomponent can play a role in triggering a biological activity on ligand binding, for example removal of the glycocomponent of gonadotrophins actually increases their receptor-binding affinity but abolishes their ability to trigger signal transduction on binding.





**Figure 2.18** Structure of two sample oligosaccharide side chains (one N-linked the other O-linked) found in glycoproteins. Man, mannose; Gal, galactose; SA, sialic acid; GlcNAc, *N*-acetylglucosamine.

- The glycocomponent may play a direct role in stabilizing the protein, for example removal of the sugar side chain from some glycoproteins such as  $\alpha$ -galactosidase results in protein aggregation and precipitation.
- The glycocomponent often plays a role in regulating a protein's serum half-life, for example sugar side chains of many glycoproteins are capped at the end with a sialic acid sugar residue, which prolongs the protein's half-life in the body. Removal of the sialic acid cap exposes other sugar residues underneath, particularly galactose residues, thus prompting quick product removal from the blood via binding to galactose-specific receptors on liver cells.

## 2.9.2 Proteolytic processing

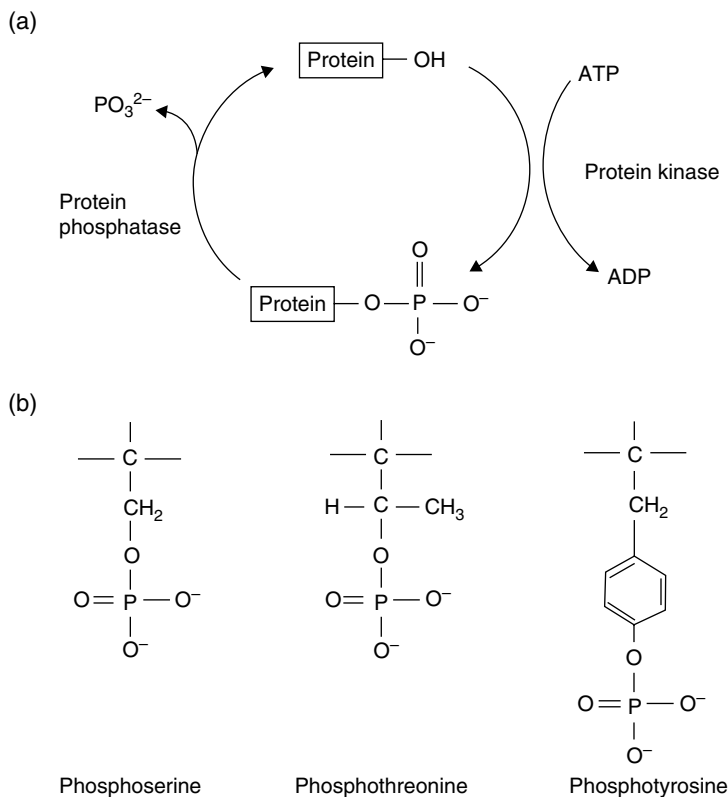
Proteolytic processing refers to limited and specific proteolytic cleavage of a polypeptide subsequent to its synthesis. Virtually all proteins, be they derived from prokaryotic or eukaryotic sources, destined for export from the cell contain a signal sequence at their N-terminal end. The signal sequence itself is cleaved off as the protein translocates across the membrane, releasing the mature polypeptide. For example, human EPO (see Chapter 8) is initially synthesized as a 193 amino acid polypeptide, of which the first 27 amino acids (residues 1–27) represent

a signal sequence. Signal sequences are usually characterized by a positively charged N-terminal region, followed by a hydrophobic region and finally a neutral but polar region. Various bioinformatic tools (which can be accessed via ExPASy) have been developed which can predict the presence of such signal sequences in sequence data.

In addition to playing a role in protein targeting, proteolysis can modulate the biological activity of many proteins. The pre-cleaved ('pro') form of such proteins are generally inactive, with activation occurring on proteolysis. Examples include the mammalian digestive enzymes trypsin, chymotrypsin and pepsin. These are initially synthesized and stored in the pancreas as 'pro' or 'zymogen' precursors. Additional examples include a range of blood clotting factors (Chapter 6) and insulin (Chapter 8). Proteolytic activation is very specific and is generally irreversible.

## 2.9.3 Phosphorylation

Reversible phosphorylation represents yet another form of PTM, and is undertaken primarily in eukaryotes but also in prokaryotes. The phosphate group donor is most often ATP and phosphorylation/dephosphorylation of the target protein is undertaken by substrate-specific protein kinase and protein phosphatase enzymes (Figure 2.19). The site



**Figure 2.19** (a) Reversible phosphorylation of a protein substrate via a kinase/phosphatase mechanism. (b) Phosphate groups are usually attached to the protein via the hydroxyl groups of serine, threonine or tyrosine residues.

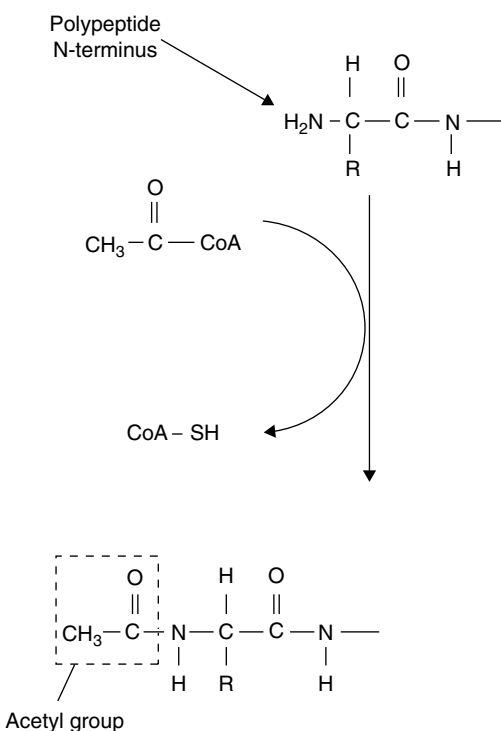
of phosphorylation is usually the hydroxyl group of either serine, threonine or tyrosine residues (Figure 2.19), although the side chains of aspartate, lysine and histidine can also sometimes be phosphorylated. Again the exact site phosphorylated exhibits both a sequence-specific characteristic and a likely requirement for a characteristic three-dimensional shape.

In the vast majority of cases phosphorylation directly affects the biological activity of the target protein, with phosphorylation/dephosphorylation functioning as a reversible on/off switch. In some cases (e.g. the enzyme glycogen phosphorylase), phosphorylation results in activation, whereas in other cases (e.g. the enzyme glycogen synthase), phosphorylation results in inactivation. Protein phosphorylation events are known to regulate a wide variety of cellular processes, including metabolism, transcription, translation and protein degradation, as well as cellular differentiation, signalling and

proliferation. In a few instances, phosphorylation does not play a regulatory role, for example the phosphorylation of the milk protein casein (see Chapter 14) is of nutritional rather than functional importance.

### 2.9.4 Acetylation, acylation and amidation

Acetylation (the addition of an acetyl group,  $\text{CH}_3\text{CO}$ ) is the most common PTM associated with eukaryotic proteins. Acetylation of the N-terminus is characteristic of approximately 50% of cytoplasmic proteins in yeast and over 80% of human cytoplasmic proteins. The acetyl group donor is usually acetyl-CoA, and the reaction is catalysed by *N*-acetyltransferase enzymes (Figure 2.20). In some cases *N*-terminal acetylation appears to occur before the polypeptide is completely synthesized, while in



**Figure 2.20** Acetylation of a polypeptide N-terminal amino group as catalysed by an *N*-acetyltransferase enzyme. The acetyl group donor is usually acetyl-CoA.

other instances acetylation occurs post-translationally. N-terminal acetylation is also characteristic of some prokaryotic and archael proteins.

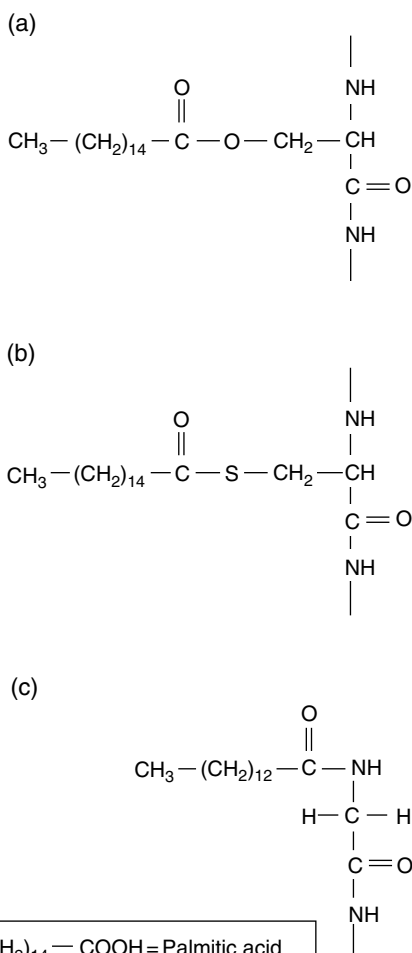
Acetylation of some proteins may also occur via the amine group found in the side chains of constituent lysine residues. This can modulate target protein activity, and such reversible acetylation reactions are characteristic of many cytoskeletal proteins, transcription factors and molecular chaperones. For example, the 393 amino acid human tumour suppressor protein P53 is subject to reversible acetylation towards its C-terminus, at Lys<sup>382</sup>, an event which enhances its transcriptional activity. Histones are also subject to this PTM. Histone acetylation typically promotes the recruitment of additional effector protein, the relaxation of chromatin structure and an increase in transcription rates.

Protein acylation refers to the direct covalent attachment of fatty acids to a polypeptide backbone. The fatty acids most commonly found in association with acylated polypeptides are the 16-carbon satu-

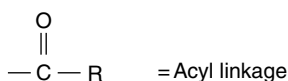
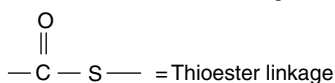
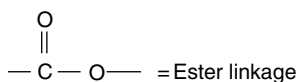
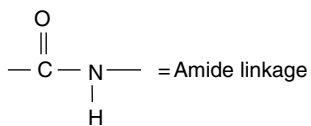
rated palmitic acid and the 14-carbon saturated myristic acid. Palmitic acid is usually covalently linked via an ester or thioester bond to either a cysteine, serine or threonine residue, while myristic acid is invariably covalently attached to an N-terminal glycine residue via an amide bond (Figure 2.21). Acylated polypeptides appear to be ubiquitous in eukaryotes and are also found in many viruses.

Myristoylation (covalent attachment of myristic acid) appears to occur co-translationally (i.e. before polypeptide synthesis is complete) and is promoted by the enzyme myristoyl-CoA:protein N-myristoyltransferase. Enzyme-based attachment of palmitic acid is a more complex post-translational event, probably involving several enzymes. A wide range of protein types are acylated, including various cytoplasmic and membrane proteins, as well as viral structural proteins (Table 2.8). The exact function of the fatty acid component is not fully defined in most cases. Initially, it was believed to play a role in anchoring/allowing proteins to interact with biological membranes, but this is probably not its main function. The fatty acid component may play a more important role in promoting protein-protein interactions. Many such acylated polypeptides are components of multi-subunit protein complexes, and in several cases removal of the fatty acid moiety negatively affects subunit interactions and/or biological activity of the multi-subunit complexes. The fatty acid component of various acylated viral caspid proteins also appears to stabilize interactions between these structural proteins. Although palmitic and myristic acids are most commonly found in association with acylated proteins, additional lipid-based substances may also be present, including stearic acid, phosphatidylinositol and farnesyl groups.

Amidation refers to the replacement of a protein's C-terminal carboxyl group with an amide group. It is a PTM more characteristic of peptides as opposed to polypeptides, and amidated peptides such as oxytocin, vasopressin and calcitonin are used therapeutically. Despite its relatively widespread occurrence, in higher eukaryotes in particular, its exact biological functions remain less than precisely refined. It may contribute to peptide stability, activity or both. It appears to be an



Key:

 $\text{CH}_3 - (\text{CH}_2)_{14} - \text{COOH} = \text{Palmitic acid}$ 
 $\text{CH}_3 - (\text{CH}_2)_{12} - \text{COOH} = \text{Myristic acid}$ 


**Figure 2.21** Linkage of (a) palmitic acid via an ester bond to a serine residue, (b) palmitic acid via a thioester bond to a cysteine residue, and (c) myristic acid to an N-terminal glycine residue via an amide bond.

**Table 2.8** Representative examples of some acylated proteins. The fatty acid moiety attached and the location of eukaryotic-derived acylated proteins are also listed.

Protein	Cellular location	Fatty acid
cAMP-dependent protein kinase	Cytoplasm	Myristic acid
Cytochrome $b_5$ reductase	ER and mitochondria	Myristic acid
$G_i$ and $G_0$ $\alpha$ -subunits	Plasma membrane	Myristic acid
Insulin receptor	Plasma membrane	Palmitic acid
Interleukin 1 receptor	Plasma membrane	Palmitic acid
Transferrin receptor	Plasma membrane	Palmitic acid
Rhodopsin	Disc membranes in retina	Palmitic acid
P55 and P28	HIV	Myristic acid
VP4	Picornaviruses	Myristic acid
P19 (gag)	HTLV I	Myristic acid
HA	Influenza virus	Palmitic acid
gE	Herpes simplex virus	Palmitic acid

important determinant in the binding of several regulatory peptides to their corresponding receptors.

## 2.9.5 Sulfation

Sulfation is a PTM that entails the attachment of a sulfate ( $\text{SO}_3^-$ ) group to the protein backbone, usually via target tyrosine residues, although it can sometimes occur via serine and threonine residues. Sulfation is undertaken mainly in higher eukaryotes. It is a process mediated by sulfotransferases in the Golgi network and it is predominantly associated with secretory and membrane proteins.

Sulfation does not appear to depend on the occurrence of a target tyrosine within a strictly defined consensus sequence. However, three to four acidic amino acid residues are normally found within five residues of sulfated tyrosines and elements of local secondary structure are also likely to play a determinant role.

Functionally, sulfation often plays a role in protein-protein interactions. Generally, the absence of sulfation tends to reduce rather than abolish activity.

Many chemokine and hormone cell-surface receptors are sulfated. This infers a possible role for this PTM in cellular processes, including immunity, haematopoiesis and angiogenesis. Sulfation may also play an important functional role in the docking of animal viruses to their target cells.

### 2.9.6 PTM engineering

PTMs, where they occur, usually have a profound effect on some aspect of protein structure or function. The majority of proteins used for therapeutic application (see Chapters 6–9) are subject to one or more PTMs (Table 2.9) and these PTMs can have a significant therapeutic impact.

One active focus of biopharmaceutical research and development is the tailoring (engineering) of the PTM complement of such proteins in order to improve their therapeutic characteristics. Because of its multiple potential therapeutic influences and because of its widespread occurrence, particularly on valuable biopharmaceuticals, engineering the oligosaccharide component of glycoproteins has received most attention to date. Earlier approaches to glycoengineering included:

- *in vitro* modification of the native glycocomponent (see the recombinant glucocerebrosidase enzyme ‘Cerezyme’ in Chapter 6);

- the introduction of additional glycosylation sites via site-directed mutagenesis (see the glycoengineered EPO product ‘Aranesp’ in Chapter 8);
- direct chemical conjugation of synthetic oligosaccharides to the backbone of a presynthesized protein (in experimental development).

More recently, efforts in glycoengineering research have focused on altering the glycosylation capacity of potential biopharmaceutical producer cells. As outlined in Chapter 3, glycosylated therapeutic proteins are invariably produced by recombinant means in mammalian cell lines (which undertake glycosylation reactions highly similar to those in humans). Alternative, potentially more technically and economically attractive producers such as *E. coli* and yeast or plant-based systems are either incapable of undertaking glycosylation (*E. coli*) or attach oligosaccharides to the protein which are different in sequence/structure compared with the native human glycocomponent, as shown by the following examples.

- Yeasts tend to add sugar side chains that contain high levels of mannose and which are very largely devoid of sialic acid caps. These characteristics promote rapid uptake and degradation of yeast-produced glycoproteins on their administration to humans.

**Table 2.9** Some post-translational modifications (PTMs) associated with proteins for therapeutic use. The vast majority of such proteins are (recombinant forms of) native human extracellular proteins, and as such their PTM profile is biased towards PTMs characteristic of extracellular proteins derived from higher eukaryotes. The list is representative only, with specific examples being found throughout Chapters 6–9.

Protein	Therapeutic application	PTM detail
Blood factor VIII	Haemophilia A (Chapter 6)	Glycosylation, disulfide bond formation, sulfation
Hirudin	Anticoagulant (Chapter 6)	Disulfide bond formation, sulfation
Tissue plasminogen activator (tPA)	Thrombolytic (Chapter 6)	Glycosylation, disulfide bond formation, proteolytic processing
$\alpha$ -Galactosidase	Fabry disease (Chapter 6)	Glycosylation, disulfide bond formation
Antibodies	Various (Chapter 7)	Glycosylation, disulfide bond formation
Insulin	Diabetes (Chapter 8)	Disulfide bond formation, proteolytic processing
Human growth hormone (hGH)	Dwarfism (Chapter 8)	Disulfide bond formation, phosphorylation
Erythropoietin (EPO)	Anaemia (Chapter 8)	Disulfide bond formation, glycosylation
Interferon $\beta$	Multiple sclerosis	Disulfide bond formation, glycosylation, phosphorylation

- Plants tend to hyperglycosylate proteins (compared with humans) and they add  $\alpha(1,3)$ -fucose and  $\beta(1,3)$ -xylose sugars which are immunogenic/allergenic in humans. In addition, the absence of terminal sialic acid residues in plant glycans will decrease the *in vivo* product half-life in humans.

An active area of research therefore involves efforts to re-engineer the glycosylation capacity of yeast and plant-based systems in an effort to render the glycosylation pattern of proteins produced therein suitable for administration to humans. This is an extremely complex task, as the glycosylation machinery of any cell typically consists of several dozen different glycosyltransferases and glycosidases, spanning both the endoplasmic reticulum and the Golgi. Initial glycosylation steps along this pathway occur in the endoplasmic reticulum and are quite similar across most eukaryotes. However, final 'trimming' and maturation steps, mainly occurring in the Golgi, tend to vary significantly from species to species.

Despite the complexity, significant progress has been made over the past number of years. For example, the elimination of mannosyltransferase enzymes as well as the introduction of selected mannosidase activities into some engineered cells has generated yeast capable of producing far more mammalian-like sugar chains. Similarly, the development of plant cell knockouts devoid of fucose and xylose transferase activities provides a means of producing more mammalian-like glycoproteins in plant-based systems. Glycoengineering has also been applied to mammalian cell lines, in order to tailor some specific therapeutic attribute of a glycosylated protein produced therein. Glycoengineered antibodies represent a specific example, as discussed in Chapter 7.

### 2.9.7 Protein PEGylation

The covalent attachment of synthetic chemical groups to proteins in effect represents a PTM of the protein, albeit an unnatural one. Prominent among such examples is that of PEGylation (the attachment of polyethylene glycol or PEG). PEG is a polymer of ethylene oxide, displaying a general molecular

formula of  $\text{H}(\text{OCH}_2\text{CH}_2)_n\text{OH}$ . It can be synthesized to yield either straight-chain or branched polymers, typically displaying molecular masses ranging from a few hundred to tens of thousands of daltons. Moreover, various chemically reactive groups can be attached to PEGs which allows them to be covalently coupled to various specific amino acid side chains, most notably lysine and cysteine. As such, PEG molecules can be conveniently covalently attached to proteins. PEG is non-toxic and non-immunogenic, properties conducive to therapeutic application. Its flexibility and ability to coordinate a large number of surrounding water molecules per ethylene unit results in a general shielding or protective effect on proteins to which it is attached, as well as significantly increasing their hydrodynamic volume, and hence their apparent size. Therapeutic proteins administered by injection may be PEGylated in order to reduce their immunogenicity, to protect them from degradation by serum proteases and in particular to slow the rate of renal excretion of smaller proteins.

The renal threshold for globular proteins is in the region of 40–60 kDa (a hydrodynamic radius of about 45 Å or 4.5 nm). Thus the attachment of appropriately sized PEG molecules to therapeutic proteins below this size threshold (e.g. interferons and some antibody fragments) can prevent their rapid renal clearance, and hence increase their therapeutic half-life. As a result less frequent dosage regimens are necessitated, with consequent economic savings and (usually) improved patient compliance and convenience. A number of such PEGylated products are considered in later chapters.

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# Chapter 3

## Protein sources

A prerequisite to the isolation, study and/or utilization of any protein is the identification of a suitable protein source. Proteins may be extracted from their naturally producing source material or may be produced via recombinant DNA technology (recombinant protein production). Because recombinant proteins are invariably not naturally synthesized by their genetically engineered producer cells, they are often termed 'heterologous' proteins. In contrast, proteins produced naturally by a cell source are often termed 'homologous' proteins. Advances in molecular biology and the 'omics' era has now generally rendered recombinant-based protein expression the more usual means of protein production, whether for research or applied (industrial or medical) applications.

### 3.1 Recombinant versus non-recombinant production

Chapters 6–13 provide numerous specific examples of proteins now produced commercially by recombinant DNA technology. Selected examples

are listed in Table 3.1. Protein production via recombinant means has a number of potential advantages over direct extraction from a native source material.

- *Overcomes problems of source availability:* many proteins are produced naturally at exceedingly low concentrations, rendering their production in large quantities difficult or impossible.
- *Overcomes problems of source safety:* many proteins are either produced naturally by dangerous/pathogenic species (e.g. snakes, various microbial pathogens) or are derived from sources that may also harbour pathogens (e.g. blood). Recombinant production in a non-pathogenic, non-toxic host organism circumvents such potential difficulties.
- *Facilitates predefined modification of the protein's amino acid sequence:* molecular biology techniques facilitate the straightforward alteration of the protein's amino acid sequence. Such alterations can range from single amino acid omissions/substitutions to far more extensive modification such as generating novel proteins by, for example, joining domains from different parental proteins together thereby generating a fusion protein

**Table 3.1** Some of the proteins and protein families produced by recombinant means that have found commercial application. In some instances the commercial products are produced exclusively via recombinant technology. In other cases (e.g. insulin) both non-recombinant and recombinant versions of the product are commercially available. The chapters in which the various proteins are discussed are also indicated.

Protein	Application	Chapter
Blood proteins (e.g. factors VIII and IX)	Treatment of haemophilia and other blood disorders	6
Thrombolytic agents (e.g. tissue plasminogen activator, tPA)	Treatment of heart attacks	6
Recombinant 'subunit' vaccines (e.g. hepatitis B surface antigen)	Vaccination against specific diseases	6
Engineered antibodies	Various, including cancer therapy	7
Native and engineered insulins	Treatment of diabetes mellitus	8
Human growth hormone	Treatment of short stature	8
Interferons	Treatment of cancer, viral diseases	9
Cholesterol esterase	Used to determine blood cholesterol levels	10
Chymosin	Cheese production	12
Various proteases, lipases and amylases	Various, including addition to detergents	12, 13
Phytase	Added to animal feed to degrade dietary phytic acid	13

product. Another such modification entails the attachment of affinity tag amino acid sequences to the protein, thereby facilitating affinity-based protein purification.

While this chapter largely focuses on protein production via recombinant means, it is important to remember that in some instances proteins required for both research and commercial purposes are still routinely obtained by extraction from a naturally

producing source. Some examples of commercial proteins obtained by direct extraction from a native source are provided in Table 3.2. This classical approach can be pursued for any number of reasons, including the following.

- Some proteins are produced by native production sources at very high levels, rendering direct purification convenient. In such cases, in particular if the protein is produced for commercial sale, direct extraction from the native source may be as attractive or more attractive economically than recombinant production. An example would be human serum albumin, which is present naturally in human serum at levels of 42 g/L. Such high production levels cannot be matched by current recombinant expression systems.
- Some proteins, when expressed in recombinant systems, accumulate in a partially/fully unfolded, biologically inactive form (see section 3.3.1) which cannot subsequently be renatured. Under such circumstances direct extraction from the native source may prove the only or most convenient way of obtaining the biologically active protein of interest. For example, it can be challenging to obtain biologically active levels of some proteins derived from extremophilic microorganisms (Chapter 11) via recombinant means.
- Some proteins can be quite toxic to the recombinant cells in which they are being produced, rendering the recombinant route challenging.
- Production in a recombinant system can influence specific post-translational modifications (PTMs) associated with a protein. Therefore it may be desirable to purify such a protein from its native source in order to establish its native PTM profile.
- In the case of proteins produced for some industrial applications (e.g. food processing), there may be some public resistance to the use of recombinant products, providing marketing advantage to companies producing the product by non-recombinant means.
- In the case of some proteins produced for commercial purposes, there may be patent issues surrounding the recombinant production route.

**Table 3.2** Some proteins obtained commercially from non-genetically engineered microorganisms. Sources include a range of bacteria, fungi and yeast, and the proteins have found medical, analytical and industrial uses.

Protein	Source	Application
Streptokinase	Various haemolytic streptococci	Thrombolytic agent (degrades blood clots)
Staphylokinase	<i>Staphylococcus aureus</i>	Thrombolytic agent
Tetanus toxoid	Formaldehyde-treated toxin obtained from <i>Clostridium tetani</i>	Tetanus vaccine
Asparaginase	<i>Erwinia chrysanthemi</i> or <i>E. coli</i>	Cancer (leukaemia) treatment
Glucose oxidase	<i>Aspergillus niger</i>	Determination of blood glucose levels
Alcohol dehydrogenase	<i>Saccharomyces cerevisiae</i>	Determination of blood alcohol levels
Various amylases	Various bacilli, <i>Aspergillus oryzae</i>	Degradation of starch
Various proteases	Various bacilli and aspergilli	Degradation of proteins for food, detergent and other applications
Cellulases	<i>Trichoderma</i> species, <i>A. niger</i> , various actinomycetes	Degradation of cellulose

## 3.2 Approaches to recombinant protein production

A wide variety of molecular biological approaches may be pursued in order to construct a recombinant protein source organism. The specific approach chosen will depend on a number of considerations, including:

- how much information is already known about the protein and particularly about its nucleotide coding sequence;
- the ultimate use for which the protein is required (e.g. for research or for a specific commercial application);
- to some extent the personal preferences of the genetic engineers developing the recombinant system.

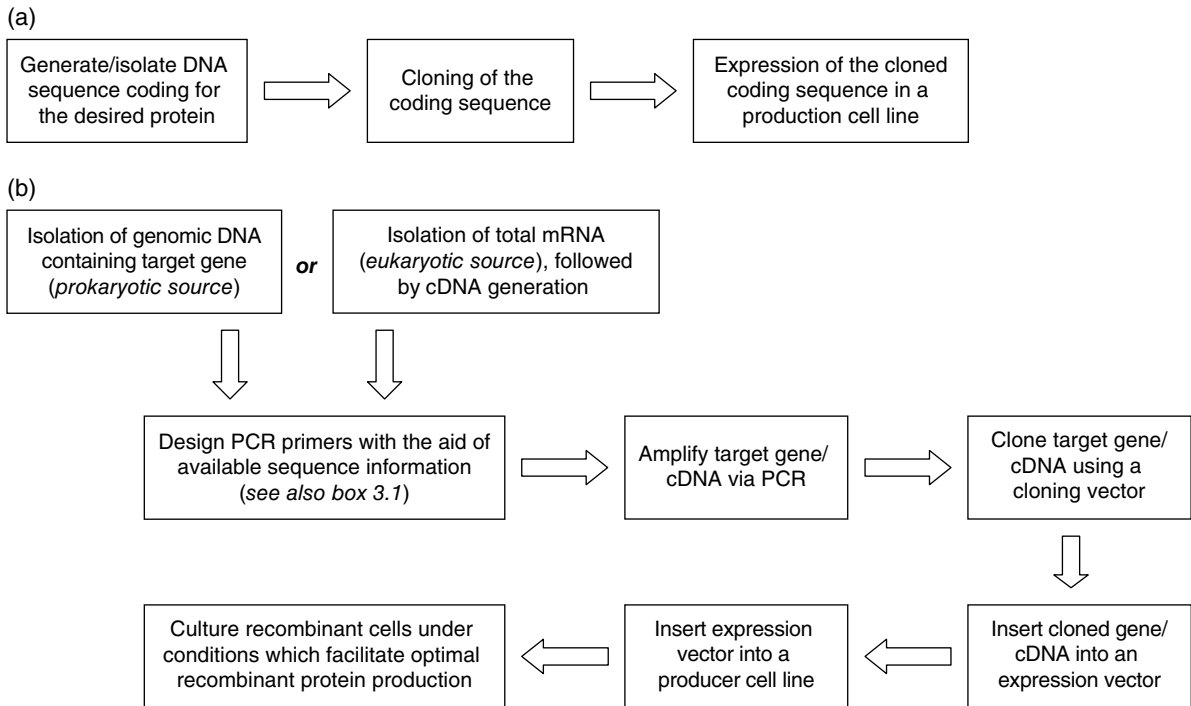
A detailed consideration of molecular biology is well beyond the scope of this book but a general approach to isolating and expressing a DNA sequence coding for a protein of interest is typically characterized by a number of stages, as outlined in Figure 3.1.

The first step entails the identification and isolation/generation of a DNA sequence coding for the protein of interest. While classical cloning techniques may be used, they have been largely superseded by approaches based on the polymerase chain reaction (PCR) (see Box 2.3). Yet an additional approach can be the direct chemical synthesis of a DNA fragment coding for the protein of interest. The latter approaches are made possible by the vast amount of DNA sequence information generated and available in sequence databases such as GenBank (see Chapter 1).

The initial steps in most modern cloning approaches involve the isolation of the template nucleotide sequence coding for the protein of interest. In the context of prokaryotic cells this generally entails the initial isolation of genomic DNA. In the case of eukaryotic cells, genomic DNA or complementary (c)DNA derived from a template mRNA sequence (see below) can constitute the starting point.

In the case of genomic DNA the isolation process typically includes:

- initial cell lysis (usually in the presence of buffers containing chelating agents, which inactivates endogenous nucleases);



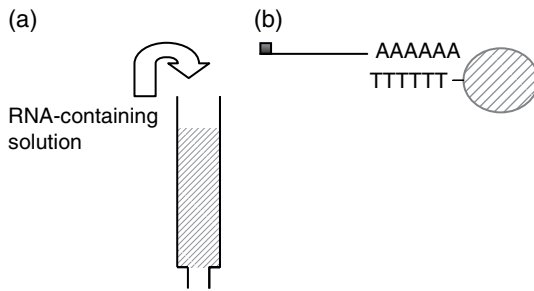
**Figure 3.1** (a) Overview of the molecular biology process by which a recombinant protein production system is developed. (b) A more detailed overview of common strategies adopted during this process. Refer to text for specific detail.

- denaturation and removal of intracellular proteins (using, for example, a protease or a detergent);
- degradation of RNA using an RNase enzyme;
- purification of the DNA (usually using organic solvent extraction, salt-based precipitation or by binding the DNA to a silica membrane or an ion-exchange resin, followed by elution in water).

If the protein of interest is derived from a eukaryotic organism, an alternative approach is to isolate mRNA and then convert it to cDNA. RNA can be conveniently extracted and purified from cells using various organic solvents and/or ion-exchange/silica gel adsorption. An alternative affinity-based approach to specifically purifying mRNA can also be used which takes advantage of the fact that such mRNA molecules contain a characteristic poly-A (polyadenylate) tail (Figure 3.2). Once the mRNA has been obtained it can be converted to

double-stranded cDNA using the enzymes reverse transcriptase and DNA polymerase. The cDNA nucleotide sequence reflects that present in the mature mRNA, which in turn reflects the actual exon-encoding protein sequences of the eukaryotic gene (see also Chapter 1, Figure 1.1). This approach is particularly important if the target eukaryotic-derived protein is to be expressed in a prokaryotic cell, as the cDNA is devoid of any intron sequences (see Figure 1.1), which are not present in prokaryotic genes.

Once the genomic DNA or cDNA is isolated, PCR (Box 2.3) allows the generation of multiple copies of the specific sequence encoding the target protein. The PCR product is then generally inserted into a cloning vector, which facilitates its convenient further amplification. Cloning vectors are themselves small DNA molecules capable of self-replication in the cell into which it is introduced, usually *Escherichia coli*. Probably the most



**Figure 3.2** Affinity-based purification of mRNA. The unpurified mRNA-containing solution is percolated through a column packed with cellulose beads (a), to which a short chain of deoxythymidylate (an oligo dT chain) has been attached. Any mRNA present is retained in the column due to complementary base pairing between its 3' poly-A tail and the immobilized oligo dT (b). Non-bound material can then be washed out of the column, with subsequent desorption of the mRNA by passing a low salt buffer through the column. The mRNA collected may then be precipitated out of solution using ethanol, followed by collection via centrifugation. An alternative and now more commonly used variation entails the direct addition of oligo (dT)-bound magnetic beads directly into the cell lysate and 'pulling out' the mRNA using a magnet. The method is rapid, thus minimizing contact time of the mRNA with degradative ribonucleases present naturally in the cytoplasm.

commonly used vectors in conjunction with *E. coli* are plasmids. Plasmids are circular extrachromosomal DNA molecules found naturally in a wide range of bacteria. They generally house several genes, often including one or more genes whose product renders the plasmid-containing cell resistant to specific antibiotic(s). One plasmid classically used in cloning experiments with *E. coli* is pUC18 (Figure 3.3).

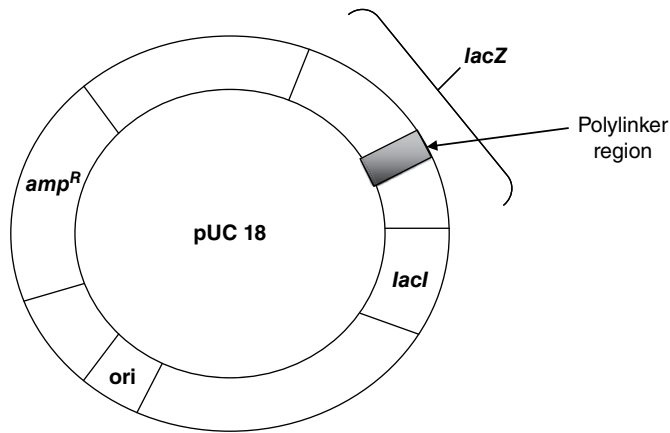
The next stage of the cloning process involves the introduction of the engineered vector into *E. coli* cells. This can be achieved by a number of different means. One approach (called transformation) involves co-incubation of the plasmids and cells in a solution of calcium chloride, initially at 0°C, with subsequent increase in temperature to 42°C. This temperature shock facilitates entry of plasmids into some cells. The *E. coli* cells are then spread out on

the surface of an agar plate and incubated under appropriate conditions in order to kill cells that have not taken up plasmid. Each individual cell will thus form a colony (clone of cells).

Cells from the appropriate colony can then be grown up in larger amounts by submerged fermentation (see Chapter 5) in order to produce larger amounts of the desired (now cloned) gene. The cells can be collected, lysed and the vector therein recovered by standard molecular techniques. The cloned gene can then be excised from the vector via treatment with an appropriate restriction endonuclease and purified by standard molecular methods. The cloned gene is also usually then directly sequenced, in order to ensure that the cloning process has not unintentionally introduced any sequence errors.

The vectors described thus far have been designed to facilitate the cloning of genomic DNA/cDNA sequences but they do not support the actual expression (i.e. transcription and translation) of the gene. Once the gene/cDNA coding for a potential target protein has been isolated, the goal next becomes one of achieving high levels of expression of this target gene. This process involves insertion of the gene into a vector that will support high-level transcription and translation. In addition to the basic vector elements (i.e. an origin of replication and a selectable marker such as an antibiotic resistance gene), expression vectors also contain all the genetic elements required to support transcription and translation (e.g. promoters, translational start and stop signals). A wide range of such expression vectors are now commercially available and obviously each is tailored to work best in a specific host cell type (e.g. bacterial, yeast, mammalian). Expression vectors are considered in Box 3.1.

Once a PCR product has been obtained it is also possible to insert it directly into an expression vector, without the inclusion of a cloning vector step (Figure 3.1). However, many expression vectors are large and replicate in cells at low copy numbers, which can make them harder to work with; in practical terms, it can be convenient to retain a repository of the cloned gene in a cloning vector that is easy to work with.

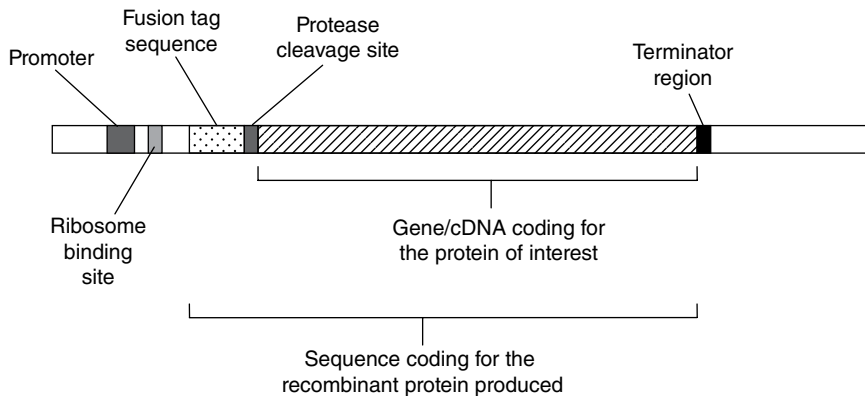


**Figure 3.3** The plasmid pUC18, which is often used for cloning purposes. The plasmid contains three genes: the ampicillin resistance gene ( $amp^R$ ); the  $lacZ$  gene, which codes for the enzyme  $\beta$ -galactosidase; and the  $lacI$  gene, which codes for a factor that controls the transcription of  $lacZ$ . Also present is an origin of replication ( $ori$ ) which is essential for plasmid replication within the cell. Note the presence of a short stretch of DNA called the polylinker region located within the  $lacZ$  gene. The polylinker (also called a multiple cloning site) contains cleavage sites for 13 different restriction endonucleases. This allows genetic engineers great flexibility to insert a DNA fragment for cloning into this area. The polylinker has been designed and positioned within the  $lacZ$  gene in a manner so as not to prevent the expression of functional  $\beta$ -galactosidase. However, if a piece of DNA for cloning is introduced into the polylinker region, the increased length does block  $\beta$ -galactosidase expression. The full sequence of the 2.69 kb plasmid is known and sequence analysis confirms the presence of multiple additional restriction endonuclease sites outside the polylinker region. There are at least six target sites for commonly used restriction enzymes within the  $amp^R$  gene.

### Box 3.1 Expression vectors

Insertion of a cDNA/gene into an expression vector, followed by insertion of the vector into a producer cell, is required to achieve recombinant protein production. A schematic diagram of the

region of a generalized expression vector which drives the production of a recombinant protein product is shown below.



The promoter region is the nucleotide sequence which RNA polymerase (the enzyme responsible for transcribing the gene into mRNA) identifies and binds. Close by is a ribosome binding site, a short nucleotide sequence which, when transcribed into the corresponding mRNA, serves as the ribosomal attachment point to the mRNA. The translation initiation codon will be a few nucleotides downstream of that point. Immediately after a fusion tag sequence is usually included. This tag codes for an amino acid sequence that will be present in the recombinant protein product. The purpose of the tag is usually to (i) enhance the solubility of the recombinant protein, as outlined later in this chapter, or (ii) act as an affinity tag that will allow the protein to be purified using an affinity-based system (outlined in Chapter 4). At the end of the tag is a further nucleotide sequence which encodes a short amino acid sequence recognized by a specific proteolytic enzyme (enabling ultimate tag removal post purification). This in turn is joined to the gene/cDNA coding for the protein of interest. After the gene/cDNA is a termination sequence, which marks the point at which transcription will stop. In addition to the regions highlighted above, the expression vector will also contain additional necessary sequences, such as an origin of replication and a selectable marker such as a gene coding for antibiotic resistance.

In many ways the promoter is the most significant component of the expression vector, as it determines not only binding of RNA polymerase but also the rate at which mRNA is synthesized. 'Strong' promoters, naturally found in association with genes whose transcribed products are required in large amounts by the cell, are generally used when constructing expression vectors. Examples of promoters incorporated in expression vectors used to produce recombinant protein in *E. coli* include:

- the *lac* promoter (normally controls transcription of the *lac* operon);
- the *trp* promoter (normally controls transcription of enzymes involved in tryptophan biosynthesis);
- the T7 promoter (specific for the RNA polymerase encoded by the T7 bacteriophage, which is far more active than native *E. coli* RNA polymerase; T7-containing expression vectors are used in conjunction with *E. coli* strains that also house the T7 RNA polymerase gene).

While some genes are constitutively (i.e. continuously) transcribed in the cells in which they are naturally found, the expression of other genes can be regulated depending on the cell's requirements at any given time. Such genes can be silenced or expressed, as required. Cells have evolved various regulatory mechanisms to achieve this, a prominent one being either direct induction or repression of gene transcription to mRNA. The molecular detail of how induction or repression of transcription occurs can vary in different cell types but, in overview, an inducible gene is one whose transcription is usually triggered by the binding of a signalling (inducer) molecule to a genetic regulatory region within or adjacent to the promoter. In the absence of the inducer, the gene is usually not transcribed, certainly to any significant extent. In this case gene repression is effectively the default situation, automatically being the case in the absence of the inducer. In other systems genes are repressed on binding to the regulatory region of a specific repressor molecule.

Recombinant protein production is achieved by culture of the expression vector-containing cells in appropriate nutrient media (Chapter 5). Successful high-level production is generally best achieved if the recombinant gene/cDNA is not expressed until the later of stages of cell growth, when the culture has reached a high cell density. Constitutive high-level expression from the earliest stages of culture is generally undesirable as:

- it will divert much of the metabolic capacity of actively growing and dividing cells into protein production;
- continual high-level transcription can negatively affect the ability of plasmid vectors to themselves

replicate, resulting in their loss from the culture cells as they divide;

- accumulation of some recombinant proteins can be toxic to the producer cells, and thus premature expression can kill the cells.

Prevention of constitutive expression of the target gene can be achieved by using expression vectors constructed to contain inducible promoter/regulatory regions. Using such systems recombinant

protein expression will only occur on addition to the culture media of the appropriate inducer molecule, which can be undertaken only when high cell density has been reached. For example, if the expression vector contains the *lac* promoter and associated regulatory region, recombinant protein production will only occur when a *lac* inducer is added to the culture medium. Isopropyl-thiogalactopyranoside (IPTG) is a commonly used such (synthetic) inducer.

### 3.3 Heterologous protein production in *E. coli*

The majority of recombinant proteins are produced in microbial systems, with *E. coli* being the most common expression system used. Some illustrative examples of commercialized *E. coli*-derived recombinant proteins are provided in Table 3.3. These, as well as other *E. coli*-derived products, are discussed throughout Chapters 6–9.

The popularity of *E. coli* as a recombinant system stems mainly from the fact that the study of prokaryotic genetics traditionally focused on it as a model system. Hence more was known about the genetic characteristics of *E. coli* than any other microorganism. Suitable plasmids were available, as were a variety of appropriate powerful and inducible promoters (Table 3.4).

The majority of proteins synthesized naturally by *E. coli* are intracellular and the majority of heterologous proteins expressed in *E. coli* accumulate in the cell cytoplasm, where they can represent 25% or more of total cellular protein (Table 3.5).

#### 3.3.1 Inclusion body formation

Extremely high levels of diverse heterologous proteins have been produced in *E. coli*. In most such cases, the resultant protein accumulates in the cytoplasm in the form of insoluble aggregates termed inclusion bodies or refractile bodies. These

**Table 3.3** Illustrative examples of proteins manufactured commercially via heterologous expression in *E. coli*. These and other examples are considered in later chapters of this book.

Product (name/ trade name)	Description	Application
Somatropin	Human growth hormone	Treatment of growth failure
Actimmune	Human interferon- $\gamma$	Chronic granulomatous disease
Intron A	Human interferon- $\alpha$	Hepatitis and various cancers
Filgrastim	Human granulocyte colony-stimulating factor	Neutropenia
Cimzia	Antibody fragment	Crohn's disease and rheumatoid arthritis
Humulin	Human insulin	Diabetes mellitus
Proleukin	Human interleukin-2	Renal cell carcinoma

aggregates are not derived from either native or fully unfolded forms of the protein but are composed of partially folded intermediates (often exhibiting stretches of intact secondary structure but little or no tertiary structure), and which are therefore obviously functionally inactive. Inclusion body formation may be triggered if high-level synthesis of recombinant protein molecules overwhelms the protein folding capacity of the cell.

Inclusion bodies may be readily viewed by dark-field microscopy (typically their diameter is in the order of 1  $\mu\text{m}$ ) and are composed predominantly



**Table 3.4** Some promoters that have been used to control expression of recombinant proteins in engineered *E. coli* cells. For example, the widely used pET expression vectors use a T7-based promoter.

Promoter	Method of induction	Comment
ara	Addition of arabinose to culture medium	Rapid induction and tight regulation, but repressed by glucose
cad	Adjustment of media to an acidic pH value	High expression levels achieved, cheap inducer
lac	Addition of isopropyl- $\beta$ -D-thiogalactoside (IPTG) to media; high temperature	Low-level expression, leaky expression under non-inducing conditions
tac	Addition of IPTG to media; high temperature	Well-characterized, high-level but leaky expression
trp	Addition of indoleacrylic acid to media	Well-characterized, high-level but leaky expression
T7	Addition of IPTG to media; high temperature	High-level expression but can have leaky expression under non-inducing conditions

**Table 3.5** Some heterologous proteins produced in *E. coli*, and the levels of expression achieved.

Protein	Level of expression achieved (per cent of total cellular protein)
Antifungal peptides	40
Insulin A chain	20
Insulin B chain	20
Human interleukin-7	46
Human granulocyte colony-stimulating factor	22
$\alpha_1$ -Antitrypsin	15
Interleukin-2	10
Tumour necrosis factor	15
Interferon- $\beta$	15
Interferon- $\gamma$	25
Calf prochymosin	8

of the expressed heterologous protein (contaminating proteins usually represent less than 15% of inclusion body mass). The most likely contributory factors to inclusion body formation include:

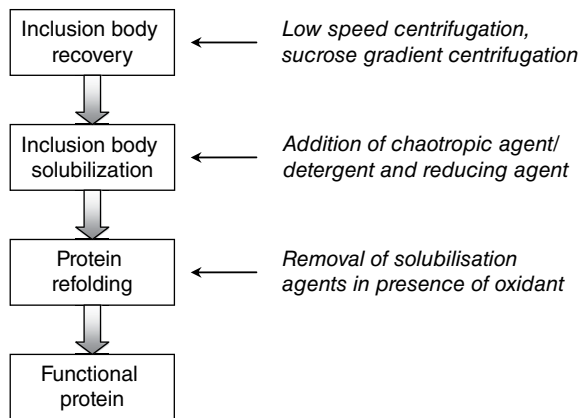
- very high local concentrations of the recombinant protein in the cytoplasm may lead to non-specific precipitation;
- the presence of insufficient chaperones/folding enzymes with subsequent aggregation of partially

folded intermediates, likely largely driven by intermolecular hydrophobic and other interactions;

- prevention of formation of disulfide linkages due to the reducing environment of the cytoplasm;
- lack of post-translational modifying enzymes may yield less stable (eukaryotic) heterologous protein products.

The formation of inclusion bodies may not be a wholly negative phenomenon. As inclusion bodies are very dense, they tend to sediment readily under the influence of a low centrifugal force. This can be exploited to quickly and effectively partially purify the aggregated protein. Typically, this is achieved by a low-speed centrifugation step, carried out immediately after cell homogenization. Inclusion bodies sediment more rapidly than cellular debris under the influence of a centrifugal force of about 500–1000  $\times g$ . The inclusion body-containing pellet can subsequently be resuspended and washed several times to reduce co-sedimenting cellular material. The carry-over of cellular debris can be problematic for several reasons;

- the presence of cell wall-associated proteases/peptidases can potentially degrade the product;
- cell debris can clog (foul) chromatography columns used to subsequently purify the recombinant protein;
- the presence of lipopolysaccharides (undesirable for therapeutic proteins; Chapter 5) in the cell envelope.



**Figure 3.4** Overview of the recovery of biologically active protein from inclusion bodies. Refer to text for further detail.

Highly pure inclusion bodies may also be recovered by sucrose density gradient centrifugation. After isolation of the inclusion body, the next step becomes one of attempting to recover fully folded and hence functionally active protein (Figure 3.4).

Denaturants are used to solubilize the aggregated polypeptides present in inclusion bodies. Denaturants usually employed include chaotropic agents such as urea or guanidinium chloride, and various detergents (e.g. sodium dodecyl sulfate), in addition to incubation under conditions of alkaline pH. Reducing agents (e.g.  $\beta$ -mercaptoethanol or cysteine) are also usually added in order to maintain cysteine residues in the protein in their reduced state, thereby preventing the formation of non-native intermolecular or intramolecular disulfide bonds. Once solubilization of the inclusion body has been achieved, the denaturant is slowly removed and replaced with buffers in which the protein is stable. Such buffers may also contain oxidizing agents if the native protein contains multiple disulfide linkages. Such 'buffer exchange' may be achieved by techniques such as dialysis, dilution, diafiltration, gel filtration chromatography, or even capture chromatography (see Chapter 4). An example of the latter approach entails the capture of the recombinant protein on an ion-exchange chromatography column or perhaps a tag affinity column if the protein has an associated affinity tag (see Chapters

1 and 4). The captured protein is subsequently eluted from the column using an appropriate buffer.

The exact conditions required to achieve maximum renaturation can vary from protein to protein and recovery of high yields of activity is not always guaranteed. This is particularly true in the case of large proteins for which correct refolding into active conformation is a complicated process. Typically, a reasonable yield of bioactive protein recovered from inclusion bodies would be in the region of 25%. Generally, however, this protein is very substantially pure (>90%).

Several approaches may be undertaken in an effort to encourage production of soluble heterologous protein in *E. coli*. Some approaches are applicable to all protein types, whereas others (e.g. inclusion of cofactors) are appropriate only in certain specific instances, which include the following.

- Empirical determination of exact optimal expression system: the precise host strain, plasmid and plasmid copy number, as well as the promoter sequence used, will influence the level of product expression and the propensity of product to accumulate as inclusion bodies.
- Growth of recombinant cells below optimal growth temperatures: this slows the growth and division rate of the cells, thereby also reducing the rate of recombinant protein synthesis. This in turn provides the protein folding machinery of the cell with a better chance of achieving full protein folding. A reduction in *E. coli* growth temperature from 37 to 30°C for example can reduce inclusion body formation for some proteins at least.
- Enhancing the endogenous production of a cofactor (or exogenously providing the cofactor) can increase soluble yields of a recombinant protein which requires the cofactor concerned.
- Co-expression of chaperones can improve soluble yield of heterologous protein.
- Expression of the desired product as a fusion protein (see below).

Fusion to a (highly soluble) native host cytoplasmic protein (Box 3.1.) enhances the solubility of some

otherwise insoluble heterologous proteins. In addition to facilitating the recovery of functionally active protein, a second benefit sometimes observed is increased expression levels of the recombinant protein construct. Two of the more common fusion partners are maltose-binding protein (a 42-kDa protein encoded by the *malE* gene of *E. coli* K12) and thioredoxin A (a 116-kDa *E. coli* cytoplasmic, highly soluble oxidoreductase enzyme). While successful in some instances, the fusion partner approach does suffer from a number of potential drawbacks, including:

- no fusion partner works universally to solubilize all proteins, and some proteins will remain insoluble no matter which tag is attached;
- subsequent proteolytic tag removal may not be efficient (due to perhaps steric considerations);
- the protein may not remain soluble once the tag is removed.

### 3.3.2 Extracellular production

Extracellular production of recombinant proteins in genetically engineered *E. coli* would have several potential advantages, including:

- ease of subsequent protein recovery (which would not require a cell homogenization step; Chapter 4);
- simplification of subsequent protein purification, as *E. coli* exports very few proteins;
- reduced likelihood of proteolytic degradation of the recombinant protein (*E. coli* produces numerous intracellular proteases but few extracellular proteases);
- increased likelihood of correct protein disulfide bond formation (disulfide bonds will not form in the cytoplasm due to its reducing chemical environment).

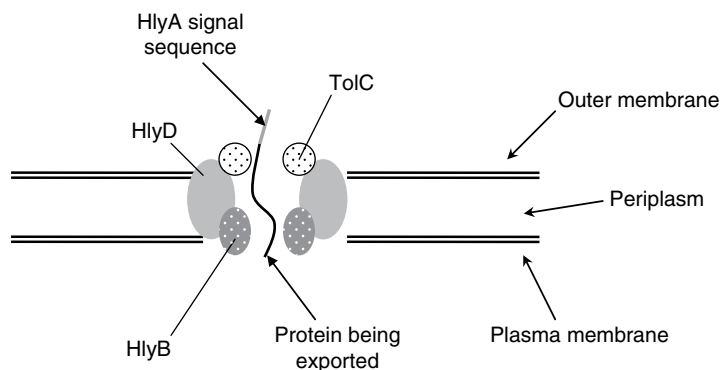
However, *E. coli* is a Gram-negative bacterium. Gram-negative bacteria contain a peptidoglycan-based cell wall outside its plasma membrane but, in addition, contain a second membrane outside the cell wall. The area between the plasma and outer membranes is known as the periplasmic

space. Extracellular protein export from Gram-negative bacteria is complex in that it requires translocation through two membranes. At least five different secretory transport systems have been identified in this regard, though not all have been elucidated in detail. Type I and type II secretory systems predominate in *E. coli*, including those strains most commonly used for recombinant protein production (K12 and B strains) and both have been used in attempts to develop engineered strains capable of exporting recombinant protein extracellularly.

The type I secretory mechanism achieves a one-step transport of exported proteins across both membranes. This transport system is composed of a number of component proteins that span inner and outer membranes (Figure 3.5). Native proteins secreted via this system have a characteristic C-terminal targeting signal peptide sequence. This signal sequence can be appended to recombinant proteins in order to facilitate their export via this transport mechanism.

The second main (type II) secretory mechanism characteristic of *E. coli* and many other Gram-negative bacteria is a two-step process, involving the initial translocation of the export protein across the plasma membrane into the periplasm, usually via the so-called SecB-dependent transport pathway. This too is peptide signal sequence dependent, facilitating its application to recombinant protein export. Further translocation of the export protein from the periplasm to the true extracellular space is dependent on a second 'secretion' transport mechanism through the outer membrane. However, recovery of recombinant protein accumulated in the periplasm can often be more efficiently achieved in practice by selective rupture of the outer membrane, releasing the contents of the periplasmic space while retaining the true intracellular contents within the still intact cell. The outer membrane can be selectively disrupted by means such as subjecting the cells to osmotic shock, limited mechanical force (ultrasound) or chemical influences (detergents or chelating agents).

Although advances in extracellular recombinant protein production from *E. coli* have been made, difficulties still remain. High-level recombinant



**Figure 3.5** Schematic diagram of the type I secretion mechanism which achieves one-step protein export from Gram-negative bacterial cells. The cell wall is omitted for clarity. At the core of the system is a complex of three different proteins, HlyB, HlyD and TolC, which cooperate to export proteins via an ATP-dependent mechanism.

protein expression usually overwhelms the secretory systems, not all proteins are excreted equally efficiently and the signal peptide can remain attached to the protein on its export (usually necessitating its subsequent removal from the purified recombinant protein). In most instances yield of true extracellular product is disappointing, often being less than 1 g/L of fermentation media. In contrast, intracellular expression will often generate protein levels of 5–10 g/L. As such, intracellular production of recombinant proteins predominates when using *E. coli*-based systems, both at research and industrial levels.

### 3.3.3 Other characteristics of heterologous protein production in *E. coli*

Another limitation of *E. coli* (and additional prokaryotic) recombinant systems is their inability to perform PTMs of recombinant eukaryotic proteins. *E. coli* does not have the ability to glycosylate, amidate or acetylate proteins for example (see Chapter 2), and this is one of the main reasons that most therapeutic proteins which display PTMs, in particular glycosylation, are generally not produced using such cells.

*E. coli*-synthesized recombinant proteins may also contain an extra amino acid residue (methionine)

**Table 3.6** Some advantages and disadvantages of *E. coli* used for the production of recombinant proteins.

#### Advantages

*E. coli* genetics well characterized, allowing straightforward genetic manipulation  
 Expression vectors available which facilitate straightforward and high-level recombinant protein expression  
 Grows rapidly and to high cell densities on relatively inexpensive fermentation media  
 Suitable fermentation technology well established  
 History of safe use for the production of many commercialized therapeutic proteins

#### Disadvantages

Poor ability to secrete recombinant proteins extracellularly in large amounts  
 Recombinant protein often accumulates intracellularly in inactive form (as inclusion bodies)  
 Unable to undertake post-translational modification of proteins  
 Presence of an additional methionine residue at the N-terminal of the recombinant protein

at their N-terminal end. This is due to the fact that translation in *E. coli* is always initiated by an N-formylmethionine residue. Although *E. coli* has the enzymatic capability to deformylate and subsequently remove this additional amino acid, removal may not be complete in the context of high-expression heterologous protein production. The presence of an extra N-terminal methionine in such recombinant proteins may alter their biological characteristics. An overview of potential advantages and disadvantages of using *E. coli* as a source of recombinant proteins is provided in Table 3.6.

### 3.4 Heterologous production in bacteria other than *E. coli*

A wide range of bacteria can serve as alternative expression systems to *E. coli*. Bacteria which are listed as GRAS (generally recognized as safe; see Chapter 5) serve as obvious attractive alternative production systems. Various proteins have been produced, at a research level at least, in food bacteria such as *Lactococcus lactis* and *Corynebacterium glutamicum*.

Heterologous protein production in species of *Streptomyces* (Gram-positive soil microbes) have also been reported. These represent non-pathogenic, relatively well characterized bacteria with the capacity to secrete large quantities of protein extracellularly. However, in most cases reported recombinant expression levels were disappointingly low (usually below 1g/L of fermentation media).

Recombinant protein production in additional Gram-positive bacteria, most notably in various bacilli, has also been undertaken. Bacilli naturally secrete various proteins in large quantities into their extracellular environment and many such proteins have found industrial use. In the region of 50% of commercially available industrial enzymes are produced by bacilli (see Chapters 12 and 13). Considerable experience has also accumulated with regard to their industrial-scale culture and their genetic manipulation. The main protein export pathways, most notably the Sec-SRP export pathway, are well understood. Traditionally, the main disadvantage of utilizing bacilli is their tendency to synthesize high levels of endogenous extracellular proteases which can potentially degrade the heterologous protein. However, various engineered strains have been developed in which the genes coding for such proteases have been removed/inactivated and such strains may be used for recombinant protein production purposes. A number of recombinant industrial enzymes are now produced using engineered bacilli (see Chapter 12, Boxes 12.3 and 12.5).

### 3.5 Heterologous protein production in yeast

Yeast cells have also become important host organisms for production of heterologous proteins. Yeasts are attractive hosts for several reasons:

- they retain many of the advantages as outlined for *E. coli* expression systems, such as rapid growth to high cell densities on inexpensive media;
- many are GRAS listed;
- many have played a central role in a range of traditional biotechnological processes such as brewing and baking and as a result a wealth of technical data has accumulated with regard to their fermentation and manipulation;
- the molecular biology of yeasts has been a focus of scientific research over a number of years;
- yeast cells, unlike bacteria such as *E. coli*, possess subcellular organelles and are thus capable of carrying out PTMs of proteins.

Initially, the majority of heterologous proteins engineered in yeast were produced in *Saccharomyces cerevisiae*. Because of its traditional industrial importance, this yeast is among the best studied of all organisms. Indeed, the first vaccine produced by recombinant DNA methods to be administered to humans (hepatitis B surface antigen; Chapter 6) was produced in this host.

Although many heterologous proteins have been successfully produced in *Saccharomyces cerevisiae* (Table 3.7), this system is also subject to a number of drawbacks (Table 3.8).

- Expression levels of heterologous proteins are often low, typically representing less than 5% of total cellular protein. Such values compare unfavourably to heterologous protein production in *E. coli*.
- Not all recombinant proteins are successfully secreted, with retention of highly expressed proteins in the endoplasmic reticulum (ER) representing a bottleneck in many cases.
- Many heterologous proteins produced and secreted by *Saccharomyces cerevisiae* are not released into

**Table 3.7** Selected proteins of therapeutic importance which have been produced (at a research level at least) by recombinant means in yeast.

Therapeutic protein	Yeast-based expression system
Hepatitis B surface antigen	<i>Saccharomyces cerevisiae</i> , <i>Pichia pastoris</i> , <i>Hansenula polymorpha</i>
Influenza viral haemagglutinin	<i>S. cerevisiae</i>
Polio viral protein, VP2	<i>S. cerevisiae</i>
Insulin	<i>S. cerevisiae</i>
Human growth hormone	<i>S. cerevisiae</i>
Antibodies/antibody fragments	<i>S. cerevisiae</i>
Human nerve growth factor	<i>S. cerevisiae</i>
Human epidermal growth factor	<i>S. cerevisiae</i>
Interferon- $\alpha$	<i>S. cerevisiae</i>
Interleukin-2	<i>S. cerevisiae</i>
Human factor XIII	<i>S. cerevisiae</i> , <i>Schizosaccharomyces pombe</i> , <i>P. pastoris</i>
Human plasmin	<i>P. pastoris</i>
Human serum albumin	<i>S. cerevisiae</i> , <i>H. polymorpha</i> , <i>Kluyveromyces lactis</i>
Hirudin	<i>S. cerevisiae</i> , <i>H. polymorpha</i>
$\alpha_1$ -Antitrypsin	<i>S. cerevisiae</i>
Tissue plasminogen activator	<i>S. cerevisiae</i> , <i>K. lactis</i>
Streptokinase	<i>P. pastoris</i>
Tumour necrosis factor	<i>P. pastoris</i>
Tetanus toxin fragment C	<i>P. pastoris</i>

**Table 3.8** Some advantages and disadvantages of heterologous protein production in yeast.

#### Advantages

Most are GRAS listed  
Proven history of use in many biotechnological processes  
Fermentation technology is well established  
Ability to carry out post-translational modifications of recombinant proteins

#### Disadvantages

Recombinant proteins usually expressed at relatively low levels  
Retention of many exported proteins in the periplasmic space  
Some post-translational modifications differ significantly from those achieved by animal cells

the culture medium but are retained in the periplasmic space. This is especially true in the case of heterologous proteins of high molecular mass. In such cases downstream processing is rendered more complicated.

- Although yeast systems have the ability to carry out PTMs such as glycosylation, the level/detail of glycosylation in particular does not closely resemble the modifications observed in native mammalian proteins. Traditional yeast-based systems are therefore not used to produce glycosylated therapeutic proteins (see Chapter 2).

While *Saccharomyces cerevisiae* remains the most popular yeast host, several other yeasts are also utilized in the production of heterologous proteins. *Kluyveromyces lactis* for example is used to produce a commercialized recombinant chymosin enzyme (see Chapter 12, Box 12.2). Additional alternatives include *Hansenula polymorpha*, *Schizosaccharomyces pombe*, *Schwanniomyces occidentalis*, *Yarrowia lipolytica* and in particular *Pichia pastoris*.

*Pichia pastoris* is among a limited number of yeasts capable of utilizing methanol as sole carbon source. Its methanol utilization pathway contains two alcohol oxidase genes (*AOX1* and *AOX2*) and *AOX1* protein levels in particular can reach over 30% of total soluble protein in the cell. *AOX1* expression is under the control of a strong and tightly regulated *AOX1* promoter, and this promoter is used in recombinant systems to drive high-level protein synthesis. Such recombinant systems are also usually designed so as to incorporate a protein export sequence on the recombinant protein, thus ensuring its extracellular export which simplifies subsequent protein recovery.

## 3.6 Heterologous protein production in fungi

Filamentous fungi populate various diverse natural habitats and exhibit great metabolic flexibility. As such, they produce a diverse range of proteins and many fungal-derived enzymes in particular have found biotechnological application. Fungal species

such as *Aspergillus niger* and *Aspergillus oryzae* naturally produce various extracellular enzymes at very high levels (up to 20 g/L in some cases when grown under optimized fermentation conditions) and many of these enzymes have been commercialized. Examples include various cellulases, amylases, proteases, phytases and lipases (see Chapters 12 and 13). Filamentous fungi also represent attractive potential hosts for heterologous gene expression for a number of reasons.

- Many are GRAS listed and have been extensively employed on an industrial scale for many decades in the production of a variety of non-recombinant enzymes as well as other primary and secondary products of metabolism (e.g. vitamins, various organic acids, antibiotics and alkaloids).
- Because of their industrial significance, large-scale fermentation systems for these fungi have been long since developed and optimized.
- They have an unparalleled natural capacity to secrete large quantities of certain proteins into the extracellular medium.
- They possess the enzymatic capability to carry out PTMs (although, as in the case of yeasts, their glycosylation capacity differs from that characteristic of mammalian cells and hence are unsuited to the production or recombinant glycosylated proteins for pharmaceutical use).

A wide variety of industrially/medically important proteins have now been produced as heterologous products in a number of fungal species, at a research level at least, most notably in various *Aspergillus* species (Table 3.9). Some have been commercialized (see Chapter 12, Box 12.8). Strong fungal promoters, for example the *A. niger* glucoamylase gene (*glaA*) promoter and the *A. nidulans* alcohol dehydrogenase gene (*alcA*) promoter, have been used in expression vectors in an attempt to maximize protein production. While the resultant protein products with suitable signal sequences have invariably been secreted into the medium in biologically active form, expression levels are often disappointing. High flux of protein synthesis through the ER, as would be characteristic of recombinant production, contributes to incorrect

**Table 3.9** Some proteins of industrial significance which have been expressed in recombinant fungal systems.

Protein	Organism
Human interferons	<i>Aspergillus niger</i> , <i>Aspergillus nidulans</i>
Bovine chymosin	<i>A. niger</i> , <i>A. nidulans</i>
Aspartic proteinase (from <i>Rhizomucor miehei</i> )	<i>Aspergillus oryzae</i>
Triglyceride lipase (from <i>Rhizomucor miehei</i> )	<i>A. oryzae</i>
Lactoferrin	<i>A. oryzae</i> , <i>A. niger</i> , <i>A. nidulans</i>
Thaumatococin	<i>A. oryzae</i> , <i>A. niger</i> , <i>Aspergillus awamori</i>
Tissue plasminogen activator	<i>A. nidulans</i>
Antibody fragments, various	<i>A. oryzae</i> , <i>A. niger</i>
Proteases, various	<i>A. oryzae</i> , <i>A. niger</i> , <i>A. awamori</i> , <i>A. nidulans</i>

protein folding, delayed folding and/or protein misassembly, triggering ER-associated protein degradation. This so-called 'ER stress' likely generates a bottleneck in terms of successful protein export.

Also contributing to low expression levels is the propensity of such filamentous fungi to produce high levels of various extracellular proteases, a consequence of their saprophytic lifestyle. Many heterologous proteins secreted extracellularly would be susceptible to proteolytic degradation. This difficulty has largely been overcome by the development of low-protease strains, generated via both classical mutagenesis and targeted gene disruption approaches (see Chapter 5).

*Trichoderma* represents another genus of filamentous fungi whose saprophytic lifestyle underpins significant metabolic diversity and the ability to naturally produce a wide range of extracellular enzymes in particular. Several species, most notably *T. reesei*, have long been used industrially for the production of non-recombinant extracellular enzymes, mainly cellulases and hemicellulases (see Chapter 12). Their overall characteristics in terms of enzyme production mirror those already discussed in the context of *Aspergillus*. A wide range of enzymes and other proteins have also been produced via recombinant

means in *Trichoderma*. However, recombinant expression levels achieved have been disappointingly low, rarely exceeding 1–2 g/L of fermentation media. In consequence, *Trichoderma* is rarely used as a host for recombinant protein expression at an industrial level, although at least one strain has found application in this regard (see Chapter 12, Box 12.4).

### 3.7 Proteins from plants

Plants represent a traditional source of a wide range of biologically active molecules. Narcotics such as opium are among the best known of such products. Crude opium consists of the dried milky exudate obtained from unripe capsules of certain species of plants mainly found in parts of Asia, India and China. The most important medical constituents of opium are the alkaloids, the best known of which is morphine. Morphine is extracted and purified from crude opium preparations, generally by ion-exchange chromatography.

For a number of reasons higher plants are not prolific producers of many commercially important proteins.

- Many industrially important proteins synthesized in plants are also found in other biological sources. In most cases, the alternative source becomes the source of choice for both technical and economic reasons (e.g. higher expression levels).
- Plant growth is seasonal in nature and hence a constant source of material is not always obtainable.
- Higher plants also tend to accumulate waste substances in structures termed vacuoles. On cell disruption these wastes, which include a number of powerful precipitating and denaturing agents, often irreversibly inactivate many plant proteins.

Despite such drawbacks, a number of industrially important proteins are obtained from plants. Two plant proteins, monellin and thaumatin, are recognized as the sweetest known naturally occurring substances. Such proteins have actual and potential uses in the food industry and are reviewed in Chapter 14.  $\beta$ -Amylases are also produced by many higher plants, the most widely used being obtained

from barley. These enzymes play an important role in the starch processing industry (see Chapter 12). However, perhaps the best-known plant-derived protein produced on an industrial scale is still the proteolytic enzyme papain.

Papain, also known as vegetable pepsin, is obtained from the latex of the green fruit and leaves of *Carica papaya*. It was first isolated and characterized in 1937. Papain is a cysteine protease (see Chapter 12). Its active site contains an essential cysteine residue which must remain in the reduced state if proteolytic activity is to be maintained. The purified enzyme exhibits broad proteolytic activity. It consists of a single polypeptide chain containing 212 amino acid residues, with a molecular mass of 23 kDa. The term ‘papain’ is applied not only to the purified enzyme but also to the crude dried latex. Papain has a variety of industrial applications, the best known of which is its use as a meat tenderizing agent. During the tenderization process, the proteolytic activity of papain is directed at collagen, which is the major structural protein in animals, representing up to one-third of all vertebrate protein. It is the collagen present in connective tissue and blood vessels which renders meat tough (Chapter 12). Papain has a relatively high optimum temperature (65°C) and retains activity at temperatures up to 90°C. Because of its thermal stability, papain maintains its proteolytic activity even during the initial stages of cooking. This enzyme has also been used in other industrially important processes, including:

- bating of animal skins;
- clarification of beverages;
- digestive aid;
- debriding agent (cleaning of wounds).

Ficin is another commercially available protease derived naturally from plant sources. It is generally extracted from the latex of certain tropical trees and, like papain, is a cysteine protease. It exhibits considerably higher proteolytic activity than papain and has similar industrial applications. Purified ficin has a molecular mass of about 25 kDa, though the term ‘ficin’ is applied not only to the purified enzyme but also to the crude latex extract. Most



large-scale industrial applications of papain and ficin do not require highly purified enzyme preparations. Plant enzymes, in particular those destined for application in the food processing industry, must be obtained only from non-toxic edible plant species.

### 3.7.1 Production of heterologous proteins in plants

Advances in recombinant DNA technology facilitates genetic manipulation not only of microorganisms but also of eukaryotic cells. Many heterologous proteins are now produced in a variety of plant-based systems, at a research level at least. Genetic manipulation of plant systems may be undertaken for a number of reasons. Introduction of foreign genes or cDNAs may be attempted in order to confer a novel function or ability on the resultant species. Novel DNA sequences may be introduced into plant cells by several means. These include use of *Agrobacterium* as a carrier or by direct injection of the DNA into certain plant cells. Using such techniques, plants can be engineered to produce

insecticides, for example, which when expressed may play a protective role. Plants may also be used to produce heterologous proteins of applied interest (Table 3.10).

Recombinant proteins produced by this strategy could be utilized in one of two ways: (i) the protein could be extracted from the plant tissue, purified (if necessary) and then used for its applied purpose; or (ii) the recombinant plant tissue could be used directly as the protein source, for example enzymes added to animal feed and which are currently produced by microbial fermentation (Chapter 13) could be expressed in plant seeds, which could then be fed directly to the animals.

Tobacco plants (*Nicotiana tabacum*) are most commonly used in the context of plant-based heterologous protein production, for a number of reasons.

- Tobacco is often used as a model system in the study of plant science and genetics and therefore its genetics are well understood.
- Various expression systems have been developed that facilitate either transient or constitutive protein production.
- It generates a high biomass yield (up to 100 tonnes leaf biomass per hectare).

**Table 3.10** Some proteins of industrial/medical interest which have been produced by recombinant means in plants.

Protein	Original source	Expressed in	Production level achieved
$\alpha$ -Amylase	<i>Bacillus licheniformis</i>	Tobacco	0.3% of soluble leaf protein
Chymosin	Calf	Tobacco	0.5% of soluble protein
Cyclodextrin glycosyltransferase	<i>Klebsiella pneumoniae</i>	Potato	0.01% of soluble tuber protein
Erythropoietin	Human	Tobacco	0.003% of soluble protein
Glucoamylase	<i>Aspergillus niger</i>	Potato	Not reported
Growth hormone	Trout	Tobacco	0.1% of soluble leaf protein
Hepatitis B surface antigen	Hepatitis B virus	Tobacco	0.007% of soluble leaf protein
Hirudin	<i>Hirudo medicinalis</i> (a leech)	Canola	1.0% of seed weight
Interferon- $\beta$	Human	Tobacco	0.00002% of fresh weight
Lysozyme	Chicken	Tobacco	0.003% of leaf tissue
Phytase	<i>Aspergillus niger</i>	Tobacco	14.4% of soluble leaf protein
Serum albumin	Human	Potato	0.02% of soluble leaf protein
Xylanase	<i>Clostridium thermocellum</i>	Tobacco	4.1% of soluble leaf protein

**Table 3.11** Major advantages and disadvantages of recombinant protein production in transgenic plants.**Advantages**

Likely economically attractive production costs  
 Ease of scale-up  
 Availability of established practices/equipment for plant harvesting/storage  
 Elimination of downstream processing requirements if the plant material containing the recombinant protein can be used directly as the protein source  
 Ability to target protein production/accumulation to specific plant tissue  
 Ability to carry out post-translational modifications  
 Plants are not known to harbour human pathogens

**Disadvantages**

Low expression levels often reported  
 Glycosylation pattern achieved usually different from that observed on animal glycoproteins  
 Lack of industrial experience/data on large-scale downstream processing of plant tissue  
 Seasonal/geographic nature of plant growth  
 Presence of toxic substances in plant cell vacuoles  
 Availability of established, alternative production systems  
 Environmental/public concerns relating to accidental environmental release/entry into food/feed chain

- It naturally produces high levels of soluble proteins.
- It is not used as a food/feed crop, minimizing the potential for accidental contamination of the food/feed chain with genetically modified plant material.
- Low-nicotine varieties are available which are suitable for the production of recombinant proteins for direct consumption, as an oral vaccine for example.

Again, recombinant protein production in plants displays both disadvantages and advantages (Table 3.11). Some of the disadvantages mirror those listed earlier in relation to direct extraction of native plant proteins for applied uses. Additional disadvantages include low expression levels, issues relating to PTM of proteins and lack of industrial experience with regard to extraction and purification of recombinant proteins from plants.

In most cases thus far reported the recombinant protein expression levels achieved have been disappointingly low (Table 3.10). In order to approach commercial viability, the recombinant protein

should accumulate in the plant tissue at levels representing at least a few per cent of total soluble proteins (TSP). To date in most cases expression levels achieved have fallen below 1% TSP. However, production levels achieved in the case of phytase (14.4% TSP) and xylanase (4% TSP) illustrate that high expression values are attainable.

Plant-based expression systems achieve glycosylation patterns which differ (in extent and composition) to those achieved by animal cells. This point is important if an altered glycosylation pattern in any way negatively influences the recombinant protein product. This is especially important in the context of therapeutically important glycoproteins, where an altered glycosylation pattern could influence product safety and/or efficacy (as described in Chapter 2, section 2.9.6).

Lack of industrial history or experience of large-scale recombinant protein production in plants is also a disadvantage from a practical standpoint. In addition, most companies producing proteins commercially do so using microbial or animal cell culture (or extraction from whole animal tissue). These companies have invested heavily in dedicated production equipment which would be redundant in the context of plant-based expression systems. Furthermore, in the context of therapeutic proteins, regulatory authorities would have to be satisfied that plant expression systems yield safe and therapeutically effective products.

Despite such potential disadvantages, a number of strong arguments can be made in favour of plant-based recombinant expression systems. Once the transgenic plants have been generated (and are expressing the recombinant product at satisfactory levels), upstream processing costs (i.e. growth of the plants) would be low. Also, scale-up of production would simply involve increasing the acreage of the crop sown. Protein production would be independent of expensive facilities/production equipment and harvesting would be straightforward using pre-existing harvesting equipment. Depending on the expression levels achieved and the variety of plant used, it has been estimated that upstream processing (i.e. growing) costs for recombinant protein synthesis in plant-based systems would be 10–50 times cheaper than costs if recombinant

*E. coli* was used. This would be economically significant, particularly if the protein is price-sensitive and subsequent downstream processing is minimal (e.g. if the harvested plant could be used directly as the recombinant protein source, without any purification). For proteins which require significant downstream processing (e.g. therapeutic proteins, which need to be purified to homogeneity), the overall savings would be far less significant. In such cases downstream processing costs would typically represent 80% or more of total production costs. Production of the recombinant protein in plants would provide no downstream processing cost benefits over other expression systems.

The ability to target expression of recombinant proteins to a specific plant tissue can also be advantageous. It could reduce the potential toxicity of the protein (for the plant) and reduce environmental/regulatory concerns (e.g. over release of the protein into the general environment via plant pollen). Targeted accumulation of the protein in plant seed is particularly attractive. The seeds of higher plants naturally contain high levels of storage protein. Seeds can be stored for extended periods after harvest, inexpensively and without causing protein degradation. In contrast, green plant tissue generally deteriorates rapidly after harvest. Recombinant production in green tissue would thus require immediate protein extraction after harvest, or storage/harvest under (expensive) refrigeration or frozen conditions.

Leu-enkephalin is an early example of a therapeutic product successfully produced in transgenic plants. This was achieved by inserting its DNA coding sequence into the gene coding for a seed storage protein termed 2S albumin. The family of 2S albumins are among the smallest seed storage proteins known, having a molecular mass in the order of 12kDa. This family of proteins are derived from a group of structurally related genes, all of which exhibit both conserved and variable sequences. The variable regions vary not only in sequence but also in length. The strategy employed to produce leu-enkephalin involved substituting part of this variable sequence with a DNA sequence coding for the five-amino-acid neurohormone. The DNA

construct was flanked on both sides by nucleotide sequences coding for tryptic cleavage sites. Expression of the altered 2S albumin gene resulted in production of a hybrid storage protein containing the leu-enkephalin sequence. The enkephalin was subsequently released from the altered protein by tryptic cleavage and purified by HPLC. Because of the incorporation of the tryptic cleavage sites, the purified product contained an extra lysine residue, which was subsequently removed by treatment with carboxypeptidase C, a proteolytic enzyme that hydrolyses only the peptide bond at the carboxyl terminus of a peptide/polypeptide. Since then, a number of larger polypeptides have also been expressed in the seeds of various plant varieties.

A small number of proteins produced by recombinant means have made it to market. These include CaroRx and human intrinsic factor. CaroRx is an antibody produced in transgenic tobacco plants. It binds to *Streptococcus mutans*, a primary causative agent of bacterial tooth decay. Product application to the tooth surface can prevent bacterial adherence, hence reducing the incidence of dental caries. Recombinant human intrinsic factor produced in the plant *Arabidopsis thaliana* can be used as a dietary supplement for the treatment of vitamin B<sub>12</sub> deficiency. Additional examples include recombinant chicken egg white avidin, which is used as a diagnostic reagent and which is produced in engineered maize seeds, as well as recombinant bovine trypsin, also produced in maize and which is used for research and processing purposes.

Concerns relating to accidental release of genetically modified crops into the environment and/or accidental entry into the food/feed chain have also accelerated the development of plant cell culture-based systems for the production of recombinant proteins. One such product (Elelyso, also known as taliglucerase alfa) became the first ever true biopharmaceutical produced in a plant-based system to gain approval for general medical use. The product is a recombinant human glucocerebrosidase used as a replacement therapy to treat Gaucher disease, a rare lysosomal storage disorder (see Chapter 6). It is produced in engineered carrot root cell culture. Additional novel plants that can be cultured under contained conditions are also being

developed as potential recombinant protein production systems, including the moss *Physcomitrella patens* and the aquatic duckweed *Lemna minor*.

### 3.8 Animal tissue as a protein source

A wide variety of commercially available proteins are obtained from animal sources. This is particularly true with regard to numerous therapeutic proteins such as insulin and blood factors. The existence of slaughterhouse facilities in which large numbers of animals are regularly processed has traditionally facilitated the collection of significant quantities of the particular tissue required as protein source.

Perhaps the best-known protein obtained from animal sources is insulin. Until the early 1980s insulin was obtained exclusively from pancreatic tissue derived from slaughterhouse cattle and pigs. The amount of insulin obtained from the pancreatic tissue of three pigs satisfies the requirements of one diabetic patient for approximately 10 days. The increasing worldwide incidence of diabetes raised fears that one day demand for insulin supplies could exceed supply from slaughterhouse sources. This is no longer of concern, however, as potentially unlimited supplies of insulin are now produced by recombinant means. Some additional examples of

commercially available hormones obtained by direct extraction from animal sources are listed in Table 3.12.

Most industrially significant proteins obtained from human and other animal sources are destined for therapeutic use. One disadvantage concerning such sources relates to the potential presence of pathogens in the raw material. The large numbers of haemophiliacs who contracted AIDS from HIV-infected blood transfusions in the 1980s stand as testament to this fact. Outbreaks of bovine spongiform encephalopathy (BSE or mad cow disease) in cattle herds from various countries serve as another example. A number of precautions must thus be taken when animal tissue is used as a protein source. The most obvious involves the use of tissue obtained only from disease-free animals. Downstream processing procedures employed in purifying the protein of interest must also be validated, thereby showing that the purification steps employed can eliminate the pathogens that may be present in the starting material (see Chapter 5).

Many pathogens, in particular viral pathogens, exhibit marked species specificity. Thus therapeutic proteins obtained from a particular animal species are not usually administered to other animals of that same species. For example, purified follicle-stimulating hormone used to superovulate cattle is usually sourced from porcine and not bovine pituitary glands.

**Table 3.12** Some proteins of industrial/medical significance which have been traditionally obtained from animal sources. Many of the listed examples may now also be obtained via recombinant production.

Protein	Source	Application
Insulin	Porcine/bovine pancreatic tissue	Treatment of diabetes mellitus
Glucagon	Porcine/bovine pancreatic tissue	Reversal of insulin-induced hypoglycaemia
Follicle-stimulating hormone (FSH)	Porcine pituitary glands Urine of postmenopausal women	Induction of superovulation in animals Treatment of (human) reproductive dysfunction
Human chorionic gonadotrophin	Urine of pregnant women	Treatment of reproductive dysfunction
Erythropoietin	Urine	Treatment of anaemia
Blood factors	Human plasma	Treatment of haemophilia
Polyclonal antibodies	Human/animal blood	Various diagnostic/therapeutic applications
Chymosin (rennin)	Stomach of (unweaned) calves	Cheese manufacture

### 3.9 Heterologous protein production in transgenic animals

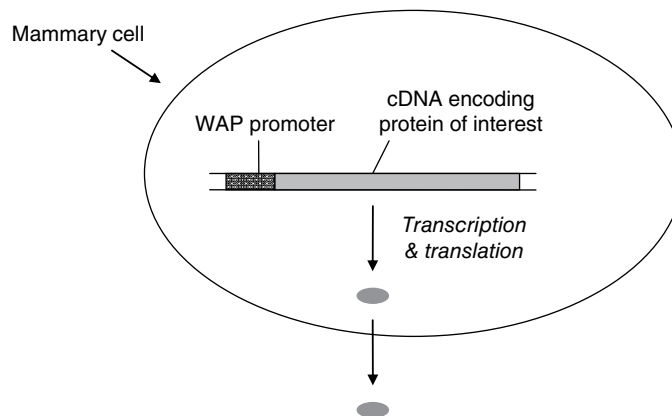
Initial experiments introducing foreign genes into the genome of animals focused on attempts to improve various animal characteristics. It was hoped that, for example, animal growth rates could be dramatically improved by genomic integration of extra copies of the growth hormone gene. Consequently, one goal of such 'molecular farming' is the introduction of specific functional genes into animals, thereby conferring on them desirable characteristics such as more efficient feed utilization, improved growth characteristics or generation of leaner meat.

Another goal of such transgenic technology is to confer on the transgenic animal the ability to produce large quantities of industrially important proteins. This has been achieved with varying degrees of success in mice, goats, sheep and cattle. Initial successes in expressing high levels of growth hormone in transgenic animals highlighted some potential problems associated with this technique. In many cases it was found that chronically high circulatory levels of growth hormone (significantly outside the normal physiological range) resulted in many adverse physiological effects. Elevated circulatory levels of many proteins of potential

therapeutic value would also almost certainly promote similar adverse effects on normal transgenic animal metabolism. Specific animal tissues were also targeted as heterologous protein expression sites. One such tissue target is the mammary gland. By targeting expression of the foreign gene to the mammary gland, the heterologous protein may be secreted directly into the milk and thus physically removed from the animal's circulatory system. Expression of the gene of interest can be targeted to this tissue by fusing the gene to the signal sequence of a milk protein.

The earliest success involving the production of a heterologous protein of considerable therapeutic potential was recorded in the mid-1980s, when the production of human tissue plasminogen activator (tPA, see Chapter 6) in the milk of transgenic mice was reported (Figure 3.6). This was achieved by injecting into mice embryos a DNA construct consisting of the promoter and upstream regulatory sequence from the mouse whey acidic protein gene fused to the gene coding for human tPA. Whey acidic protein is the most abundant whey protein found in mice milk. Biologically active tPA was recovered from the milk of the resultant transgenic mice.

While various pharmaceutically important proteins have been produced in the milk of transgenic animals (Table 3.13), only two such products have gained regulatory approval for general medical use.



**Figure 3.6** Generalized schematic diagram of the production of a recombinant protein in the milk of transgenic mice. Refer to text for details.

**Table 3.13** Some proteins of therapeutic use which have been expressed in the milk of transgenic animals.

Protein	Produced in	Use
$\alpha_1$ -Antitrypsin	Sheep	Emphysema (some forms)
Factor IX	Sheep	Haemophilia B
Fibrinogen	Sheep	Blood disorders
Antithrombin III	Goats	Blood disorders
Human C1 esterase inhibitor	Rabbits	Angioedema
Various monoclonal antibodies	Goats	Various, including <i>in vivo</i> tumour detection
Human serum albumin	Cows	Plasma volume expander

- ATryn, a recombinant human antithrombin molecule, is used to prevent blood clotting in antithrombin-deficient patients undergoing surgery. It is produced in the milk of transgenic goats.
- Ruconest, a recombinant form of human C1 esterase inhibitor, is used to treat acute angioedema (caused by a genetic mutation in the C1 esterase inhibitor gene). It is produced in the milk of transgenic rabbits.

Apart from tPA and  $\alpha_1$ -antitrypsin, various other heterologous proteins have been successfully produced in milk. Such production systems have a number of potential advantages over alternative production methods such as animal cell culture (see section 3.10). Desirable features include the following.

- *High production capacities.* During a typical 5-month lactation period, one sheep for example can produce 2–3 L of milk daily. If the recombinant protein is expressed at a level of 1 g/L, a single sheep could produce in excess of 20 g product per week.
- *Ease of collection of source material.* This only requires the animal to be milked. Commercial automated milking systems are readily available. Such systems require only moderate design alterations as they are already designed to maximize hygienic standards during the milking process.

- *Low capital investment requirements and low operational costs.* Traditional production methods yielding recombinant proteins require considerable expenditure on fermentation equipment. Using this technology such costs are reduced to raising and maintaining the transgenic herds.
- *Ease of production scale-up.* Producer animal numbers can be expanded by breeding programmes.

However, the approach also suffers from some disadvantages:

- yields of heterologous proteins are often extremely variable, in some cases less than 1 mg/L having been recorded;
- availability of alternative expression systems, including animal cell culture, which are technically more straightforward.

### 3.10 Heterologous protein production using animal cell culture

Animal cell culture represents an important source of many proteins, virtually all of which are destined for healthcare application. Almost 40% of all recombinant therapeutic proteins approved to date for general medical use are produced in such cells. Monoclonal antibodies are among the best-known examples (Table 3.14).

The culture of mammalian cells is technically more challenging and economically more costly than microbial cell culture, ultimately because they are larger, more complex and more fragile (because they lack a cell wall). Specifically, when compared with microbial cells, animal cells:

- grow more slowly (the doubling time of many bacteria can be as little as 20–30 minutes, whereas it is more typically 18–30 hours in the case of mammalian cells);
- reach lower cell densities in culture (typically in the  $10^6$ /mL range as opposed to  $10^9$ /mL range in the case of *E. coli*);

**Table 3.14** Some recombinant pharmaceutical proteins approved for general medical use which are produced commercially via animal cell culture.

Protein	Produced in	Medical application
Factor VIII	CHO cells, BHK cells	Haemophilia A
Factor IX	CHO cells	Haemophilia B
tPA	CHO cells	Heart attacks
FSH	CHO cells	Infertility
Erythropoietin	CHO cells	Anaemia
Interferon- $\beta$	CHO cells	Multiple sclerosis
Several monoclonal antibodies	Various	Various, including prevention of kidney transplant rejection and localization or treatment of tumours <i>in vivo</i>

CHO, Chinese hamster ovary; BHK, baby hamster kidney; tPA, tissue plasminogen activator; FSH, follicle-stimulating hormone.

- require a far more complex cocktail of nutrients in the culture media;
- achieve lower levels of recombinant protein production.

Therefore, microbial-based expression systems will invariably be chosen ahead of mammalian cell culture-based expression systems for recombinant protein production as a default option. However, microbial systems are either incapable of undertaking protein post-translational modification (bacteria) or undertake PTMs with altered structural detail (e.g. yeast/fungal glycosylation patterns), when compared with mammalian systems. Moreover, many microbial systems (e.g. *E. coli*) often fail to achieve high-level extracellular production of larger or multi-subunit proteins. Thus, the use of mammalian-based expression systems can be desirable/necessary to produce biologically active, larger, complex proteins, particularly if they are glycosylated. Many therapeutically important proteins fall into this category (see Chapters 6–9).

The most common mammalian cell lines used in the industrial-scale production of recombinant therapeutic proteins include Chinese hamster ovary (CHO) cells, various murine myeloma cell lines (in particular NS0 and SP2/0), as well as baby hamster kidney derived (BHK) cells. The availability of high expression vectors and the optimization of cell culture media and conditions now facilitate mammalian

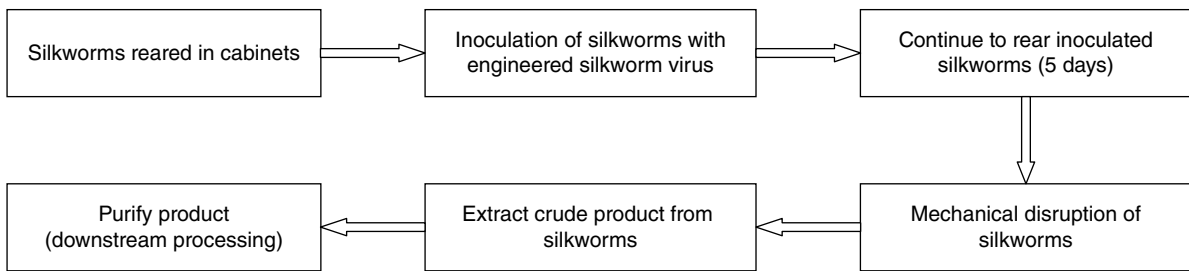
cell-based extracellular recombinant expression levels approaching 5 g/L, a 50-fold increase over the typical expression levels achievable in such systems 20 years ago.

### 3.11 Insect cell culture systems

A wide range of proteins have been produced at laboratory scale, at least in recombinant insect cell culture systems. The approach generally entails the infection of cultured insect cells with an engineered baculovirus (viral family that naturally infects insects) carrying the gene coding for the desired protein placed under the influence of a powerful viral promoter. Among the systems most commonly employed are:

- the silkworm virus, *Bombyx mori* nuclear polyhedrosis virus (BmNPV) in conjunction with cultured silkworm cells (i.e. *Bombyx mori* cells) or;
- the virus *Autographa californica* nuclear polyhedrosis virus (AcNPV), in conjunction with cultured armyworm cells (*Spodoptera frugiperda* cells).

Baculovirus/insect cell-based systems display a number of potential production advantages.



**Figure 3.7** Overview of the industrial production of the IFN- $\omega$  product Vibragen Omega. Refer to text for details.

- High-level intracellular recombinant protein expression. The use of powerful viral promoters such as those derived from the viral polyhedrin or P10 genes can drive recombinant protein expression levels to 30–50% of total intracellular protein.
- Insect cells can be cultured more rapidly and using less expensive media compared with mammalian cell lines.
- Human pathogens (e.g. HIV) do not generally infect insect cell lines.

However, a number of disadvantages are also associated with this production system.

- Targeted extracellular recombinant production generally results in low-level extracellular accumulation of the desired protein (often in the mg/L range). Extracellular production simplifies subsequent downstream processing.
- Post-translational modifications, in particular glycosylation patterns, can be incomplete and/or can differ very significantly from patterns associated with native human glycoproteins. Insect cell-synthesized sugar side chains tend to be high in mannose, devoid of sialic acids and are often simple short structures.

Therapeutic proteins successfully produced on a laboratory scale in insect cell lines include hepatitis B surface antigen, interferon- $\gamma$  and tPA. To date two therapeutic products produced by such means have been approved for human use: Cervarix is a subunit cervical cancer vaccine (Chapter 6), while Provenge is a colony-stimulating factor fusion protein used to treat prostate cancer.

Two recombinant veterinary vaccines (Bayovac CSF E2 and Porcilis Pesti) are also produced using insect cell-based production systems. Both contain the E2 surface antigen protein of classical swine fever virus as active ingredient. The vaccines are administered to pigs in order to immunize against classical swine fever.

An alternative insect cell-based system used to achieve recombinant protein production entails the use of live insects. Most commonly live caterpillars or silkworms are injected with the engineered baculovirus vector, effectively turning the whole insect into a live bioreactor. One veterinary biopharmaceutical, Vibragen Omega, is manufactured using this approach and an overview of its manufacture is outlined in Figure 3.7. Briefly, whole live silkworms are introduced into pre-sterile cabinets and reared on laboratory media. After 2 days, each silkworm is inoculated with engineered virus using an automatic microdispenser. This engineered silkworm polyhedrosis virus harbours a copy of cDNA coding for feline interferon- $\omega$ . During the subsequent 5 days of rearing, a viral infection is established and hence recombinant protein synthesis occurs within the silkworms.

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# Chapter 4

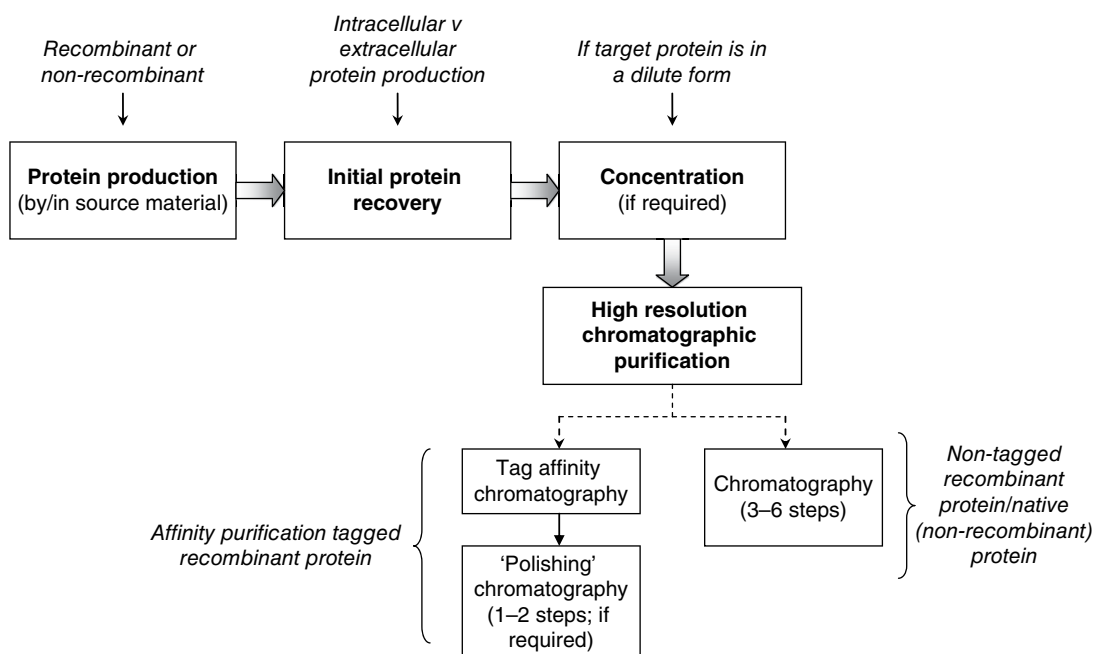
## Protein purification and characterization

As outlined in the previous chapter, proteins can be obtained from a wide variety of sources. Despite such diversity of origin, proteins (both native and recombinant) derived from any such source are usually purified using a similar overall approach and similar techniques (Figure 4.1). The exact details of the purification scheme for any given protein will depend on a number of factors, including:

- level of expression of target protein;
- exact source material chosen and location of the target protein (extracellular or intracellular);
- presence of a fusion tag on a recombinant protein designed to facilitate affinity purification;
- physicochemical characteristics of the protein;
- purpose of purification.

The source material chosen will dictate the range and type of contaminants present in the starting material as well as the amount of target protein present therein. In most instances recombinant production using a powerful expression system ensures high-level protein expression in comparison with target protein levels present in the native (non-recombinant) source material.

Extracellular versus intracellular production will also influence the initial stages of the purification strategy applied: if the protein is an intracellular one, initial protein recovery requires cellular disruption. The level of expression of the target protein will also bear on the purification protocol. Low-level expression, for example, may necessitate extraction of very large quantities of source material, which subsequently requires significant concentration before chromatographic purification. On the other hand, high-level expression may render redundant an initial concentration step. The physicochemical properties of the protein will have an obvious influence on the purification protocol. Proteins are fractionated from each other on the basis of differences in physicochemical characteristics such as solubility, size, surface hydrophobicity and charge (Table 4.1). In the case of most recombinant proteins purification affinity tags are attached, via protein engineering, in order to confer some very pronounced physicochemical characteristic on the protein (see also Box 3.1). This generally renders separation of the target protein from contaminants more straightforward, usually simplifying the purification protocol required.



**Figure 4.1** Generalized approach used to extensively purify a protein.

**Table 4.1** Chromatographic techniques most commonly applied to protein purification protocols. The basis of separation is listed in each case.

Technique	Basis of separation
Ion-exchange chromatography	Differences in protein charge at a given pH
Gel filtration chromatography	Differences in mass/shape of different proteins
Affinity chromatography	Biospecific interaction between a protein and an appropriate ligand
Hydrophobic interaction chromatography	Differences in surface hydrophobicity of proteins
Chromatofocusing	Separates proteins on the basis of their isoelectric points
Hydroxyapatite chromatography	Complex interactions between proteins and the calcium phosphate-based media. Not fully understood

An additional factor that significantly influences the purification protocol is the purpose for which the protein is being purified. If this is purely academic, purification to homogeneity is usually the most important goal, with issues such as the number of steps required, duration of procedure, cost and percentage yield of final product being of secondary importance. If the protein is being purified for a commercial application, economic as well as technical factors will be of concern, and the

protein will generally be purified only to the minimal level required (see Chapter 5). The quantity of protein required will also influence the protocol developed. Academic purposes usually require the generation of pure protein in the milligram range in order to support functional/structural studies. Proteins destined for applied purposes, on the other hand, are usually required in much greater quantities (Table 4.2), and the level of purity demanded will vary according to application.

**Table 4.2** Typical quantities of any given protein required to meet the indicated academic/applied purposes.

Purpose	Quantity of protein typically required
Mass spectrometry studies	Nanograms
Electrophoretic analysis	Micrograms
General functional/physicochemical studies	Milligrams to grams
Specialized commercial proteins used for research-related applications	Hundreds of milligrams to hundreds of grams (moderately purified)
Commercial enzymes/antibodies used for <i>in vitro</i> diagnostic purposes	Grams to kilograms (moderately/highly purified)
Proteins used for <i>in vivo</i> therapeutic purposes: treatment of low-incidence diseases (e.g. Gaucher disease)	Hundreds of grams to tens of kilograms (highly purified)
Proteins used for <i>in vivo</i> therapeutic purposes: treatment of high-incidence diseases (e.g. diabetes, haemophilia) or when high-level dosages are typically administered (e.g. most monoclonal antibodies)	Tens to hundreds of kilograms (highly purified)
Bulk industrial enzymes	More than 1000 kg (usually unpurified)
Milk-derived proteins produced by/for bulk food applications	More than 100,000 kg (purity varies with production method)

## 4.1 Protein detection and quantification

The ability to detect and quantify (i) total protein concentration and (ii) target protein levels is an essential prerequisite to the purification and characterization of any protein. Various methods may be used to determine protein concentration. The most commonly employed such methods, along with their mode of action, advantages and disadvantages, are summarized in Table 4.3. Choice of assay method is made on the basis of assay sensitivity required, presence of interfering agents in the sample and, to a certain extent, personal preference. Although difficult to generalize, absorbance at 280 nm as well as the bicinchoninic acid (BCA), Bradford and fluorescence-based assays (NonyOrange and FluoroProfile) are typically the more commonly employed quantification methods.

Detection and quantification specifically of the protein of interest may be undertaken by one of two general methods: bioassay or immunoassay. As the name implies, bioassay involves direct measurement of the biological (functional) activity of the target protein. It is defined as the quantitative measurement

of a response following the application of a stimulus (i.e. the target protein) to a biological system. The 'biological system' is most commonly microbial, animal or plant cells/tissue, or whole animals/plants, as appropriate. Some examples of specific bioassays are provided in Table 4.4.

Bioassays are generally comparative; in addition to the test sample, a 'standard' (i.e. a sample containing a known quantity of target analyte) is required. The standard will have been assigned a specific number of units of biological activity per milligram, and such standards are often commercially available. Direct comparison of the magnitude of the biological response induced by the test sample to that induced by the standard allows assignment of activity levels present in the former.

Bioassays directly detect or quantify the true biological activity of the target protein. However, assay procedures can be costly and of prolonged duration (hours/days). Furthermore, as the response measured is generated by a biological system, high inherent imprecision in the results is often a feature, often resulting in standard deviations of  $\pm 20$ –30%. The bioassay should of course also measure a biological response which is triggered uniquely by the target protein.

**Table 4.3** The various methods most commonly used to detect and quantify protein levels in a biological sample. Notable characteristics of each method are also listed.

Method	Sensitivity	Mode of action	Advantages	Disadvantages
Absorbance at 280 nm (UV method)	0.02–3.0 mg/mL	R-groups of aromatic amino acids (tyrosine, tryptophan and, to a lesser extent, phenylalanine) absorb at 280 nm	Simple and fast, non-destructive to sample	Low sensitivity, identical concentrations of different proteins will yield different absorbance values (reflection of different aromatic amino acid content)
Absorbance at 205 nm (far-UV method)	1.0–100 µg/mL	Peptide bond absorbs at this wavelength	Simple, fast, sensitive, non-destructive to sample	Not all UV-Vis spectrophotometers are capable of measuring absorbance at 205 nm. Most commonly used buffers also absorb at this wavelength, although if used at low strength (<10 mmol/L) this may not cause a problem
Acid digestion (ninhydrin method)	20–50 µg	Protein hydrolysed to constituent amino acids by incubation with H <sub>2</sub> SO <sub>4</sub> at 100°C. Amino acid content quantified by subsequent reaction with ninhydrin, forming a derivative which absorbs at 570 nm	Sensitive. Phenolic/aromatic compounds do not interfere with assay	Uses hazardous chemicals (H <sub>2</sub> SO <sub>4</sub> at 100°C), extended total assay times (up to 20 hours)
Bicinchoninic acid (BCA) method	20–100 µg/mL	Copper-containing reagent which, when reduced by protein, reacts with bicinchoninic acid yielding a derivative that absorbs maximally at 562 nm	Sensitive and convenient, less susceptible to assay interference than some other methods	Cost (expensive reagent). Departure from exact assay protocol (e.g. duration, temperature, reagent concentration) can result in variable results
Bradford method	150–750 µg/mL	Based on reversible pH-dependent binding of Coomassie Brilliant Blue G-250 dye to protein. Absorbance maximum at 595 nm	Moderately sensitive, easy and fast to run	Alkaline pH/buffers will interfere with assay. Subject to interference by detergents (e.g. sodium dodecyl sulfate)
Dry weight method	Minimum of 2–4 mg per sample	Heat protein-containing sample to 106°C for 4–6 hours (minimum), weigh dry content	Straightforward method. No chemical reagents required	Poor sensitivity. Sample must contain no non-volatile, non-protein substances
FluoroProfile® protein quantification	50 ng/mL to 200 µg/mL	Based on fluorescence of a natural product (epicocconone) when it interacts with protein	Very sensitive, large linear dynamic range	Some buffer components (CHAPS, Tris), detergents and NaCl can interfere
Fluorescence emission method	5–50 µg/mL	Based on fluorescence properties of aromatic amino acid residues in the protein	Sensitive, simple, fast, non-destructive to sample	Identical concentrations of different proteins can yield different absorbance readings due to varying aromatic amino acid content
Hartree–Lowry method	30–150 µg/mL	Combination of copper and phosphomolybdic/phosphotungstic acid reacts quantitatively with proteins, displaying an absorbance maximum at 750 nm	Good sensitivity	Assay somewhat laborious. Subject to interference by detergents and chelating agents
NanoOrange® protein quantitation assay	10 ng/mL to 10 µg/mL	Based on fluorescence of a merocyanine dye when it interacts with detergent-coated protein	Excellent sensitivity and protein to protein variability	Subject to interference by some reducing agents, salts, detergents and some buffer constituents
Silver binding procedure	150 ng/mL to 20 µg/mL	Based on binding of silver ions to proteins	Extremely sensitive	Chelating agents, detergents and reducing agents interfere with assay
Trichloroacetic acid precipitation method	Varies, depending on detection method used	Entails addition of TCA to protein sample in order to precipitate it, followed by resuspension of the protein and assay by one of the above methods	Used to remove (non-TCA precipitable) interfering agents from a protein sample before quantification	Laborious

**Table 4.4** Example bioassays designed to detect/quantify the indicated protein.

Protein	Bioassay description
Interleukin-2	Ability to promote the proliferation of activated T-lymphocytes
Interferon- $\alpha$	Ability to inhibit the cytopathic effect of certain viruses (e.g. vesicular stomatitis virus) on certain human cell lines
Granulocyte colony-stimulating factor	Ability to promote proliferation of certain animal cell lines
Tumour necrosis factor	Ability to induce cytotoxic effect on certain animal cell lines (e.g. murine fibroblast cell lines)
Erythropoietin	Ability to stimulate the proliferation of the TF-1 animal cell line
Polypeptide antibiotics	Ability to prevent growth of various indicator microorganisms

Enzymes represent a key family of proteins that continue to be a focus of scientific and applied interest (see Chapters 6 and 10–13). Assay of enzyme activity is usually achieved by incubating the catalytic protein with its substrate for a specific period of time under defined environmental conditions (of temperature and pH). Catalytic activity is calculated by monitoring either the rate of substrate consumption or product generation. Two (related) units of enzyme activity are most commonly used.

- One unit (U) of enzyme activity is the amount of that enzyme that will catalyse the transformation of 1  $\mu\text{mol}$  of substrate per minute (or where more than one bond of each substrate molecule is attacked, 1 microequivalent of the group concerned per minute) under the chosen assay conditions. A variation of this definition refers to 1 U of activity as being the amount of enzyme required to produce 1  $\mu\text{mol}$  of product per minute under the assay conditions.
- The katal (kat) is the SI unit of enzyme activity: 1 kat is defined as that catalytic activity which will raise the rate of reaction by 1 mol/s in a specified assay system.

Enzyme assays directly detect and quantify the functional activity of these catalytic proteins.

Immunoassays (Chapter 10) may also be used to detect and quantify a protein. In this instance antibodies raised against the protein of interest must be available. An important variation of this is the use of antibodies raised against an affinity purification fusion tag if present on a recombinant protein. Immunoassays display a number of positive

characteristics, including a high degree of specificity and sensitivity, short assay duration and conduciveness to automation. Their major disadvantage is that they do not measure the protein's actual biological activity. Modifications to the protein (e.g. partial proteolysis or partial denaturation) that may decrease or abolish its biological activity will not affect the immunoassay result if the modification does not alter the three-dimensional structure of the specific part of the protein to which the antibodies bind. In most instances therefore it is preferable to detect and quantify a protein by direct measurement of its biological activity. If technical or economic considerations render this approach unattractive for routine use, progression of protein purification could be followed by immunoassay. A bioassay could then be carried out on at least the final purified product, and if possible also after particularly crucial purification steps.

## 4.2 Initial recovery of protein

The initial step of any purification procedure involves recovery of the protein from its source material. The complexity of this step depends largely on whether the protein of interest is intracellular or extracellular. Many proteins produced by fermentation of microorganisms or by animal cell culture (see Chapter 5) are secreted into the media. Initial product recovery in such cases involves the separation of the whole cells from the cell growth media by filtration or centrifugation. The protein of interest is present in the cell-free medium, often in very dilute form.

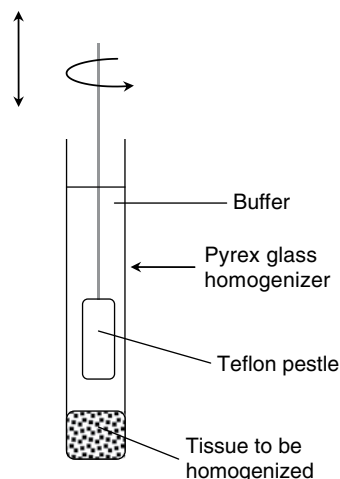
In the case of intracellular microbial proteins, cell harvesting from the culture medium is followed by resuspension of the cells in buffer (a pH-controlled aqueous solution) or water with subsequent cell disruption. Resuspension of microbial cell pastes can often be achieved by simple stirring, although in some cases a more vigorous approach using mechanical mixing is required. Such cell pastes are resuspended in much smaller volumes than the original volume of fermentation broth from which they were prepared. Reduced volumes facilitate more efficient handling during subsequent purification steps.

Most proteins obtained from animal or plant tissue are intracellular in nature. The initial step in processing such material obviously involves collection of the appropriate tissue required. Specific examples include the collection of pituitary glands from which hormones such as follicle-stimulating hormone (FSH) and luteinizing hormone (LH) may be purified, collection of blood from which various blood proteins are obtained, or collection of internal organs such as liver and kidneys from which various enzymes and other proteins of interest may be obtained. If purification is not scheduled to begin directly after harvest of source material, the material can be stored frozen.

### 4.2.1 Cell disruption: animal and plant cells

If the protein required is an intracellular one, collection of the source cells or tissue is followed by their disruption. Most mammalian cells and tissues are relatively easily disrupted. Animal cells, unlike their bacterial or plant counterparts, are devoid of a protective cell wall. Most techniques rely on physical disruption of the cell membrane. A well-known example is that of the Potter homogenizer (Figure 4.2). Disruption is achieved in this case by shear forces generated between a rotating plastic (Teflon) pestle and the inside wall of a glass test tube-like container.

Additional methods by which some animal cell types can be disrupted include osmotic shock and freeze-thaw cycles. Disruption via osmotic shock entails placing the cells in a buffer of high osmotic



**Figure 4.2** Schematic diagram of the Potter homogenizer, commonly used to homogenize gram-level quantities of animal tissue such as liver and brain.

pressure (e.g. a buffer containing 15–25% sucrose). This results in migration of water from inside the cell into the extracellular sucrose medium. After a suitable time (usually 1 hour or less) the cells are then transferred into a weak buffer or distilled water (i.e. a solution of low osmotic pressure). As a result water floods back into the cell, often rupturing the plasma membrane. As the name suggests, freeze-thaw cycles involve freezing the cells, followed by thawing. This cycle is repeated if necessary. Cell rupture is usually prompted by damage to the plasma membrane as a result of intracellular ice crystal growth during the freezing process. This method is not widely used.

Efficient homogenization of plant tissue is more challenging, mainly because of the presence of the outer cell wall. Disruption is often achieved by physical means, in which the plant material is subjected to homogenization by rapidly rotating blades. The Waring blender (which is somewhat similar in design to a domestic food blender) is often used. This approach can also be used to disrupt animal tissue, particularly tough tissue such as muscle.

### 4.2.2 Microbial cell disruption

Disruption of microbial cells is also rendered difficult due to the presence of the microbial cell



**Table 4.5** Some chemical, physical and enzyme-based techniques that may be employed to achieve microbial cell disruption.

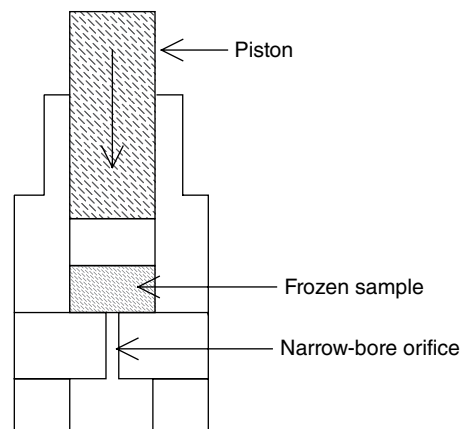
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Treatment with chemicals
Detergents
Antibiotics
Solvents (e.g. toluene, acetone)
Chaotropic agents (e.g. urea, guanidine)
Exposure to alkaline conditions
Sonication
Homogenization
Agitation in the presence of abrasives (usually glass beads)
Treatment with lysozyme

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wall. Despite this, a number of very efficient systems exist which are capable of disrupting large quantities of microbial biomass (Table 4.5). Disruption techniques such as sonication or treatment with the enzyme lysozyme are usually confined to laboratory-scale operations, due either to technical limitations of scale or on economic grounds. Large-scale cell disruption by chemical means has been employed successfully in some instances. Chemicals utilized include a variety of detergents and antibiotics, solvents such as toluene or acetone, and treatment under alkaline conditions or with chaotropic agents such as urea or guanidine.

Protein extraction procedures employing detergents are effective in many instances, but suffer from a number of drawbacks. The mode of detergent action primarily involves solubilization of the cell's membrane. Ionic detergents such as sodium lauryl sulfate are more efficient than non-ionic detergents such as polysorbates. The major disadvantage associated with the utilization of any detergent system is that detergents often induce protein denaturation and precipitation. This obviously limits their usefulness. Many other chemicals, including various solvents or incubation under alkaline conditions, also suffer from this disadvantage. Furthermore, even if the chemicals employed do not adversely affect the protein, their presence may impair a subsequent purification step (e.g. the presence of detergent can prevent proteins from binding to a hydrophobic interaction column). In addition, the presence of such materials in the



**Figure 4.3** Diagrammatic representation of a cell homogenizer. This is one of a number of instruments routinely used to rupture microbial cells and, in some cases, animal/plant tissue.

final preparation, even in trace quantities, may be unacceptable if for example it interferes with protein quantification (see Table 4.2) or if the protein is destined for administration to humans or animals. Detergent-based cell disruption systems have been successfully employed in a number of specific cases. Triton, for example, has been used to render *Nocardia* cells permeable in the large-scale extraction and purification of cholesterol oxidase (see Chapter 10).

Disruption of microbial cells (and some animal/plant tissue types) is most often achieved by mechanical methods such as microbial homogenization, or by vigorous agitation with abrasives. During microbial homogenization a cell suspension is typically forced through an orifice of very narrow internal diameter at extremely high pressures. This generates extremely high shear forces. As the microbial suspension passes through the outlet point, it experiences an almost instantaneous drop in pressure to normal atmospheric pressure. The high shear forces and subsequent rapid pressure drop act as very effective cellular disruption forces, and result in the rupture of most microbial cell types (Figure 4.3). In most cases a single pass through the homogenizer results in adequate cell breakage, but it is also possible to recirculate the material through the system for a second or third pass.

An additional method often employed to achieve microbial cell disruption, both at laboratory level and on an industrial scale, involves cellular agitation in the presence of glass beads. In such bead mills, the microorganisms are placed in a chamber together with a quantity of glass beads, typically of 0.2–0.3 mm in diameter. This mixture is then shaken/agitated vigorously, resulting in numerous collisions between the microbial cells and the glass beads. It also results in the grinding of cells between the rotating beads. These forces promote efficient disruption of most microbial cell types. Operational parameters such as ratio of cells to beads and the rate or duration of agitation may be adjusted to achieve optimum disruption of the particular cells in question. Laboratory systems can homogenize several grams of microbial cells in minutes. Industrial-scale bead milling systems can process in excess of 1000 L of cell suspension per hour. Cooling systems minimize protein inactivation by dissipating the considerable heat generated during this process.

## 4.3 Removal of whole cells and cell debris

On completion of the homogenization step cellular debris and any remaining intact cells can be removed by centrifugation or filtration. As previously mentioned these techniques are also used to remove whole cells from the medium during the initial stages of extracellular protein purification.

### 4.3.1 Centrifugation

Centrifugation achieves accelerated sedimentation by the application of a centrifugal force to the sample (in this case the cellular homogenate). In practice this is undertaken by placing the sample in a rapidly rotating rotor (Figure 4.4) that is turning at a specific angular velocity ( $\omega$ ). Under such circumstances the components of the sample each experience a centrifugal force ( $F_c$ ) which is proportional to the

product of the substance's mass ( $m$ ) and the distance ( $r$ ) from the centre of rotation ( $F_c = \omega^2 rm$ ).

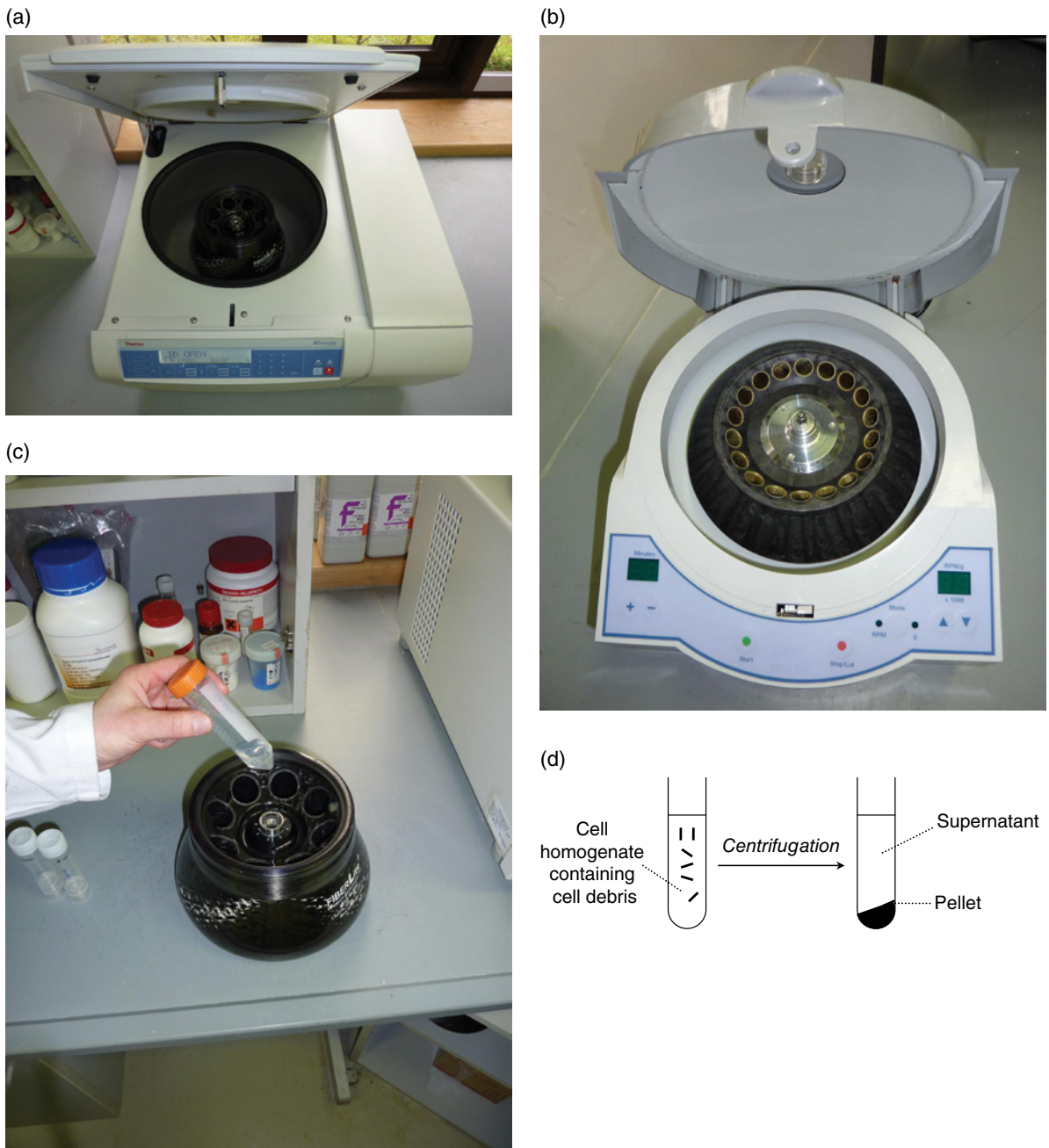
Centrifugation remains the method of choice for achieving cell and cellular debris separation, both at laboratory scale and on an industrial scale. However, a number of factors, most notably the high capital and running costs, have led many to investigate alternative means of sedimenting cells or cellular debris. The most popular alternative method is filtration.

### 4.3.2 Filtration

Both whole cells and cell debris may be removed from solution by filtration. Either depth filters or (far more commonly) membrane filters may be used. Depth filters consist of randomly orientated fibres (usually manufactured from glass fibre or cellulose) that form an irregular network of channels or mesh-like structures. Such filters retain particles not only on their surface but also within the depth of the filter. Depth filtration is sometimes used to remove whole cells from fermentation media. Such filters are also used to remove or reduce levels of cellular debris, denatured protein aggregates, or other precipitates from solution.

Membrane filtration, also termed microfiltration, is achieved using thin membrane-like sheets of polymeric substances such as cellulose acetate or nitrate, nylon or PTFE (polytetrafluoroethylene), in which very small pores have been generated. Pore sizes generally range between 10 and 0.02  $\mu\text{m}$ . Membrane filters of pore diameter 0.2–0.45  $\mu\text{m}$  will retain all microbial cells. The retention of particles or microbial cells occurs only on or in the surface layer of the filter. The material to be filtered is applied to the filtration system under pressure in order to achieve satisfactory flow rates. Membrane filtration enjoys increasing popularity as the system of choice for removing cell and cellular debris from solutions. The method is efficient and requires relatively simple equipment.

Microfiltration is most often employed to sterilize a protein solution. Removal of all microbial cells is achieved by use of an 'absolute' 0.2- $\mu\text{m}$  filter. Membrane filters may be classified as 'absolute' or 'nominal'. Absolute filters are guaranteed to remove



**Figure 4.4** Laboratory-scale centrifuges (a, b), a centrifuge rotor (c) and a schematic representation of the centrifugation process (d). Centrifugation of a cell homogenate allows collection of the cellular debris as a 'pellet' at the base of the sample container (the centrifuge tube), while the clarified liquid ('supernatant') contains the intracellular contents.

all particles larger than the indicated filter pore size (i.e. they are 100% effective). Nominal filters, on the other hand, while effective, may not be 100%, so although these are considerably cheaper than

absolute filters, they should not be used for critical operations such as sterilization. Sterile filtration on a laboratory scale is usually achieved by passing the protein solution directly through a 0.22- $\mu\text{m}$  filter.

In summary, filtration techniques may be used at a number of stages during a protein purification process:

- to achieve separation of whole cells from fermentation media;
- to remove whole cells and cell debris after cell disruption;
- to achieve a reduction in or totally eliminate microbial species from the product stream at later stages in a purification process.

In some instances, such separations may also be achieved by centrifugation. Although centrifugation represents the traditional method, filtration is increasingly being used, particularly on an industrial scale. As discussed in section 4.4.3, another form of filtration, ultrafiltration, can be used to concentrate protein solutions.

### 4.3.3 *Aqueous two-phase partitioning*

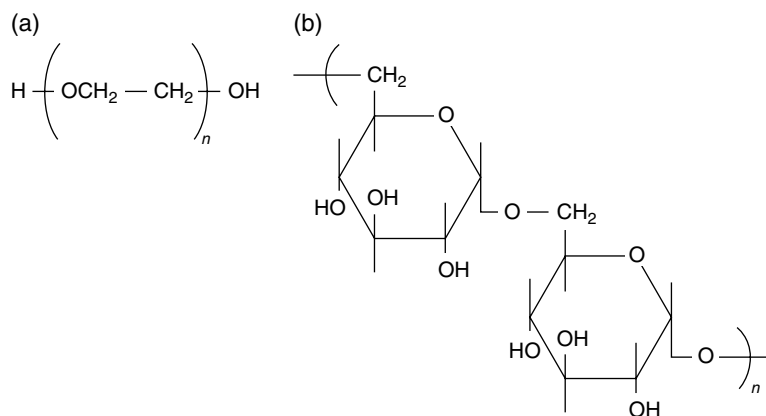
The technique of aqueous two-phase partitioning may be used to separate whole cells or cell debris from soluble protein. It can also be used to achieve a limited degree of protein purification and concentration. Although studied for many years, it is used to a far more limited extent than centrifugation or filtration. This is mainly due to a less than complete understanding as to how the technique works at a molecular level.

Aqueous two-phase partitioning is based on the fact that many water-soluble (aqueous) polymers are incompatible with each other, or with salt solutions which are of high ionic strength. Thus, if one such polymer is mixed with a salt solution or with a second (incompatible) polymer, two phases are formed on standing. Such partitioning systems may be employed to separate proteins from cell debris or other impurities. The debris partitions to the lower, more polar and more dense phase, while soluble proteins tend to partition into the top, less polar and less dense phase. Subsequent separation of the two phases achieves effective separation of cellular debris from soluble protein.

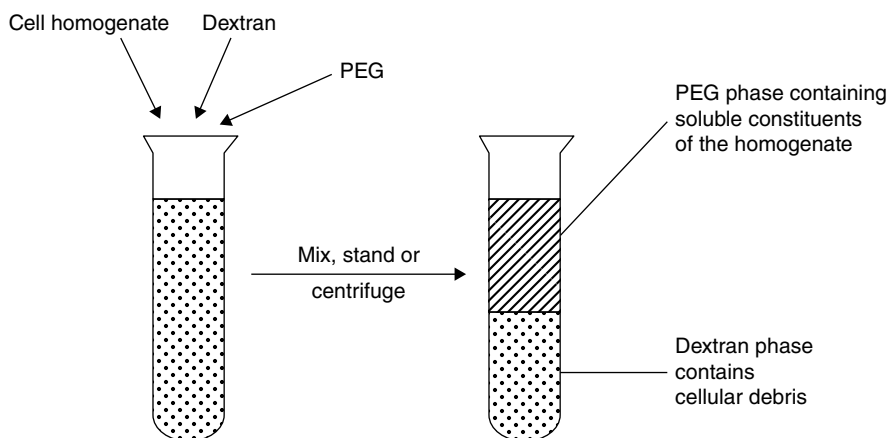
The most commonly employed polymers are polyethylene glycol (PEG, a polymer consisting of ethylene molecules linked by ether bonds) and dextran (a polymer consisting of repeating glucose residues linked by  $\alpha 1 \rightarrow 6$  bonds (Figure 4.5)). The most common polymer-salt system is based on PEG and a phosphate salt (normally sodium or potassium phosphate). If solutions of PEG and dextran are mixed with a cell homogenate under appropriate conditions, the protein components partition into the upper PEG phase whereas cellular debris accumulate in the lower dextran phase. In this way, effective separations can be achieved on prolonged standing. If the mixture is one of PEG and phosphate, the proteins tend to accumulate in the upper PEG-rich phase whereas the debris accumulates in the lower phosphate-rich phase (Figure 4.6).

When two such incompatible water-soluble moieties are mixed, subsequent phase separation occurs at a slow rate. However, the rate of phase separation is increased by applying a centrifugal force. Thus phase separation is accelerated by employing a subsequent centrifugation step. Once the phase separation has been completed, the phase containing the protein of interest is subjected to further processing. Centrifugation is normally used to accelerate phase separation. Application of the phase containing the protein of interest to an ultrafiltration system (see section 4.4.3) results in an immediate concentration of the protein product. Alternatively, further purification may be achieved by employing a second phase extraction step, in which the partition coefficient of the (new) system is altered. This may be achieved by, for example, altering the molecular mass of the polymers used (various PEG preparations may be obtained with molecular masses ranging from 200 to 20,000 Da), or by changing the ionic strength of the solutions. During separation of this second phase system, some of the protein species remain in the PEG phase while others will partition to the lower phase, depending on the conditions employed. In this way, a limited purification of the protein of interest may be attained.

A high degree of protein localization in one phase may be obtained by attaching a ligand which specifically binds the protein of interest to the least polar



**Figure 4.5** Molecular structure of (a) polyethylene glycol ( $n$  is usually between 4 and 200) and (b) a fragment of the dextran backbone.



**Figure 4.6** Principle of an aqueous two-phase purification system as applied to separation of cellular debris from soluble proteins. PEG, polyethylene glycol.

polymer of the two-phase system (normally PEG). This technique is termed affinity partitioning. In this case conditions are employed that promote partitioning of most contaminant proteins in addition to nucleic acids and cell debris to the more polar phase. However, the protein of interest will partition into the less polar phase due to the presence of the ligand in this phase. Affinity ligands can be specific (e.g. a substrate or inhibitor of an enzyme of interest) or non-specific (e.g. various dyes which bind a number of protein types). Affinity ligands are discussed in more detail in section 4.5.4.

Aqueous two-phase partitioning is a potentially useful clarification/partial purification technique for a number of reasons:

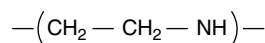
- it is a gentle method, having little or no adverse effect on the biological activity of most proteins;
- many of the polymers used exhibit protein stabilizing properties;
- the yield of protein activity recovered is generally high;
- few if any technical difficulties arise during process scale-up.

The major disadvantage associated with this technique relates to the lack of understanding of the molecular mechanisms involved in the partitioning process. Development of a two-phase process to achieve effective partitioning is wholly empirical. Without an intimate understanding of the underlying principles involved, a rational approach to designing such systems cannot be undertaken. Technical-grade dextran is also quite expensive. This may be overcome by employing crude dextran preparations, or substituting dextran with other polymers such as polyvinyl alcohol (PVA) or polyvinyl pyrrolidone (PVP). The disadvantages outlined above, coupled with the availability of alternative established separation techniques such as centrifugation or filtration, has thus far limited the utilization of this technique.

#### 4.3.4 Removal of nucleic acid and lipid

In some cases it is necessary to remove or destroy the nucleic acid content of a cell homogenate prior to subsequent protein purification. Liberation of large amounts of nucleic acids often significantly increases the viscosity of the cellular homogenate. This generally renders the homogenate more difficult to process, particularly on an industrial scale. Significant increases in viscosity place additional demands on the method of cell debris removal employed. Increased centrifugal forces for longer time periods may be required to efficiently pellet cell debris in such solutions. If a filtration system is employed to remove cellular debris, increased viscosity will also adversely affect flow rate and filter performance. Increased viscosity due to liberation of nucleic acids during homogenization is often most noticeable when prokaryotic organisms are used, as the DNA in such organisms is not bounded by an intracellular protective membrane, the nuclear membrane, as is the case in eukaryotic cells.

The inclusion of a specific nucleic acid removal step is not required for all purification procedures. Inclusion or exclusion of such a step depends on the extent to which liberated nucleic acids affect the viscosity of particular suspensions and on the intended



**Figure 4.7** Molecular structure of the repeat unit of polyethylenimine.

use of the final protein product. Effective nucleic acid removal is particularly important when purifying any protein destined for therapeutic use. Regulatory authorities generally insist that the nucleic acid content present in the final preparation be, at most, a few picograms per therapeutic dose (see Chapter 5).

Effective removal of nucleic acids during protein purification may be achieved by precipitation, or by treatment with nucleases. A number of cationic (positively charged) molecules are effective precipitants of DNA and RNA; they complex with, and precipitate, the negatively charged nucleic acids. The most commonly employed precipitant is polyethylenimine, a long-chain cationic polymer (Figure 4.7). The precipitate is then removed, together with cellular debris, by centrifugation or filtration. The use of polyethylenimine during purification of proteins destined for therapeutic applications is often discouraged, as small quantities of unreacted monomer may be present in the polyethylenimine preparation. Such monomeric species may be carcinogenic. If polyethylenimine is utilized in such cases, the subsequent processing steps must be shown to be capable of effectively and completely removing any of the polymer or its monomeric units that may remain in solution.

Nucleic acids may also be removed by treatment with nucleases, which catalyse the enzymatic degradation of these biomolecules. Indeed nuclease treatment has become the most popular method of nucleic acid removal during protein purification. The treatment is efficient, inexpensive and, unlike many of the chemical precipitants used, nuclease preparations themselves are innocuous and do not compromise the final protein product.

Some cell or tissue types (most notably of animal origin) contain an appreciable level of lipid. Removal of the lipid layer from the crude protein solution before further purification is desirable as (i) it is a contaminant and (ii) it can interfere with subsequent purification steps (e.g. clog

chromatographic columns). The lipid layer can be removed by passage of the solution through glass wool or a cloth of very fine mesh size.

## 4.4 Concentration

During the initial stages of some protein purification procedures the protein of interest is present in dilute form. This is particularly true in the case of many native (non-recombinant) proteins which are produced extracellularly. Under such circumstances it is desirable to concentrate the protein solution such that reduced volumes can be more speedily and conveniently handled during subsequent purification steps. Methods most commonly used to achieve concentration on a laboratory scale include precipitation, ion-exchange chromatography and ultrafiltration.

### 4.4.1 Concentration by precipitation

Protein precipitation can be promoted by agents such as neutral salts, organic solvents, high molecular mass polymers, or by appropriate pH adjustments (Table 4.6). Concentration is achieved by collecting the precipitated protein and redissolving it in a much smaller volume than that in which it was dissolved originally. Concentration by precipitation is one of the oldest concentration methods known. Ammonium sulfate is likely the most common protein precipitant utilized. This neutral salt is particularly popular due to its high solubility, inexpensiveness, lack of denaturing properties towards most proteins, and its stabilizing effect on many proteins.

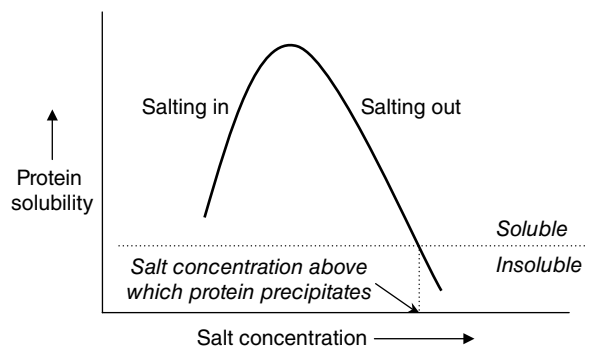
The addition of small quantities of neutral salts to a protein solution often increases protein solubility – the ‘salting-in’ effect. However, increasing salt concentrations above an optimal level leads to destabilization of proteins in solution and eventually promotes their precipitation. This is known as ‘salting out’ (Figure 4.8). At high concentrations, such salts effectively compete with the protein molecules for water of hydration. This promotes

**Table 4.6** Various methods/techniques that may be used to precipitate proteins from solution.

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Addition of neutral salts, e.g. ammonium sulfate
Addition of organic solvents, e.g. ethanol or acetone
Addition of organic polymers, e.g. polyethylene glycol
Affinity precipitation (addition of a ligand, often an antibody, which precipitates the target protein from solution on the basis of biospecific molecular interactions)
Adjustment of solution pH (some proteins precipitate out of solution at their isoelectric point)
Selective denaturation: approach can be used to precipitate contaminant proteins from the protein of interest, if the latter is more stable than the former in the presence of some denaturing influence (e.g. extremes of pH or elevated temperature)

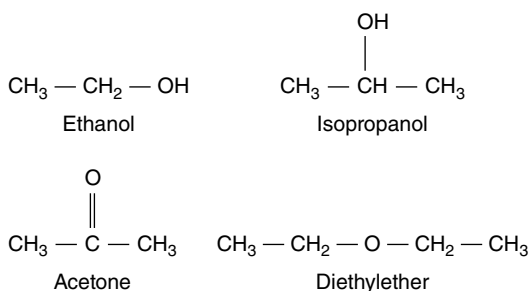
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**Figure 4.8** Effect of salt concentration on protein solubility. Increases in salt concentration from low initial values often increase protein solubility (salting in). Further increases above an optimal value will result in destabilization of the protein, and eventually its precipitation from solution (salting out).

increased protein–protein interactions, predominantly interactions between hydrophobic patches on the surface of adjacent protein molecules. Such increased protein–protein interactions eventually result in protein precipitation.

Addition of various organic solvents to a protein solution can also promote protein precipitation. Added organic solvents lower the dielectric constant of an aqueous solution. This in turn promotes increased electrostatic attraction between bodies of opposite charge in the solution, in this case proteins. Such increasing interactions between proteins of opposite charges eventually leads to their precipitation. Organic solvents frequently used to promote



**Figure 4.9** Structure of the organic solvents most commonly used as protein precipitants.

precipitation include ethanol, isopropanol, acetone and diethylether (Figure 4.9). Protein precipitation using organic solvents must be carried out at temperatures at or below 0°C in order to prevent protein denaturation. As such solvents depress the freezing point of aqueous solutions, it is usually feasible to maintain the solution temperature several degrees below 0°C.

Precipitation may also be promoted by the addition of organic polymers such as PEG. The addition of such polymers, however, often dramatically increases the viscosity of the resultant solution, making recovery of the precipitate more difficult. Recovery is normally achieved by centrifugation or by filtration. The precipitate can subsequently be redissolved in a smaller volume of resuspending liquid and, in this way, it is effectively concentrated.

Concentration by precipitation has also played an important role in the downstream processing of many industrially important proteins. The technique is relatively straightforward to perform and requires only a limited amount of equipment. In many cases, precipitation also achieves some degree of protein purification (different protein types generally require different concentrations of a precipitant to effect their removal from solution). High recoveries of biological activity are also usually recorded. There are also a number of disadvantages associated with this technique, particularly at industrial scale.

- Many of the precipitants used are highly corrosive, in particular towards stainless steel equipment such as centrifuge rotors.
- Precipitation is often quite inefficient if employed under circumstances where the initial protein

concentration is low. In such cases, low recoveries of protein may be recorded.

- Some precipitants, such as acetone and diethylether, are highly flammable and hence are hazardous to work with. Others such as ethanol are quite expensive.
- Many precipitants (e.g. most organic solvents) must be disposed of carefully after use.
- In many cases all traces of the precipitant present in the precipitate must be removed before further processing. For example, ammonium sulfate must be removed from resuspended salt-mediated precipitates before the resultant protein solution can be applied to an ion-exchange column. This can be achieved by dialysis or diafiltration (see section 4.4.4).

#### 4.4.2 Concentration by ion exchange

At any given pH, proteins display a positive, negative, or zero net charge. Using this parameter of molecular distinction, different protein molecules can be separated from one another by judicious choice of pH, ionic strength and ion-exchange materials. Positively charged proteins will bind to cation exchangers whereas negatively charged proteins will bind to anion exchangers. Elution of such bound protein may be easily achieved by subsequent irrigation with a solution of high ionic strength (e.g. buffer-containing a salt such as NaCl or KCl added to a final concentration of up to 0.5 mol/L). Ion exchange offers an effective and relatively inexpensive method of achieving initial concentration (by eluting the protein off the ion-exchange column in a much smaller volume than initially applied). This may also result in a limited degree of protein purification.

Batchwise adsorption of proteins present in dilute solutions is easily achieved by the direct addition of the ion exchanger to the solution in question. Examples of such 'dilute' protein solutions include (i) fermentation broths or cell culture media containing extracellular proteins from which the whole cells have been removed or (ii) cell homogenates from which cell debris has been removed. The ion-exchange material may be recovered by centrifugation, and is



then generally placed in a filter funnel or stainless steel vessel. Such holding vessels contain an outlet covered by a mesh that ensures retention of the ion-exchange material. Bound proteins are then eluted from the ion exchanger by addition of a suitable solution at high ionic strength. The protein-containing eluate is collected and subjected to further processing. The ion-exchange material can be regenerated and reused for the next purification cycle.

If the protein of interest is negatively charged, anion exchangers containing functional groups such as aminoethyl or diethylaminoethyl moieties may be employed. In the case of positively charged proteins, cation exchangers containing functional groups such as carboxymethyl moieties are used (see section 4.5.2). These functional groups are covalently linked to porous beads usually made from cross-linked dextran, agarose or composites of such materials.

Ion-exchange techniques are relatively inexpensive and robust and the materials can easily be regenerated. Very high levels of protein recovery are generally recorded. Batch adsorption/elution also results in considerable clarification of the resultant protein solution. Many impurities that have adverse effects on solution characteristics either do not bind to the ion exchanger or do not subsequently elute from such exchangers under the conditions employed to elute the target protein. Undesirable impurities include particulate material that previous clarification steps failed to remove, various lipid and/or carbohydrate molecules, in addition to partially denatured or aggregated protein. Effective clarification of such protein solutions is desirable as it prevents fouling of columns during the subsequent purification steps. Such initial ion-exchange treatments also lead to some degree of protein purification, as only other molecules with similar charge characteristics will bind and subsequently co-elute with the protein of interest.

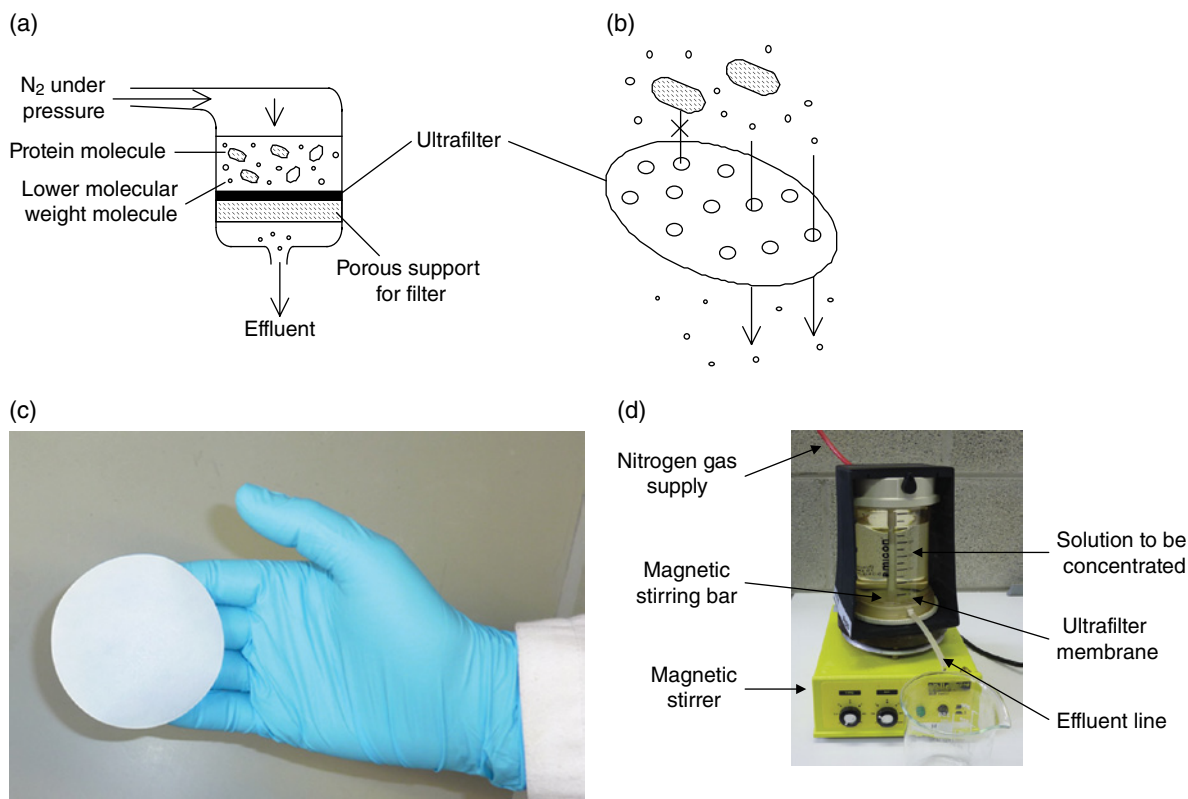
### 4.4.3 Concentration by ultrafiltration

Protein solutions may be quickly and conveniently concentrated by ultrafiltration (Figure 4.10) and this method of concentration is the one most widely

applied, both on a laboratory and industrial scale. As previously discussed, the technique of microfiltration may be used to remove whole cells or cell debris from solution. Membrane filters employed in the microfiltration process generally have pore diameters ranging from 0.1 to 10  $\mu\text{m}$ . Such pores, while retaining whole cells and large particulate matter, fail to retain most macromolecular components such as proteins. In the case of ultrafiltration membranes, pore diameters normally range from 1 to 20 nm. These pores are sufficiently small to retain proteins of low molecular mass. Ultrafiltration membranes with molecular mass cut-off points ranging from 1 to 300 kDa are commercially available. Membranes with molecular mass cut-off points of 3, 10, 30, 50 and 100 kDa are most commonly used.

Traditionally, ultrafilters have been manufactured from cellulose acetate or cellulose nitrate. Several other materials such as polyvinyl chloride and polycarbonate are now also used in membrane manufacture. Such plastic-type membranes exhibit enhanced chemical and physical stability when compared with cellulose-based ultrafiltration membranes. An important prerequisite in manufacturing ultrafilters is that the material utilized exhibits low protein adsorptive properties. No matter which material is utilized the pore size obtained is not absolutely uniform. The molecular mass cut-off point quoted for any such filter is thus best regarded as a nominal value. For protein work it is advisable to use a membrane whose stated molecular mass cut-off point is 5 kDa or more lower than the molecular mass of the protein of interest. It is also important to realize that the molecular mass cut-off point quoted applies to globular proteins. The overall shape of the protein of interest affects its ultrafiltration characteristics. For example, if the protein is somewhat elongated, it may not be retained by an ultrafilter whose cut-off point is significantly lower. Extensive post-translational modifications, in particular glycosylation, may also affect ultrafiltration behaviour.

Ultrafiltration is often carried out on a laboratory scale using a stirred cell system (Figure 4.10). The flat membrane is placed on a supporting mesh at the bottom of the cell chamber, and the material to be concentrated is then transferred into the cell. Application of pressure, usually nitrogen gas, ensures



**Figure 4.10** Ultrafiltration separates molecules on the basis of size and shape. (a) Diagrammatic representation of a typical laboratory-scale ultrafiltration system. The sample (e.g. crude protein solution) is placed in the ultrafiltration chamber, where it sits directly above the ultrafilter membrane. The membrane in turn sits on a macroporous support that provides it with mechanical strength. Pressure is then applied (usually in the form of an inert gas) as shown. Molecules larger than the pore diameter (e.g. large proteins) are retained on the upstream side of the ultrafilter membrane. However, smaller molecules, particularly water molecules, are easily forced through the pores, thus effectively concentrating the protein solution (b). Membranes can be manufactured that display different pore sizes, i.e. have different molecular mass cut-off points. (c) An ultrafilter membrane. (d) A laboratory stirred cell ultrafilter unit in operation.

adequate flow through the ultrafilter. Molecules of lower molecular mass than the filter cut-off pore size (e.g. water, salt and low-molecular-mass compounds) all pass through the ultrafilter, thus concentrating the molecular species present whose molecular mass is significantly greater than the nominal molecular mass cut-off point. Concentration polarization (the build-up of a concentrated layer of molecules directly over the membrane surface which are unable to pass through the membrane) is minimized by a stirring mechanism operating close to the membrane surface. If unchecked, concentration polarization would result in a lowering of the flow

rate. Additional ultrafilter formats used on a laboratory scale include cartridge systems, within which the ultrafiltration membrane is present in a highly folded format. In such cases the pressure required to maintain a satisfactory flow rate through the membrane is usually generated by a peristaltic pump.

Another membrane configuration is that of the hollow fibre. In this case, the hollow cylindrical cartridge casing is loaded with bundles of hollow fibres. Hollow fibres have an outward appearance somewhat similar to a drinking straw, although their internal diameters may be considerably smaller. In this configuration, the liquid to be filtered is pumped

through the central core of the hollow fibres. Molecules of lower molecular mass than the membrane rated cut-off point pass through the walls of the hollow fibre. The permeate, which emerges from the hollow fibres all along their length, is drained from the cartridge via a valve. The concentrate emerges from the other end of the hollow fibre and is collected by an outlet pipe; this is referred to as the retentate. The permeate is then normally discarded while the retentate, containing the protein of interest, is processed further. The retentate may be recycled through the system if further concentration is required.

Ultrafiltration has become prominent as a method of protein concentration for a variety of reasons:

- the method is very gentle, having little adverse effect on bioactivity of the protein molecules;
- high recovery rates are usually recorded (some ultrafiltration manufacturers claim recoveries of over 99%);
- processing times are rapid compared with alternative methods of concentration;
- little ancillary equipment is required;
- ultrafiltration may also achieve some degree of protein purification (on the basis of differences in molecular mass).

One drawback relating to this filtration technique is its susceptibility to rapid membrane clogging. Viscous solutions also lead to rapid decreases in flow rates and prolonged processing times. Ultrafiltration may also be used to remove low-molecular-mass molecules from protein solutions by diafiltration.

#### 4.4.4 *Diafiltration*

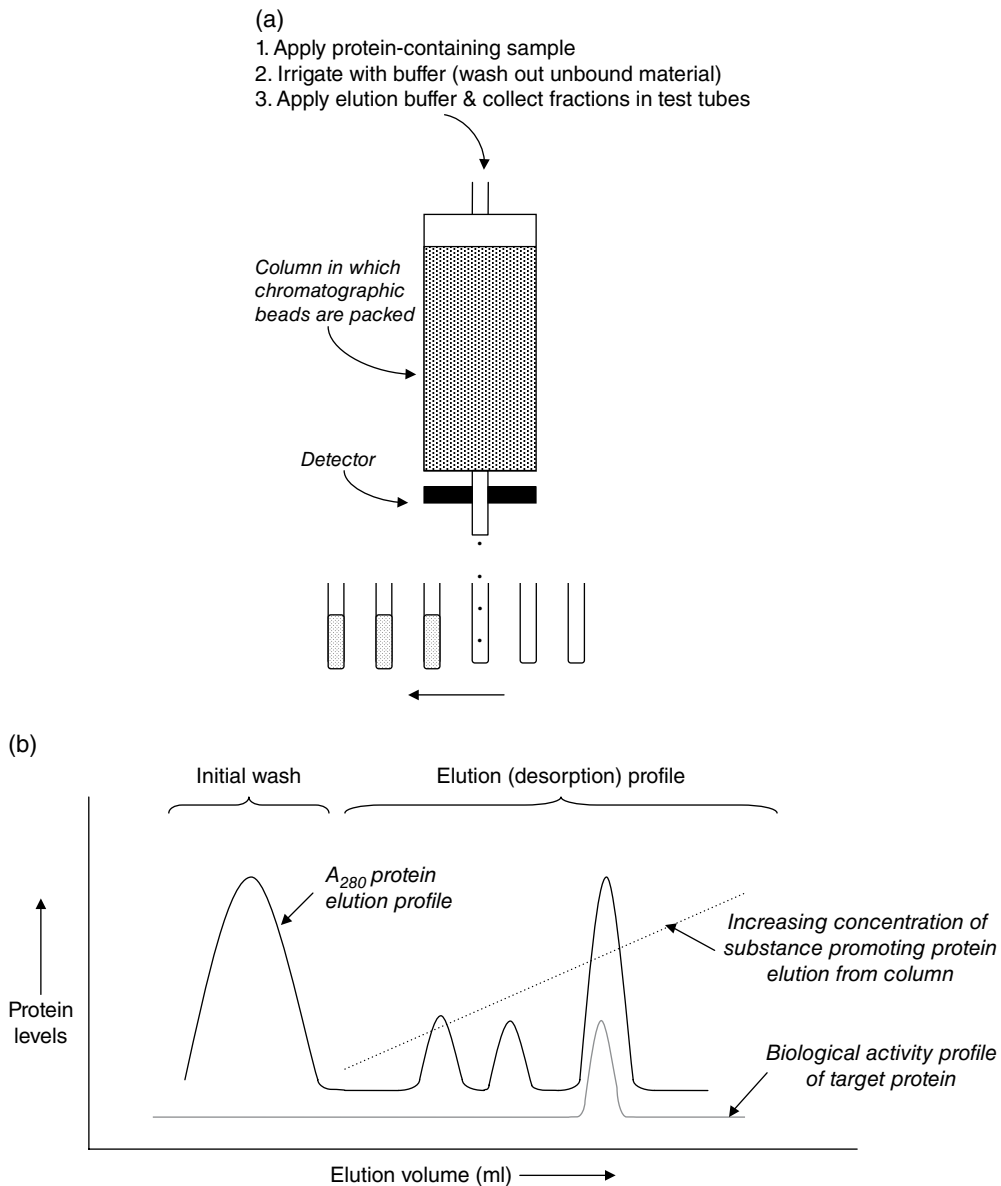
Diafiltration is a process whereby an ultrafiltration system is utilized to reduce or eliminate low-molecular-mass molecules from a solution. In practice this normally entails the removal of salts, ethanol and other solvents, buffer components, amino acids, peptides, added protein stabilizers or other molecules. Diafiltration is generally preceded by an ultrafiltration step to initially reduce process

volumes. The actual diafiltration process is identical to that of ultrafiltration except for the fact that the level of reservoir is maintained at a constant volume. This is achieved by the continual addition of solvent lacking the low-molecular-mass molecules which are to be removed. By recycling the concentrated material and adding sufficient fresh solvent to the system such that five times the original volume has emerged from the system as permeate, over 99% of all molecules that freely cross the membrane will have been removed from the solution. Removal of low-molecular-mass contaminants from protein solutions may also be achieved by dialysis or gel filtration chromatography. However, diafiltration is emerging as the method of choice, as it is quick, efficient and utilizes the same equipment as ultrafiltration.

## 4.5 Chromatographic purification

Once the protein is recovered from its producer source and concentrated as necessary, it can be purified to homogeneity. In other words all contaminant proteins (and other potential contaminants such as DNA) must be removed. Purification is generally achieved by column chromatography.

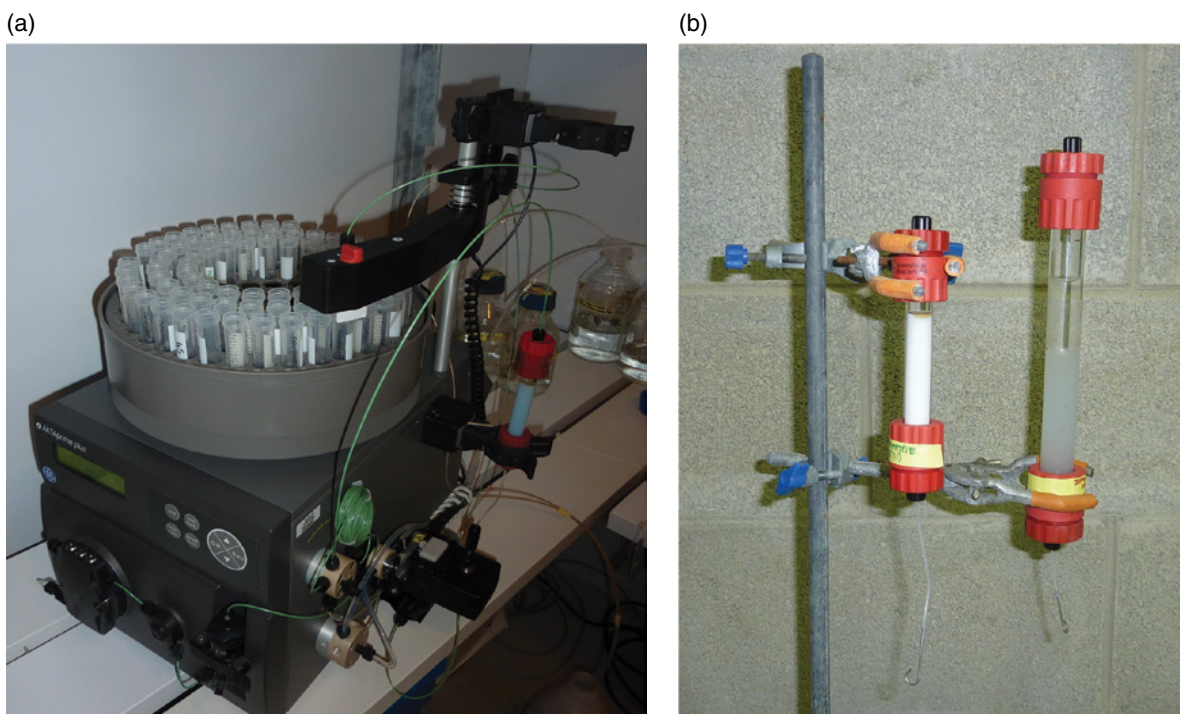
As outlined in Chapter 1, column chromatography refers to the separation of different protein types from each other according to their differential partitioning between two phases: a solid stationary phase (the chromatographic beads, usually packed into a cylindrical column) and a mobile phase (usually a buffer). With the exception of gel filtration, all forms of chromatography used in protein purification protocols are adsorptive in nature. The protein mix is applied to the column (usually) under conditions which promote selective retention of the target protein. Ideally this target protein should be the only one retained on the column, but this is rarely attained in practice. After sample application the column is washed ('irrigated') with mobile phase in order to flush out all unbound material. The composition of the mobile phase is then altered in order to promote desorption of the bound protein.



**Figure 4.11** (a) Typical sequence of events during an adsorption-based protein purification chromatographic step. Fractions collected during an initial column wash (to wash unbound material out of the column) and during the elution of bound protein (usually triggered by an increasing concentration of desorbing molecules included in the elution running buffer) are usually assayed for (i) total protein (by measuring absorbance at 280 nm) and (ii) target protein bioactivity. In the case illustrated (b), a large amount of unbound protein emerges from the column during the initial wash, while three different protein peaks are subsequently eluted from the column. Only the last peak contains the protein of interest.

Protein exit from the column is usually continuously monitored by an in-line monitor that measures absorbance at 280 nm (see Table 4.2). Fractions of eluate are collected in test tubes, which are then

assayed for the protein of interest (Figure 4.11). The fractions containing the target protein are then pooled and subjected to the next step in the purification process.



**Figure 4.12** (a) A basic laboratory-scale protein purification system and (b) two chromatography columns.

Individual protein types possess a variety of characteristics that distinguish them from other protein molecules. Such characteristics include size and shape, overall charge, the presence of surface hydrophobic groups and the ability to bind various ligands. Quite a number of protein molecules may be similar to one another if compared on the basis of any one such characteristic. All protein types, however, present their own unique combination of characteristics, a protein chromatographic 'fingerprint'. Various chromatographic techniques have been developed that separate proteins from each other on the basis of differences in such characteristics (see Table 4.1). Utilization of any one of these methods to exploit the molecular distinctiveness usually results in a dramatic increase in the purity of the protein of interest. A combination of methods may be employed to yield highly purified protein preparations.

In general, a combination of two to six different chromatographic techniques are employed in a typical purification procedure (see Figure 4.1). Gel filtration and ion-exchange chromatography are among the most common. Affinity chromatography

is employed wherever possible as its high biospecificity facilitates the achievement of a very high degree of purification. The use of affinity systems based on affinity tags has become standard practice in the purification of most recombinant proteins. The operation of modern chromatographic systems is highly automated and is usually computer controlled (Figure 4.12).

#### 4.5.1 *Size exclusion chromatography (gel filtration)*

Size exclusion chromatography, also termed gel permeation or gel filtration chromatography, separates proteins on the basis of their size and shape. As most proteins fractionated by this technique have approximately similar molecular shape, separation is often described as being on the basis of molecular mass.

Fractionation of proteins by size exclusion chromatography is achieved by percolating the protein-containing solution through a column packed with a porous gel matrix in bead form. As the sample

travels down the column, large proteins cannot enter the gel beads and hence are quickly eluted. The progress of smaller proteins through the column is retarded as such molecules are capable of entering the gel beads. The internal structure of the matrix beads could be visualized as a maze, through which proteins small enough to enter the gel must pass. Various possible routes through this maze are of varied distances. All proteins capable of entering the gel are thus not retained within the gel matrix for equal time periods. The smaller the protein, the more potential internal routes open to it and thus, generally, the longer it is retained within the bead structure. Protein molecules are therefore usually eluted from a gel filtration column in order of decreasing molecular size.

In most cases the gel matrices utilized are prepared by chemically cross-linking polymeric molecules such as dextran, agarose, acrylamide and vinyl polymers. The degree of cross-linking controls the average pore size of the gel prepared. Most gels synthesized from any one polymer type are thus available in a variety of pore sizes. The higher the degree of cross-linking introduced, the smaller the average pore size and the more rigid the resultant gel bead. Highly cross-linked gel matrices have pore sizes that exclude all proteins from entering the gel matrix. Such gels may be used to separate proteins from other molecules that are orders of magnitude smaller, and are often used to remove low-molecular-mass buffer components and salts from protein solutions (Figure 4.13).

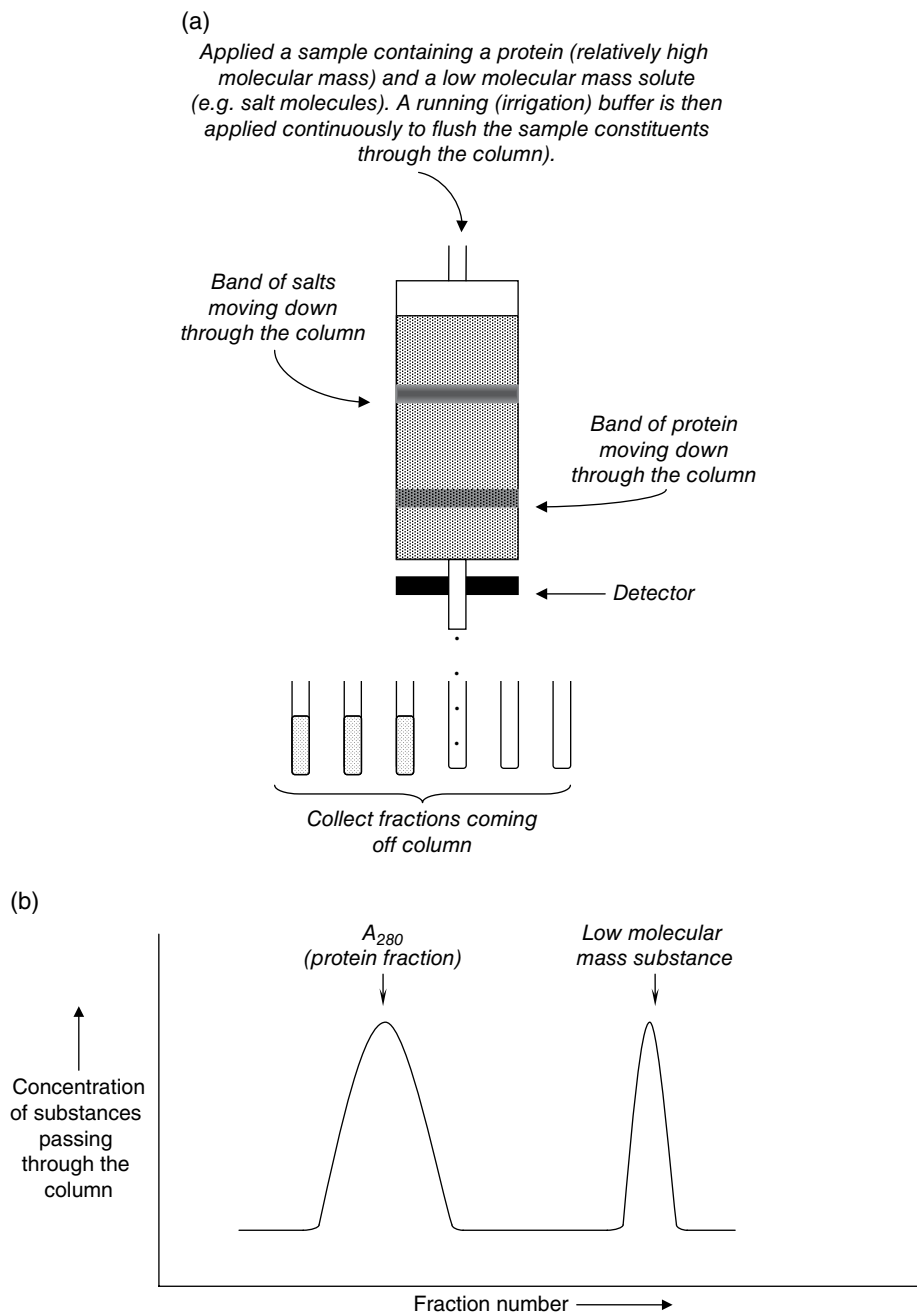
Size exclusion chromatography is rarely employed during the initial stages of protein purification. Small sample volumes must be applied to the column in order to achieve effective resolution. Application volumes are usually in the range of 2–5% of the column volume. Furthermore, columns are easily fouled by a variety of sample impurities. Size exclusion chromatography is thus often employed towards the end of a purification sequence, when the protein of interest is already relatively pure and is present in a small concentrated volume. After sample application, the protein components are progressively eluted from the column by flushing with an appropriate buffer. In many cases, the eluate from the column passes through a detector. This facilitates

immediate detection of protein-containing bands as they elute from the column. The eluate is normally collected as a series of fractions. While size exclusion chromatography is an effective fractionation technique, it generally results in a significant dilution of the protein solution relative to the starting volume applied to the column.

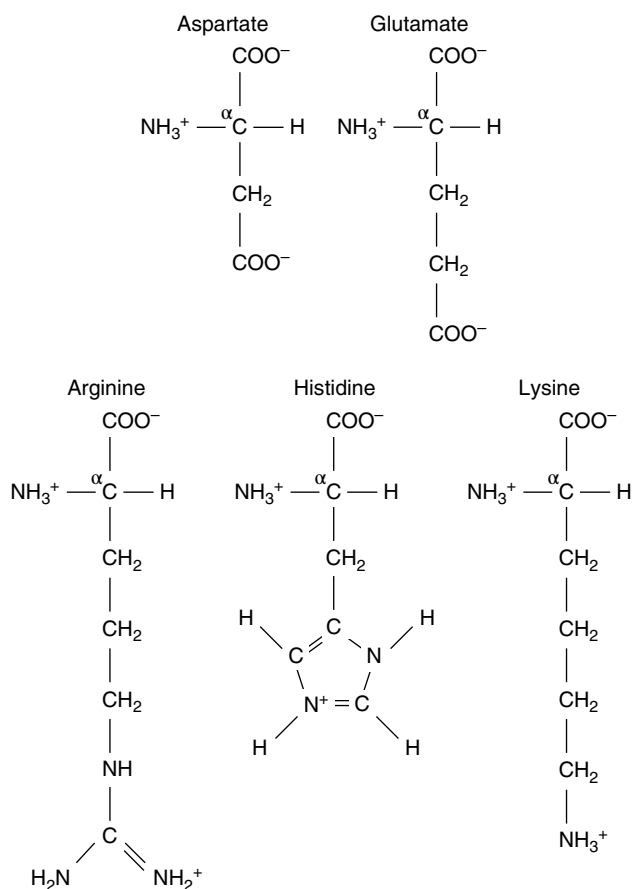
### 4.5.2 Ion-exchange chromatography

Several of the 20 amino acids that constitute the building blocks of proteins exhibit charged side chains. At pH 7.0, aspartic and glutamic acid have overall negatively charged acidic side groups, while lysine, arginine and histidine have positively charged basic side groups (Figure 4.14). Protein molecules therefore possess both positive and negative charges, largely due to the presence of varying amounts of these seven amino acids (N-terminal amino groups and the C-terminal carboxyl groups also contribute to overall protein charge characteristics). The net charge exhibited by any protein depends on the relative quantities of these amino acids present in the protein, and on the pH of the protein solution. The pH value at which a protein molecule possesses zero overall charge is termed its isoelectric point (pI). At pH values above its pI a protein will exhibit a net negative charge, whereas at pH values below the pI a protein will exhibit a net positive charge (see also Chapter 1, Figure 1.5).

Ion-exchange chromatography is based on the principle of reversible electrostatic attraction of a charged molecule to a solid matrix which contains covalently attached side groups of opposite charge (Figure 4.15). Proteins may subsequently be eluted by altering the pH or by increasing the salt concentration of the irrigating buffer. Ion-exchange matrices that contain covalently attached positive groups are termed anion exchangers. These will adsorb anionic proteins, such as proteins with a net negative charge. Matrices to which negatively charged groups are covalently attached are termed cation exchangers, adsorbing cationic proteins such as positively charged proteins. Positively charged functional groups (anion exchangers) include



**Figure 4.13** The application of gel filtration chromatography to separate proteins from molecules of much lower molecular mass. The mobile phase (the 'running buffer') will be devoid of the molecular species to be removed from the protein. Highly cross-linked porous beads are used, which exclude all protein molecules. However, the lower molecular mass substances can enter the beads and their progress down through the column will therefore be retarded (a). The earlier fractions collected will contain the proteins while the latter fractions will contain the low-molecular-mass contaminants (b). In practice this 'group separation' application of gel filtration chromatography is mainly used to separate proteins from salt (e.g. after an ammonium sulfate precipitation step) or for buffer exchange.



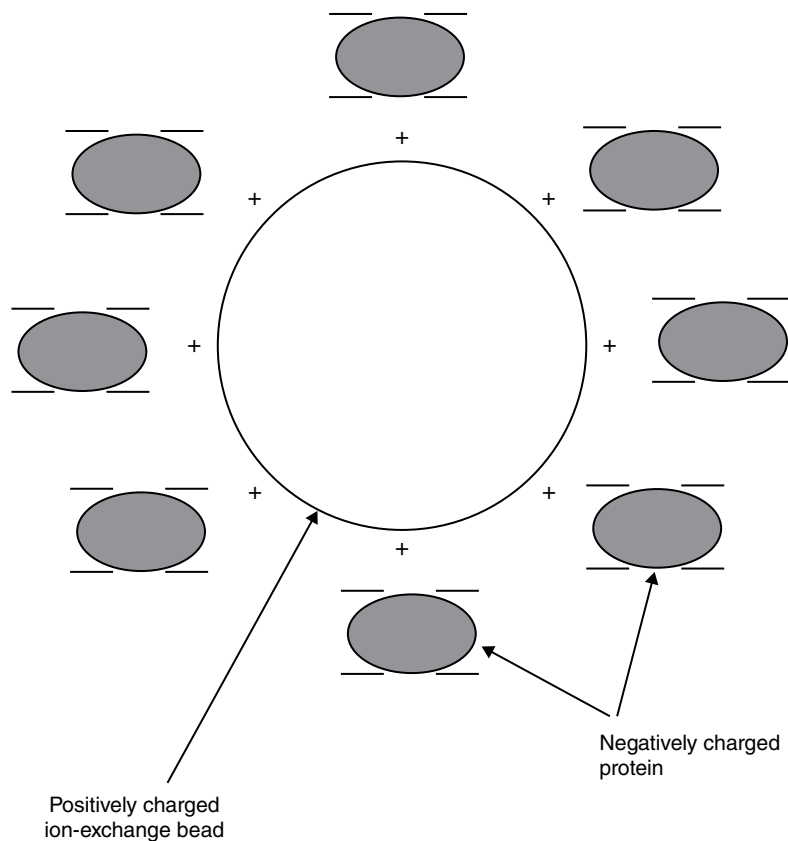
**Figure 4.14** Structure of amino acids having overall net charges at pH 7.0. In proteins the charges associated with the  $\alpha$ -amino and  $\alpha$ -carboxyl groups in all but the terminal amino acids are not present, as these groups are directly involved in the formation of peptide bonds.

species such as aminoethyl and diethylaminoethyl groups. Negatively charged groups attached to suitable matrices forming cation exchangers include sulfo and carboxymethyl groups (Table 4.7).

During the cation-exchange process positively charged proteins bind to the negatively charged ion-exchange matrix by displacing the counter-ion (often  $\text{H}^+$ ) which is initially bound to the resin by electrostatic attraction. Elution may be achieved using a salt-containing irrigation buffer. The salt cation, often  $\text{Na}^+$  of  $\text{NaCl}$ , in turn displaces the protein from the ion-exchange matrix. In the case of negatively charged proteins, an anion exchanger is obviously employed, with the protein adsorbing to the column by replacing a negatively charged counter-ion.

The vast majority of purification schemes, certainly in which non-affinity-tagged recombinant proteins are being purified, employ at least one ion-exchange step – it represents the single most popular chromatographic technique in the context of protein purification. Its popularity is based on the high level of resolution achievable, its straightforward scale-up (for industrial application), together with its ease of use and ease of column regeneration. In addition, it leads to a concentration of the protein of interest. It is also one of the least expensive chromatographic methods available. At physiological pH values most proteins exhibit a net negative charge. Anion exchange chromatography is therefore most commonly used.





**Figure 4.15** Principle of ion-exchange chromatography, in this case anion exchange chromatography. The chromatographic beads exhibit an overall positive charge. Proteins displaying a net negative charge at the pH selected for the chromatography will bind to the beads due to electrostatic interactions.

### 4.5.3 Hydrophobic interaction chromatography

Of the 20 amino acids commonly found in proteins, eight are classified as hydrophobic due to the non-polar nature of their side chains (R groups, Figure 4.16). Most proteins are folded such that the majority of their hydrophobic amino acid residues are buried internally in the molecule, and hence are shielded from the surrounding aqueous environment (see Chapter 2). Internalized hydrophobic groups normally associate with adjacent hydrophobic groups. However, a minority of hydrophobic amino acids are present on the protein surface and hence are exposed to the outer aqueous environment. Different protein molecules differ in the number and type of hydrophobic amino acids on their surface, and hence

their degree of surface hydrophobicity. Hydrophobic amino acids tend to be arranged in clusters or patches on the protein surface. Hydrophobic interaction chromatography fractionates proteins by exploiting their differing degrees of surface hydrophobicity. It depends on the occurrence of hydrophobic interactions between the hydrophobic patches on the protein surface and hydrophobic groups covalently attached to a suitable matrix.

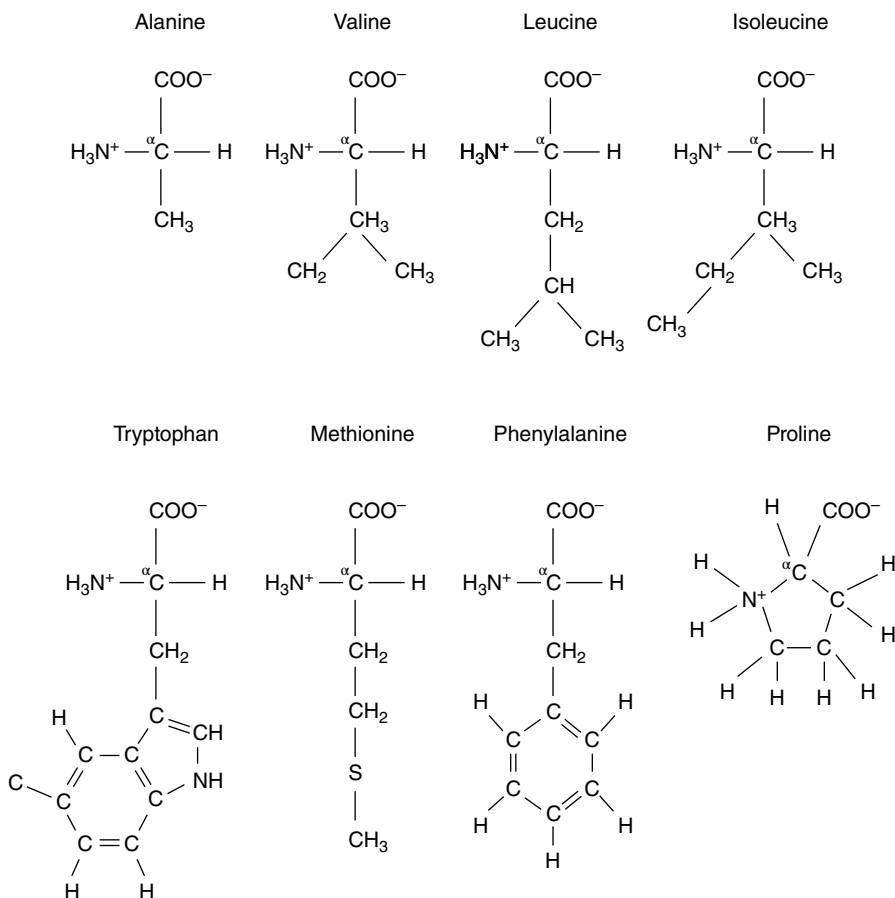
In hydrophobic interaction chromatography, the most popular beads (resins) are cross-linked agarose gels to which hydrophobic groups have been covalently linked. Specific examples include octyl- and phenyl-Sepharose gels that contain octyl and phenyl hydrophobic groups, respectively (Figure 4.17). Protein separation by hydrophobic interaction chromatography depends on interactions between the

**Table 4.7** Functional groups commonly attached to chromatographic beads in order to generate cation or anion exchangers.

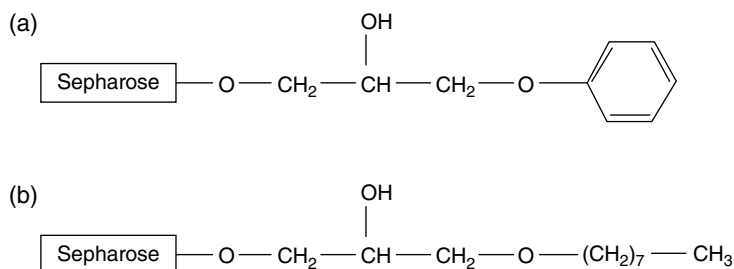
Group name	Group structure	Exchanger type
Diethylaminoethyl (DEAE)	$-\text{O}-(\text{CH}_2)_2-\text{NH}^+-$ $(\text{CH}_2-\text{CH}_3)_2$	Anion exchanger
Quaternary ammonium (Q)	$-\text{CH}_2-\text{NH}^+(\text{CH}_3)_3$	Anion exchanger
Quaternary aminoethyl (QAE)	$-\text{O}-(\text{CH}_2)_2-\text{N}^+(\text{C}_2\text{H}_5)_2$ $-\text{CH}_2-\text{CHOH}-\text{CH}_3$	Anion exchanger
Carboxymethyl (CM)	$-\text{O}-\text{CH}_2-\text{COO}^-$	Cation exchanger
Methylsulfonate (S)	$-\text{CH}_2-\text{SO}_3^-$	Cation exchanger
Sulfopropyl (SP)	$-\text{CH}_2-\text{CH}_2-\text{CH}_2\text{SO}_3^-$	Cation exchanger

protein itself, the gel matrix and the surrounding aqueous solvent. Increasing the ionic strength of a solution by the addition of a neutral salt (e.g. ammonium sulfate or sodium chloride) increases the hydrophobicity of protein molecules. This may be explained (albeit somewhat simplistically) on the basis that the hydration of salt ions in solution results in an ordered shell of water molecules forming around each ion. This attracts water molecules away from protein molecules, which in turn helps to unmask hydrophobic domains on the surface of the protein.

Protein samples are therefore best applied to hydrophobic interaction columns under conditions of high ionic strength. As they percolate through the



**Figure 4.16** Structural formulae of the eight commonly occurring amino acids that display hydrophobic characteristics.



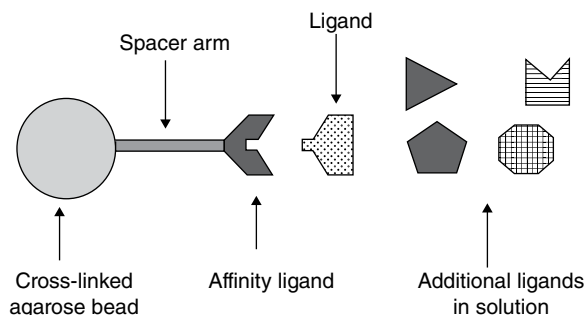
**Figure 4.17** Chemical structure of (a) phenyl and (b) octyl Sepharose, widely used in hydrophobic interaction chromatography.

column, proteins may be retained via hydrophobic interactions. The more hydrophobic the protein, the tighter the binding. After a washing step, bound protein may be eluted utilizing conditions which promote a decrease in hydrophobic interactions. This may be achieved by irrigation with a buffer of decreased ionic strength, inclusion of a suitable detergent, or lowering the polarity of the buffer by including agents such as ethanol or ethylene glycol.

Reverse-phase chromatography may also be used to separate proteins on the basis of differential hydrophobicity. This technique involves applying the protein sample to a highly hydrophobic column, often a C18 column (in which the chromatographic beads have the highly hydrophobic  $(\text{CH}_2)_{17}\text{CH}_3$  groups covalently attached). Elution is subsequently promoted by decreasing the polarity of the mobile phase. This is normally achieved by the introduction of an organic solvent. Elution conditions are harsh, and generally result in denaturation of many proteins.

#### 4.5.4 Affinity chromatography

Affinity chromatography is perhaps the most powerful and highly selective method of protein purification available. This technique relies on the ability of most proteins to bind specifically and reversibly to other compounds, often termed ligands (Figure 4.18). A wide variety of ligands may be covalently attached to an inert support matrix, and subsequently packed into a chromatographic column. In such a system, only the protein molecules that selectively bind to the immobilized ligand will be retained on the



**Figure 4.18** Schematic representation of the principle of biospecific affinity chromatography. The chosen affinity ligand is chemically attached to the support matrix (agarose bead) via a suitable spacer arm. Only those ligands in solution that exhibit biospecific affinity for the immobilized species will be retained.

column. Washing the column with a suitable buffer will flush out all unbound molecules.

Elution of bound protein from an affinity column is achieved by altering the composition of the elution buffer, such that the affinity of the protein for the immobilized ligand is greatly reduced. A variety of non-covalent interactions contribute to protein–ligand interaction. In many cases, changes in buffer pH, ionic strength, inclusion of a detergent or agents such as ethylene glycol that reduce solution polarity may suffice to elute the protein. In other cases, inclusion of a competing ligand promotes desorption. Competing ligands often employed include free substrates, substrate analogues or cofactors. Use of a competing ligand generally results in more selective protein desorption than does the generalized approach, such as alteration of buffer pH or ionic strength. In some cases, a combination of such

elution conditions may be required. Identification of optimal desorption conditions often requires considerable empirical study.

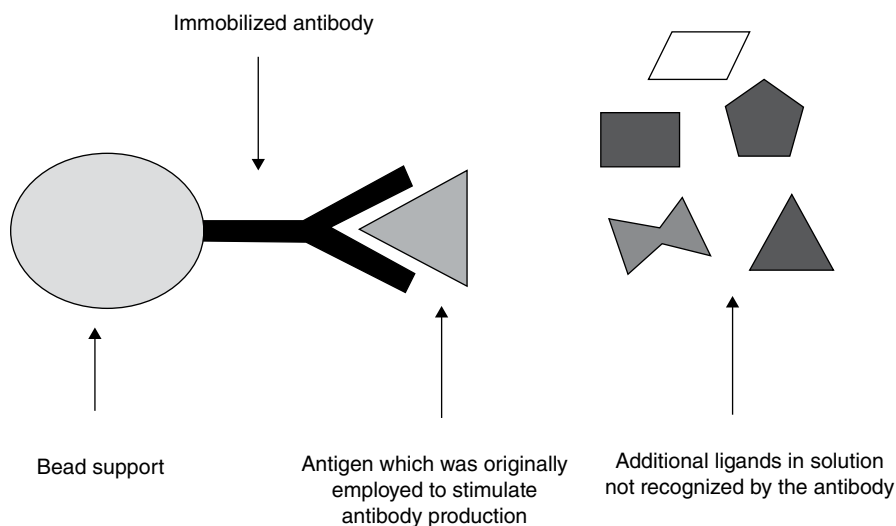
Affinity chromatography offers many advantages over conventional chromatographic techniques. The specificity and selectivity of biospecific affinity chromatography cannot be matched by other chromatographic procedures. Increases in purity of over 1000-fold with almost 100% yields are often reported, at least on a laboratory scale. Incorporation of an affinity step could thus drastically reduce the number of subsequent steps required to achieve protein purification. Despite such promise, biospecific affinity chromatography does display some practical limitations.

- Many biospecific ligands are extremely expensive and often exhibit poor stability.
- Many of the ligand-coupling techniques are chemically complex, hazardous, time-consuming and costly;
- Any leaching of coupled ligands from the matrix also gives cause for concern for two reasons: (i) it effectively reduces the capacity of the system and (ii) leaching of what are often noxious chemicals into the protein products is undesirable (particularly if the protein is being purified with a view to its industrial application).

#### 4.5.4.1 Immunoaffinity purification

Immobilized antibodies may be used as affinity adsorbents for the antigens that stimulated their production (Figure 4.19). Antibodies, like many other biomolecules, may be immobilized on a suitable support matrix by a variety of chemical coupling procedures.

Immunoaffinity chromatography is among the most highly specific of all forms of biospecific chromatography. The high affinity with which an antibody normally binds its ligand can often make subsequent ligand desorption from the column difficult. Desorption can require conditions that result in partial denaturation of the bound protein. This is often achieved by alteration of buffer pH or by employing chemical disrupting agents such as urea or guanidine. One of the most popular elution methods involves irrigation with a glycine-HCl buffer at pH 2.2–2.8. In some cases, elution is more readily attainable at alkaline pH values. Specific examples have been documented in which protein elution was performed under relatively mild conditions, such as a change of buffer system or an increase in ionic strength, although such examples are exceptional. The inclusion of an immunoaffinity step in the purification of recombinant blood factor VIII used to treat haemophilia (see



**Figure 4.19** Principle of immunoaffinity chromatography. Only antigen that is specifically recognized by the immobilized antibody will be retained on the column.

Chapter 6) is one example of the industrial use of this technique.

#### 4.5.4.2 Protein A chromatography

Most species of *Staphylococcus aureus* produce a protein known simply as protein A. This protein consists of a single polypeptide chain of molecular mass 42 kDa. Protein A binds the Fc region (the constant region) of IgG (see Chapter 7) obtained from human and many other mammalian species with high specificity and affinity. Immobilization of protein A on chromatography beads provides a powerful affinity system that may be used to purify IgG. However, there is considerable variation in the binding affinity of protein A for various IgG subclasses obtained from different mammalian sources. In some cases another protein, protein G, may be used instead of protein A. Most immunoglobulin molecules that bind to immobilized protein A do so under alkaline conditions, and may subsequently be eluted at acidic pH values.

#### 4.5.4.3 Lectin affinity chromatography

Lectin affinity chromatography may be used to purify a range of glycoproteins. Lectins are a group of proteins synthesized by plants, vertebrates and a number of invertebrate species. Especially high levels of lectins are produced by a variety of plant seeds. Plant lectins are often termed phytohaemagglutinins. All lectins have the ability to bind certain monosaccharides (e.g.  $\alpha$ -D-mannose,  $\alpha$ -D-glucose, D-N-acetylgalactosamine) and the sugar specificity for many are known (Table 4.8). Among the best-known and most widely used lectins are concanavalin A (Con A), soybean lectin (SBL) and wheat germ agglutinin (WGA).

Glycoproteins generally bind to lectin affinity columns at pH values close to neutrality. Desorption may be achieved in some cases by alteration of the pH of the eluting buffer. However, the most common method of desorption involves inclusion of free sugar molecules for which the lectin exhibits a high affinity in this elution buffer, i.e. the inclusion of a competing ligand.

Although lectin affinity chromatography may be utilized to purify a variety of glycoproteins, it

**Table 4.8** Some lectins commonly used in immobilized format for the purification of glycoproteins. The sugar specificity is listed, as are the free sugars used to elute the bound glycoprotein.

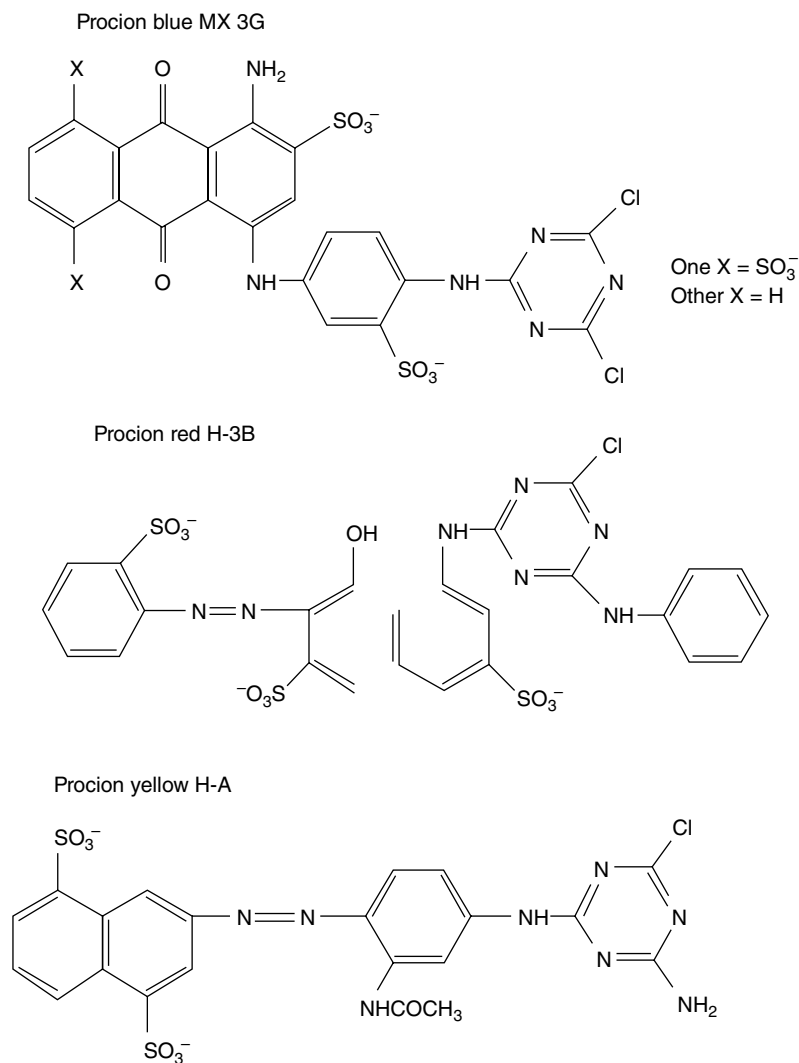
Lectin	Source	Sugar specificity	Eluting sugar
ConA	Jack bean seeds	$\alpha$ -D-mannose, $\alpha$ -D-glucose	$\alpha$ -D-methyl mannose
WGA	Wheat germ	N-acetyl- $\beta$ -D-glucosamine	N-acetyl- $\beta$ -D-glucosamine
PSA	Peas	$\alpha$ -D-mannose	$\alpha$ -D-methyl mannose
LEL	Tomato	N-acetyl- $\beta$ -D-glucosamine	N-acetyl- $\beta$ -D-glucosamine
STL	Potato tubers	N-acetyl- $\beta$ -D-glucosamine	N-acetyl- $\beta$ -D-glucosamine
PHA	Red kidney bean	N-acetyl-D-galactosamine	N-acetyl-D-galactosamine
ELB	Elderberry bark	Sialic acid or N-acetyl-D-galactosamine	Lactose
GNL	Snowdrop bulbs	$\alpha$ 1 $\rightarrow$ 3 Mannose	$\alpha$ -methyl mannose
AAA	Freshwater eel	$\alpha$ -L-fucose	L-fucose

has not been widely employed for a number of reasons:

- most lectins are quite expensive;
- crude protein sources containing one glycoprotein usually contain multiple glycoproteins and in most such instances lectin-based affinity systems will result in the co-purification of several such glycoproteins;
- limited application of this approach means it has little track record, particularly on an industrial scale.

#### 4.5.4.4 Dye affinity chromatography

The development of dye affinity chromatography may be attributed to the observation that some proteins exhibit anomalous elution characteristics when fractionated on gel filtration columns in the presence of blue dextran. Blue dextran consists of a triazine dye (Cibacron Blue F3G-A) covalently linked to the high-molecular-mass sugar dextran. The discovery that some proteins bind the triazine



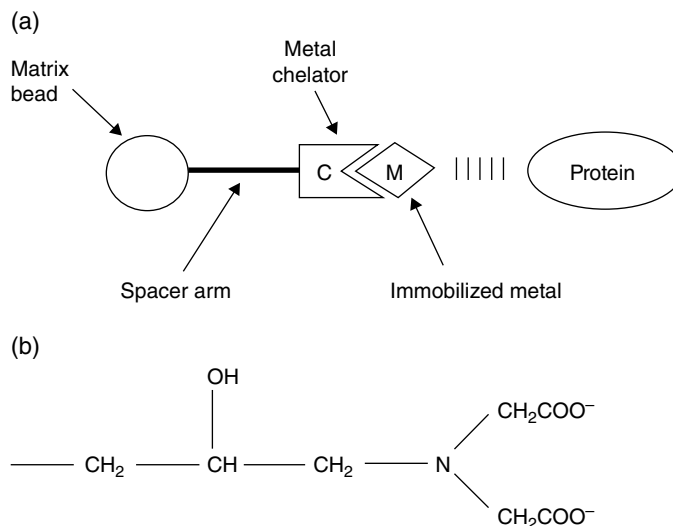
**Figure 4.20** Some monochloro and dichloro triazine dyes commonly used as affinity ligands in dye affinity chromatography.

dye led to its use as an affinity adsorbent by immobilization on an agarose matrix. A variety of other triazine dyes (Figure 4.20) also bind certain proteins and hence have also been used as affinity adsorbents. Dye affinity chromatography displays some positive general characteristics.

- The dyes are readily available in bulk and are relatively inexpensive.
- Chemical coupling of the dyes to the matrix is usually straightforward, often requiring no

more than incubation under alkaline conditions at elevated temperature. The use of noxious coupling chemicals such as cyanogen bromide is avoided.

- The dye–matrix bead linkage is relatively resistant to chemical, physical and enzymatic degradation. In this way ligand leakage from the column is minimized and is easily recognizable if it does occur due to the dye's colour.
- The protein binding capacity of immobilized dye adsorbents is also high and exceeds the binding



**Figure 4.21** Schematic representation of the basic principles of metal chelate affinity chromatography. (a) Certain proteins are retained on the column via the formation of coordinate bonds with the immobilized metal ion. (b) The structure of the most commonly used metal chelator, iminodiacetic acid (IDA).

capacity normally exhibited by natural biospecific adsorption ligands.

- Elution of bound protein is also relatively easily achieved.

However, a major potential disadvantage is that it is not possible to accurately predict if a specific protein will be retained on a dye affinity column, or what conditions will allow optimum binding/elution. Such information must be derived by empirical study. An understanding of the specific interactions that allow many apparently unrelated proteins to bind to dye affinity ligands, while other proteins are not retained, is usually lacking. The presence of negatively charged sulfonate groups lends triazine dyes an ion-exchange character. These dyes also contain aromatic groups that can lend them some degree of hydrophobicity. Hydrophobic interactions along with charged-based interactions therefore may play some role in protein adsorption. The dyes can also hydrogen bond with proteins.

#### 4.5.4.5 Immobilized metal ion affinity chromatography

Immobilized metal ion affinity chromatography (IMAC) is a pseudoaffinity protein purification

technique first developed in the 1970s. The mode of adsorption relies on the formation of weak coordinate bonds between basic groups on a protein surface with metal ions immobilized on chromatographic beads (Figure 4.21). The affinity media is synthesized by covalent attachment of a metal chelator to the chromatographic bead via a spacer arm. Chelating agents such as iminodiacetate (IDA) are capable of binding a number of metal ions (e.g. Fe, Co, Ni, Cu, Zn, Al), and binding effectively immobilizes the ion on the bead. The affinity gel is normally supplied without bound metal, so the gel can be 'charged' with the metal of choice (by flushing the column with a solution containing a salt of that metal, e.g. CuSO<sub>4</sub> in the case of copper). The metal ions most commonly used are Zn<sup>2+</sup>, Ni<sup>2+</sup> and Cu<sup>2+</sup>. Basic groups on protein surfaces, most notably the side chain of histidine residues, are attracted to the metal ions, forming the weak coordinate bonds. Elution of bound proteins is undertaken by lowering the buffer pH (this causes protonation of the histidine residues, which are then unable to coordinate with the metal ion). Alternatively, a strong competitor complexing agent (e.g. the chelating agent EDTA) can be added to the elution buffer.

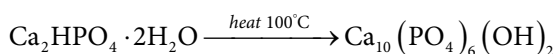
IMAC finds most prominent application in the affinity purification of recombinant proteins to

which a histidine tag has been attached (described later). As protein binding occurs via the histidine residues this technique is no more inherently useful for the purification of metalloproteins than for the purification of non-metalloproteins (a common misconception, given its name).

### 4.5.5 Chromatography on hydroxyapatite

Hydroxyapatite occurs naturally as a mineral in phosphate rock and also constitutes the mineral portion of bone. It also may be used to fractionate proteins by chromatography.

Hydroxyapatite is prepared by mixing a solution of sodium phosphate ( $\text{Na}_2\text{HPO}_4$ ) with calcium chloride ( $\text{CaCl}_2$ ). A white precipitate known as brushite is formed. Brushite is then converted to hydroxyapatite by heating to  $100^\circ\text{C}$  in the presence of ammonia.



Brushite

Hydroxyapatite

The underlying mechanism by which this substance binds and fractionates proteins is poorly understood. Protein adsorption is believed to involve interaction with both calcium and phosphate moieties of the hydroxyapatite matrix. Elution of bound species from such columns is normally achieved by irrigation with a potassium phosphate gradient.

### 4.5.6 Chromatofocusing

Fractionation by chromatofocusing separates proteins on the basis of their isoelectric points. This technique basically involves percolating a buffer of one pH through an ion-exchange column which is pre-equilibrated at a different pH. Because of the natural buffering capacity of the exchanger, a continuous pH gradient may be set up along the length of the column. In order to achieve maximum resolution a linear pH gradient must be constructed. This necessitates the use of an eluent buffer and exchanger

that exhibit even buffering capacity over a wide range of pH values. The range of the pH gradient achieved will obviously depend on the pH at which the ion exchanger is pre-equilibrated and the pH of the eluent buffer. The sample is applied, usually in the running buffer, whose pH is lower than that of the pre-equilibrated column. After sample application, the column is constantly percolated with a specially formulated buffer that establishes an increasing pH gradient down the length of the column.

On sample application, negatively charged proteins immediately adsorb to the anion exchanger, while positively charged proteins flow down the column. Because of the increasing pH gradient formed, such positively charged proteins will eventually reach a point within the column where the column pH equals their own pI values (i.e. the pH at which the protein has an overall net charge of zero). Immediately on further migration down the column such proteins become negatively charged, as the surrounding pH values increase above the protein's pI value, so they bind to the column. Overall, therefore, on initial application of the elution buffer, all protein species will migrate down the column until they reach a point where the column pH is marginally above their isoelectric points. At this stage they bind to the anion exchanger. Proteins of differing isoelectric points are thus fractionated on the basis of this parameter of molecular distinction.

The pH gradient formed is not a static one. As more elution buffer is applied, the pH value at any given point along the column is continually increasing. Thus any protein which binds to the column will be almost immediately desorbed as once again it experiences a surrounding pH value above its pI, and becomes positively charged. Any such desorbed protein flows down the column until it reaches a further point where the pH value is marginally above its pI value, and it again rebinds. This process is repeated until the protein emerges from the column at its isoelectric point. To achieve best results, the pI value of the required protein should ideally be in the middle of the pH gradient generated. Chromatofocusing can result in a high degree of protein resolution, with protein bands being eluted as tight peaks. This technique is



particularly effective when used in conjunction with other chromatographic methods during protein purification. Most documented applications of this method still pertain to laboratory-scale procedures. Scale-up to industrial level is somewhat discouraged by economic factors, most notably the cost of the eluent required.

### 4.5.7 High performance liquid chromatography

Most of the chromatographic techniques described thus far are usually performed under relatively low pressures where flow rates through the column are generated by low-pressure pumps (low-pressure liquid chromatography or LPLC; see Figure 4.12). Faster separation times coupled to improved resolution can generally be achieved by high performance liquid chromatography (HPLC; see Chapter 1).

In the context of protein purification/characterization, HPLC may be used for analytical or preparative purposes. Most analytical HPLC columns available have diameters ranging from 4 to 4.6 mm and lengths from 10 to 30 cm (Figure 4.22). Preparative HPLC columns currently available have much wider diameters, typically up to 80 cm, and can be longer than 1 m. Various chemical groups may be incorporated into the matrix beads, so

techniques such as ion exchange, gel filtration, affinity, hydrophobic interaction and reversed-phase chromatography are all applicable to HPLC.

Many small proteins, particularly those which function extracellularly (e.g. insulin, growth hormone and various cytokines), are quite stable and may be fractionated on a variety of HPLC columns without significant denaturation or decrease in bio-activity. In contrast, many larger proteins are relatively labile and loss of activity due to protein denaturation may be observed on high-pressure fractionation.

At both preparative and analytical levels HPLC exhibits several important advantages as compared with low-pressure chromatographic techniques (see also Chapter 1).

- HPLC offers superior resolution due to the reduction in bead particle size. The diffusional distance inside the matrix particles is minimized, resulting in sharper peaks than those obtained when low-pressure systems are employed.
- Because of increased flow rates HPLC systems also offer much improved fractionation speeds, typically in the order of minutes rather than hours.
- HPLC is amenable to a high degree of automation.

An alternative chromatographic system to HPLC is also available. Termed FPLC or fast protein liquid

(a)



(b)



**Figure 4.22** A typical laboratory HPLC system (a) and a selection of typical HPLC chromatographic columns (b).

chromatography, this technique employs operating pressures significantly lower than those used in conventional HPLC systems. Lower pressures allow use of matrix beads based on polymers such as agarose. FPLC chromatographic columns are constructed of glass or inert plastic materials. Conventional HPLC columns are manufactured from high-grade stainless steel. Despite their operation at lower pressures, they still combine high resolution with enhanced speed of operation compared with traditional low-pressure systems.

#### 4.5.8 Purification of recombinant proteins

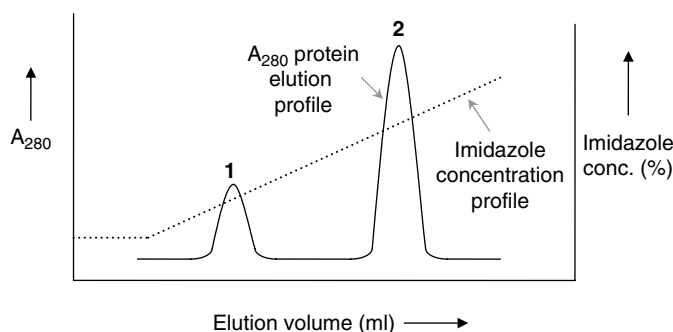
As already outlined in this chapter (and see also Box 3.1), recombinant proteins are often engineered to contain an affinity purification fusion tag. Such proteins can be very substantially or fully purified by a single chromatographic step using chromatographic beads to which a ligand that specifically and selectively binds the affinity tag has been attached.

Several affinity purification tag systems have been developed, the more common of which are the poly-histidine tag and the glutathione *S*-transferase (GST) tag. Inclusion of a poly-histidine tag in a fusion protein allows affinity-based purification

using IMAC (see Figure 4.21), as the electron donor groups on the imidazole ring portion of the histidine side chains form coordination bonds with the immobilized transition metal (usually  $\text{Ni}^{2+}$ ) immobilized on the IMAC column. Moreover, bound protein can be conveniently eluted from the column by including free imidazole in the elution buffer (Figure 4.23). An alternative means of elution is to lower the pH of the eluting buffer (causing protonation of the histidine). Poly-histidine tags can be composed of two to ten consecutive histidine residues, with six-residue tags being most commonly used. The presence of the tag can also provide a convenient means of detecting or quantifying the recombinant protein (Box 4.1).

GST is a much larger affinity tag, being a 26-kDa protein produced naturally by the parasite *Schistosoma japonicum*, and binds with high selectivity to the tripeptide glutathione. GST-tagged proteins therefore bind to affinity columns containing immobilized glutathione, and can subsequently be eluted from the column by the inclusion of free glutathione in the elution buffer.

Tags can be attached at the N-terminus or C-terminus of the target protein. This flexibility becomes important in cases where local structural characteristics at one or other end of the protein



**Figure 4.23** Schematic diagram of an elution profile from an IMAC column onto which a His-tagged unpurified protein extract has been loaded and unbound protein subsequently washed through. In this case elution is promoted via application of an increasing gradient of imidazole concentration in the eluting buffer, and protein elution from the column is followed by monitoring absorbance of the eluate at 280 nm. Sometimes an initial elution peak is observed at low imidazole concentrations (peak 1). This usually represents non-tagged proteins, which weakly bind the column via, for example, histidine residues naturally present on their surface. His-tagged proteins bind more tightly to the column and are therefore eluted only at higher imidazole concentrations (peak 2).

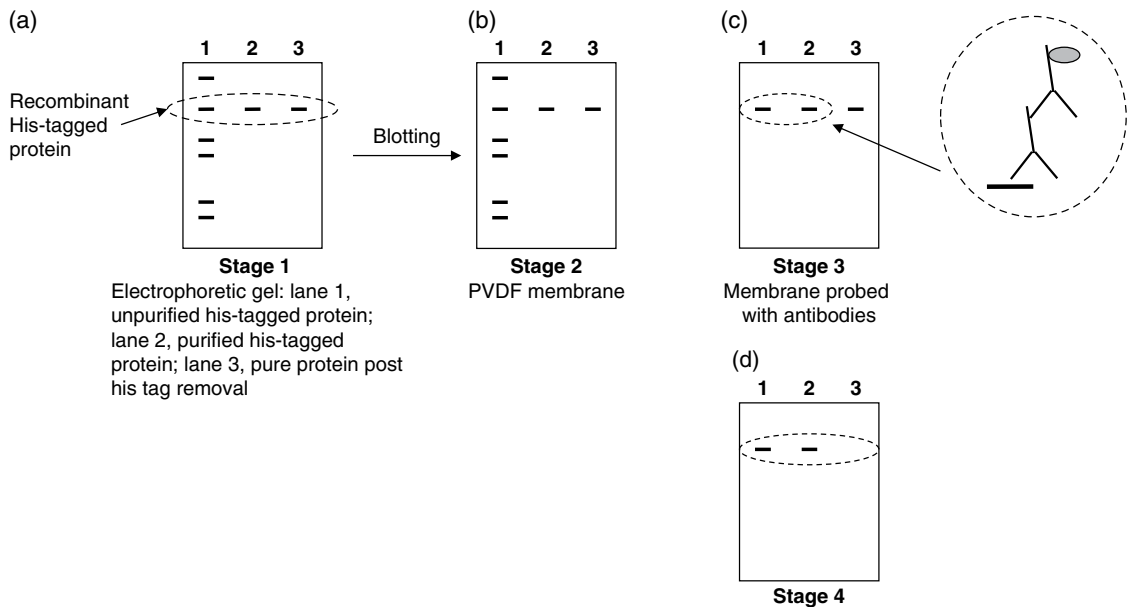
**Box 4.1 Detecting His-tagged proteins and verifying tag removal**

The presence of a histidine (His) tag, in addition to facilitating convenient protein purification, can also facilitate convenient detection and quantification of the tagged protein. Traditionally this entailed the application of antibodies raised against the tag itself. The antibodies can be used in different formats, for example they can be used to develop an immunoassay-based approach (see Chapter 10) to detect and quantify the tagged protein in solution. Alternatively the antibodies can be used as probes for post-electrophoretic protein analysis. This approach, termed Western blotting or immunoblotting, is outlined below.

Western blotting involves initial separation of the proteins in the sample to be analysed via electrophoresis (see Chapter 1). Non-denaturing, denaturing (SDS-PAGE) and two-dimensional electrophoresis have all been used (stage 1 in the diagram below). The separated protein bands are then transferred from the electrophoretic gel onto a thin membrane made from nitrocellulose or polyvinylidene difluoride (PVDF) by a process known as electroblotting (stage 2). The membrane surface is probed using an anti-His

tag antibody, which will bind any His-tagged protein on the membrane surface (stage 3). After a washing step (to remove unbound antibody), the membrane is incubated with a second antibody that recognizes the bound antibody. The second antibody has a detection tag attached (such as an enzyme capable of generating a chemiluminescent signal; stage 3), which allows visualization of the His-tagged protein (stage 4). This approach can also be used to verify that the tag has been subsequently successfully removed post purification (stage 4 in diagram and see main text).

A more recent approach to His tag detection entails the use of fluorescent stains that can be applied directly to the electrophoretic gel for tag detection. This approach is more convenient than the Western blot approach described above. The detection agent is composed of a fluorescent (or other easily detectable) tag conjugated to a  $\text{Ni}^{2+}$ :nitrilotriacetic acid (NTA) complex. The  $\text{Ni}^{2+}$  (and hence indirectly the fluorescent molecule) binds the polyhistidine tag via the histidine side chain, the same principle as used for IMAC purification.



results in complications such as partial burying of the tag in the protein's interior or where the terminal amino acid sequence might negatively affect the efficiency of subsequent tag removal.

In many cases the affinity tag does not significantly alter protein function. However, it is usually desirable or necessary to remove the tag after IMAC purification, as the presence of the tag may:

- have some effect on the overall protein structure;
- have some effect on protein function;
- be in itself potentially immunogenic (in cases of therapeutic proteins).

Removal of the tag may be carried out by chemical or enzymatic means. This is achieved by designing the tag sequence such that it contains a cleavage point for a specific protease or chemical cleavage method at the protein-tag fusion junction. Sequence-specific proteases often employed to achieve tag removal include thrombin (factor IIa), factor Xa, enterokinase and TEV (tobacco etch virus) protease (Box 4.2).

Although there are several methods that can achieve tag removal, most approaches suffer from some inherent or potential limitation(s).

- One essential prerequisite for any method is that the protein itself must remain intact after the cleavage treatment. The required protein therefore should not contain any peptide bonds susceptible to cleavage by the specific method chosen.
- Chemical methods are generally undertaken under harsh conditions, often requiring high temperatures or extremes of pH which could denature the protein.
- Proteolytic removal of tags in particular is often less than 100% efficient, thereby leaving a fraction of the original tag-protein fusion products still intact.
- The addition of a poly-histidine tag can sometimes trigger protein aggregation.
- Selective cleavage of the tag must be followed by subsequent separation of the tag from the protein of interest. This may require a further chromatographic step.

#### Box 4.2 TEV proteases

TEV protease is a 50-kDa proteolytic enzyme (Chapter 12) that recognizes a specific seven-amino-acid cleavage sequence in target protein substrates (Glu-X-X-Tyr-X-Gln-Y, where X represents any amino acid and Y any amino acid except proline), with cleavage occurring after the Gln residue. This cleavage sequence can easily be included in appropriate expression vectors (see Box 3.1).

At the core of the enzyme's active site is a His<sup>234</sup>Asp<sup>269</sup>Cys<sup>339</sup> catalytic triad and the enzyme is optimally active at temperatures approaching 30°C and pH 6.5–8.5. The long cleavage recognition sequence confers an advantage on TEV protease in that such a lengthy sequence is rarely found in proteins and hence the enzyme will rarely target a recombinant protein for hydrolysis. In contrast some of the alternative tag-cleaving proteases recognize shorter sequences, which are therefore statistically more likely to occur in recombinant proteins. Factor Xa, for example, displays just a four-amino-acid target recognition sequence.

The main weakness of TEV protease is that it undergoes autoproteolysis during storage, generating a truncated version of the enzyme with greatly reduced catalytic activity. Autoproteolysis occurs between Met<sup>218</sup> and Ser<sup>219</sup> in the molecule's catalytic domain.

Protein engineering has been used to overcome this weakness. Unsurprisingly, most engineering efforts centred around substitution of amino acids contributing, or immediately adjacent, to the autoproteolysis peptide bond. While a number of variants were generated that displayed little or no autoproteolysis, most also exhibited additional undesirable changes, such as greatly reduced catalytic activity. On the other hand, variants in which the Ser<sup>219</sup> was modified to a Val or Pro retained almost full catalytic activity while being essentially immune from autoproteolysis. At least one commercialized TEV-based protease is based on these findings.

An alternative strategy to tag removal is the use of self-cleaving tags, such as an intein tag. Inteins (intervening proteins, sometimes called 'protein introns') are self-excising segments found naturally within some proteins. However, such systems can still suffer from many of the same limitations as other fusion tags, including less than 100% efficient tag removal rates.

Overall, despite its potential limitations, affinity-based purification of affinity-tagged recombinant proteins will, in most instances, simplify (shorten) purification schemes and underpin high percentage yield of purified protein. Shorter purification schemes can be particularly helpful if the protein is a labile one and can reduce the overall cost of purification (Box 4.3). Moreover, the development of such affinity tags provides a single platform-based approach to the purification of virtually all proteins, which is essential to facilitating high-throughput purification of proteins for the purposes of structural proteomics (see Chapter 1).

#### 4.5.9 Purification of membrane proteins

The great majority of proteins purified and characterized to date are soluble proteins of intracellular or extracellular origin. However, up to 30% of all genome protein-encoding sequences are believed to encode membrane-associated proteins. A proportion of such proteins are relatively loosely associated with the membranes, often interacting with the membrane surface only via non-covalent means, including electrostatic attraction and hydrogen bonding. Such proteins are typically quite soluble in aqueous-based solvent and can usually be detached from the membrane via straightforward means such as changing buffer pH or increasing buffer salt concentration. Once separated from the membrane they may be purified and characterized by standard methodologies.

On the other hand, integral membrane proteins typically span the entire membrane bilayer. The surface characteristics of the transmembrane portion

of such proteins will be hydrophobic in nature. As a consequence, extraction of such proteins into aqueous buffers results in protein aggregation, via the formation of intermolecular hydrophobic interactions. This renders the purification and characterization of integral membrane proteins much more challenging, likely explaining why only about 0.5% of the tens of thousands of proteins described in the protein databank (see Chapter 1) are integral membrane proteins. Nevertheless, interest in such proteins continues to grow. Many have critical cellular roles in, for example, regulation of what enters and leaves the cell, pathways of signal transduction, and in energy generation. Moreover, such proteins represent over 60% of current drug targets. Integral membrane proteins are simply termed 'membrane proteins' for the rest of this section.

Some membrane proteins are expressed at relatively high levels, and thus it may be convenient to purify such proteins by direct extraction from their native membrane source. In other instances, however, it may be more appropriate to produce the integral membrane protein via recombinant means. Expression vectors used for recombinant soluble protein production may also be used to produce membrane proteins, and expression can be undertaken in *Escherichia coli* or other cell types. Insertion of membrane proteins into a membrane in native (non-recombinant) systems is signal sequence dependent. Typically such signal sequences consist of nine to twelve largely hydrophobic amino acid residues at the N-terminus. Such signal sequences are included in recombinant expression constructs, to ensure the protein is inserted into membranes, and the proteins are also invariably tagged with histidine at the C-terminus to facilitate subsequent affinity purification.

Even when high-level expression of functional membrane proteins is achieved, such expression generally remains orders of magnitude lower than that achieved in the case of soluble proteins. This is likely due to the fact that membrane availability for protein insertion is limiting, as well as the generally large and complex nature of many such proteins. Expression in *E. coli* can also result in inclusion body

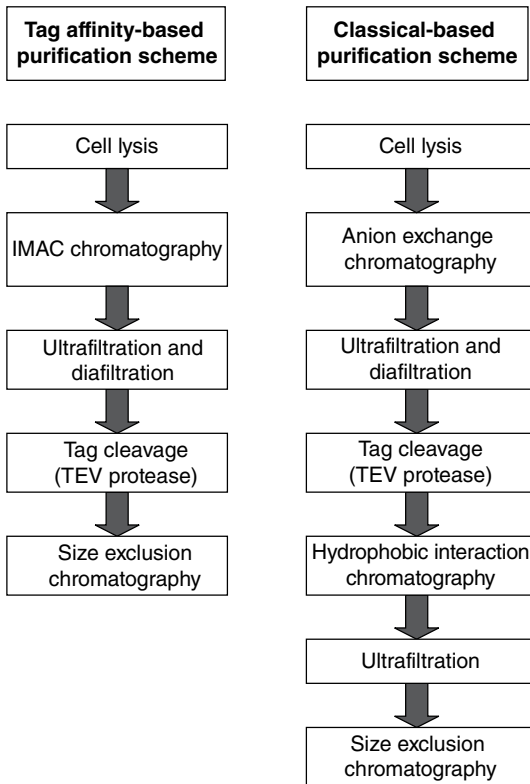
**Box 4.3 Affinity versus classical protein purification: a case study**

In general, tag affinity-based purification of recombinant proteins is faster, cheaper and simpler than classical purification schemes, as exemplified by a case study undertaken in our own laboratory. In this case, a His-tagged recombinant  $\beta$ -galactosidase enzyme was produced in an *E. coli* system. The product accumulated intracellularly

in soluble form. Crude (unpurified) soluble enzyme was extracted via cell lysis, with subsequent development of both an affinity-based (IMAC chromatography) and a classical purification scheme. Considerable empirical studies were undertaken to optimize both purification schemes. In order to generate final identical product, the affinity tag was removed as part of both affinity-based and classical purification schemes. The actual purification schemes developed, which achieved electrophoretic homogeneity, are outlined schematically.

The affinity-based system developed is composed of five processing steps, whereas purification to the same degree required seven steps when the classical approach was pursued. Moreover, the affinity-based purification process resulted in the recovery of 34% of the  $\beta$ -galactosidase present in the crude preparation (i.e. a yield of 34%), whereas the yield recorded for the classical purification scheme was 11%.

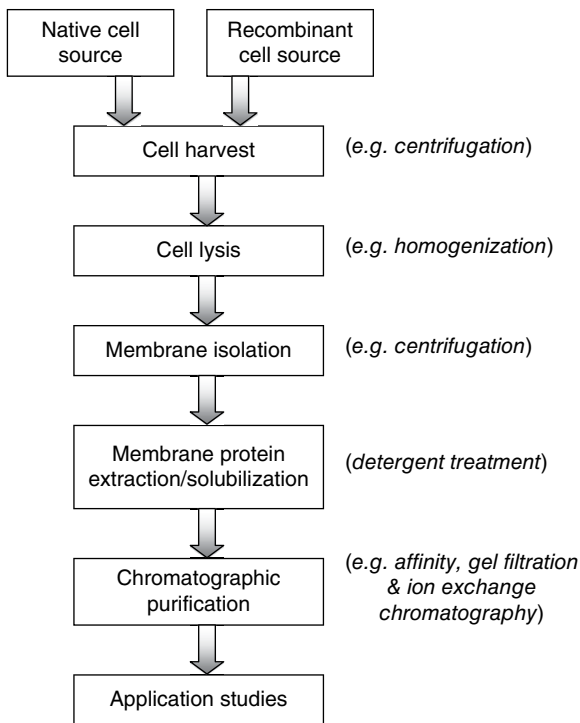
These results have a number of consequences. When calculated in terms of 'per milligram of pure protein generated', the affinity-based system, relative to the classical approach, resulted in a 60% saving in materials cost, an overall water consumption (in terms of making up buffers, etc.) saving of 75%, and a reduction in chemical oxygen demand (an environment pollution indicator) associated with waste streams generated of 60%.



formation, but successful refolding of functionally active membrane proteins from such sources is extremely challenging.

Purification of membrane proteins from native or recombinant systems invariably begins with the isolation of the cellular membrane fraction (Figure 4.24). Isolation of membrane fractions from a cellular lysate can be achieved via centrifugation. In the case of eukaryotic cells/tissue, membrane fractions

from various intracellular organelles can be obtained by first isolating the individual organelles using differential centrifugation. This normally entails fractionation of the initial lysate using stepwise, progressively higher centrifugal forces (Figure 4.25a). Such differential centrifugation usually will not achieve full purification of individual organelle/membrane types. However, further purification may be achieved by applying the sample to density gradient



**Figure 4.24** Overview of membrane protein isolation and purification.

centrifugation (Figure 4.25b), with individual organelles migrating through the gradient until they reach a point that equals their own density. Organelle isolation is of course particularly critical if destined for organelle proteomic studies. Isolation of membrane fractions from prokaryotic cells is also invariably achieved via centrifugation (Figure 4.26).

Several companies now also offer general membrane protein extraction kits. Although straightforward and convenient to use, these kits do not separate membranous structures from different organelles and some contamination by cytosolic proteins remains in the final preparations. For example, the use of one such kit involves the initial treatment of cells/tissue with a mild detergent. This permeabilizes the cells, allowing release of the soluble cytosolic proteins. A second detergent is then used to solubilize the membrane proteins.

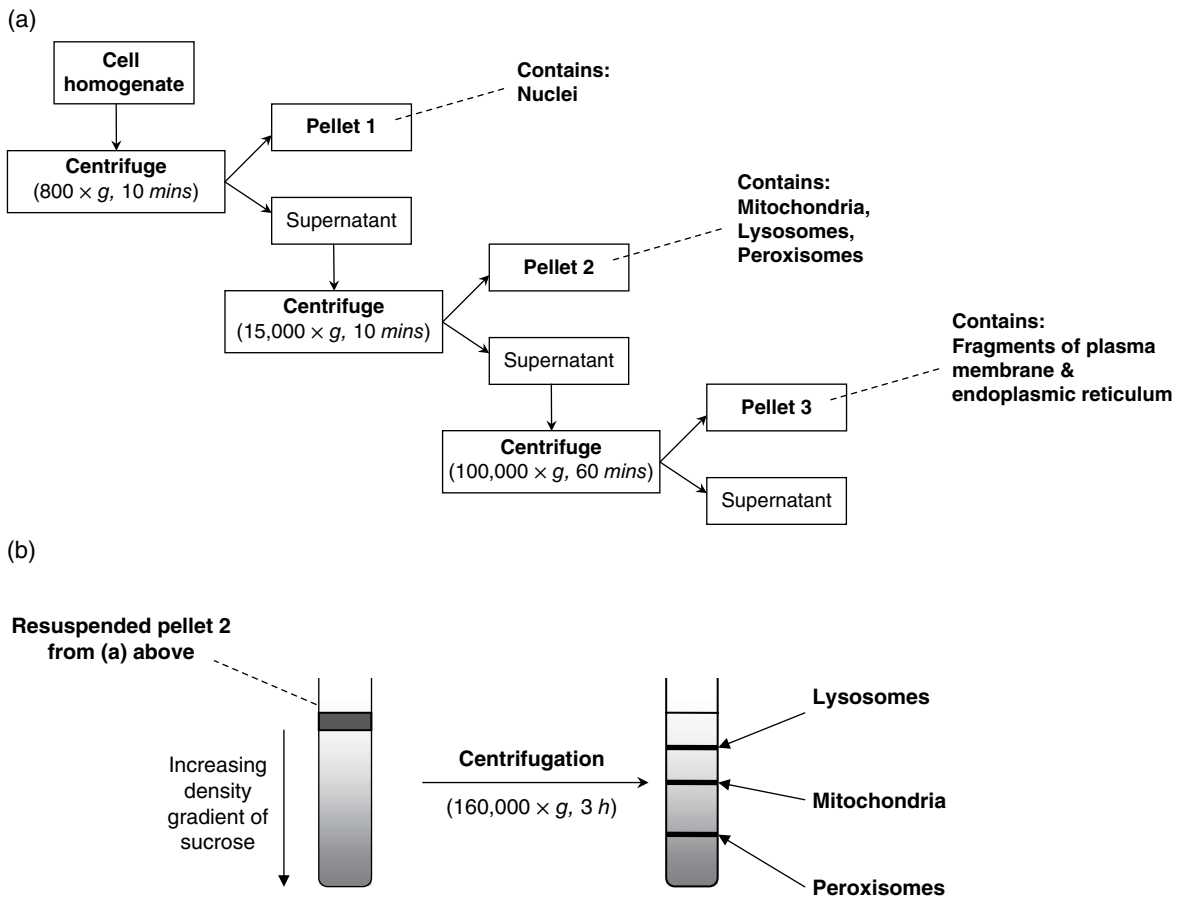
No matter how an initial membrane fraction is prepared, the subsequent step in membrane

protein recovery and purification entails solubilization of the membrane, including the proteins therein, using suitable detergents. Detergents are amphipathic substances, containing both polar (head) groups and non-polar (tail) regions. As such they are capable of interacting with hydrophobic substances, rendering them soluble in polar solutes such as water. Detergents are classified according to the chemistry of their polar head groups as non-ionic, ionic or zwitterionic (Figure 4.27).

Above a certain concentration in water (usually  $<0.2\%$ , known as the critical micellar concentration or CMC), individual detergent molecules interact with each other to form multimolecular complexes (micelles). The non-polar tails interact with each other and are buried within the micelle's interior, shielded from surrounding water. The polar heads, on the other hand, face outward from the micelle surface, freely interacting with the surrounding aqueous media.

The inclusion of a detergent at a concentration above its CMC in an aqueous extraction buffer results in membrane solubilization and detergent complexation with integral membrane proteins. This renders the proteins soluble in aqueous media (Figure 4.28). The proteins often retain their native conformation and functional activity. The exact detergent (and concentration thereof) that facilitates optimum protein extraction and retention of structural/functional integrity can vary from protein to protein and is generally determined by direct experimental screening trials.

Once solubilized, membrane proteins may then be purified using standard chromatographic modalities. The main difference (compared with purification of water-soluble proteins) is that the detergent must be included in all handling solutions during purification (e.g. running and elution buffers). Most chromatographic modalities can be applied in the presence of detergents, including IMAC-based systems used to purify histidine-tagged recombinant proteins. A potential complication in this case, however, is that most detergents negatively affect (though not necessarily totally inactivate) the proteolytic enzymes used to subsequently remove the affinity tags.



**Figure 4.25** Eukaryotic cellular organelle separation via differential centrifugation (a). Individual fractions (pellets) may then be resuspended in a small volume of buffered solution and applied to a density gradient centrifugation step in order to further purify individual organelle fractions (b).

Careful choice of ion-exchange media is important too if ionic detergents are used (anionic detergents are avoided with anion exchange columns, whereas cationic detergents are avoided in cases where a cation exchange step is applied). Hydrophobic interaction chromatography is largely unsuited to membrane protein purification schemes, as the detergent molecules prevent hydrophobic interactions forming between the protein and the chromatographic media.

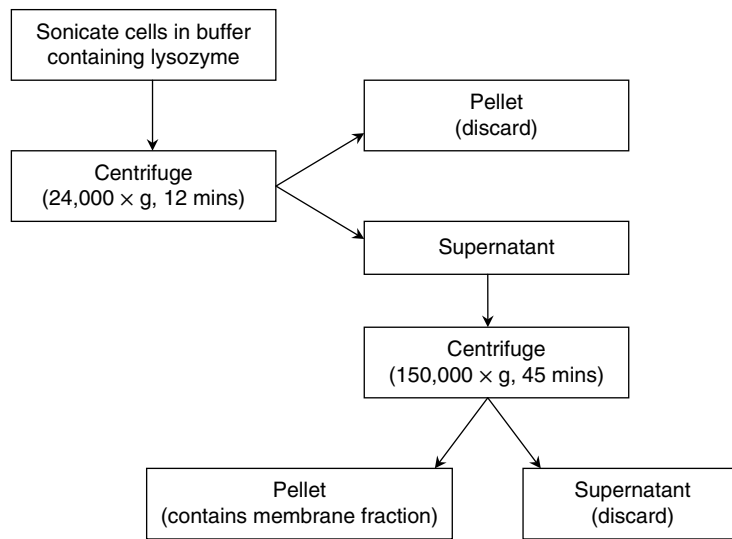
Typical purification schemes for tagged recombinant membrane proteins consist of IMAC, ion exchange and a final gel filtration step. Purification schemes for non-tagged proteins often require additional steps. Finally, although relatively

uncommon, some small stable membrane proteins may be successfully extracted using organic solvents (e.g. a chloroform/methanol mix). As in the case of detergent-mediated operations, the solvent mix must be retained for all follow-on purification steps.

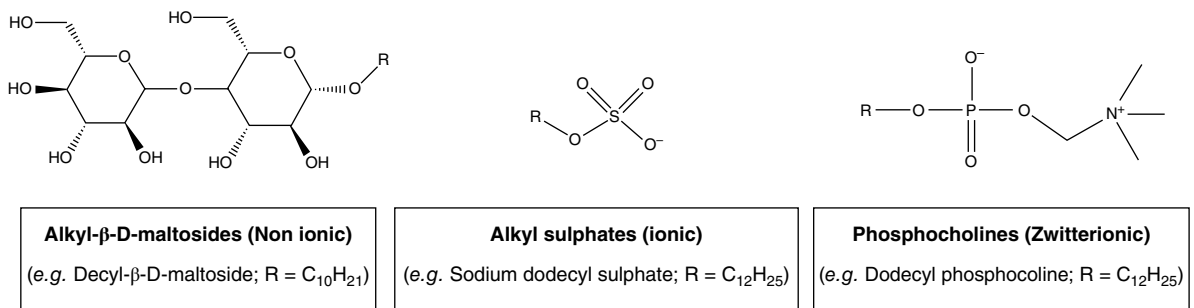
## 4.6 Protein inactivation and stabilization

Proteins may be subjected to a wide range of influences that results in loss of their biological activity. Influences can be chemical, physical or biological in





**Figure 4.26** Typical protocol used to isolate the membrane fraction from *E. coli* cells.



**Figure 4.27** Examples of non-ionic, ionic and zwitterionic detergents. The chemical detail of the polar head group is shown. The non-polar tail group is represented by 'R' in each case. Generally, non-ionic detergents disrupt protein-lipid interactions but not protein-protein interactions, in contrast to both ionic and zwitterionic detergents.

nature and can destroy biological activity by inducing denaturation, by covalently modifying the protein, or by partially degrading it (Table 4.9). In other words, any influence that alters the protein's native structure will likely influence its biological activity (in the vast majority of cases, its biological activity will be decreased or abolished).

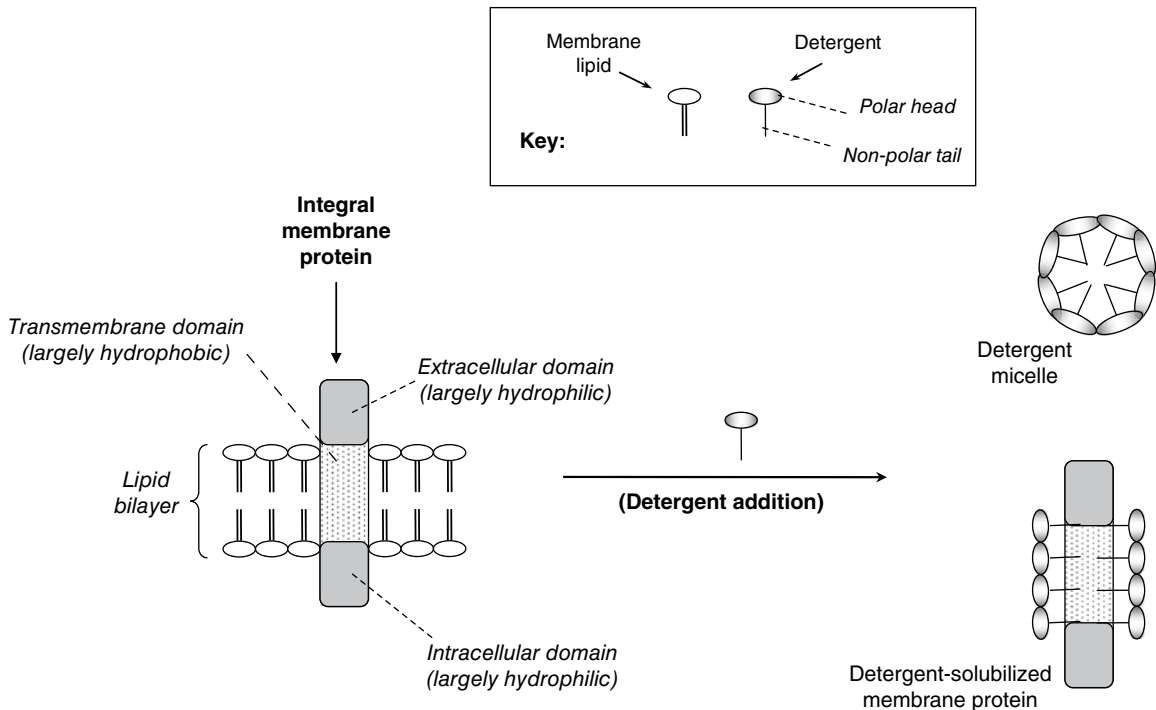
Loss of biological activity can occur during:

- protein recovery from its producer source;
- the protein purification procedure;
- subsequent to purification, during protein storage.

Proteins vary widely in terms of their susceptibility to any given inactivating influence, and their relative

stability/labability in any given circumstance ultimately depends on their structure. Although exceptions exist, as a general principle extracellular proteins tend to be more inherently stable than intracellular proteins. Extracellular proteins have evolved to function in a largely uncontrolled extracellular environment, whereas intracellular proteins have evolved to function in a far more regulated environment:

- Intracellular pH is generally maintained within narrow parameters (usually between pH 6 and 7).
- The cell membrane regulates entry of substances into the cell, and intracellular compartmentalization (eukaryotic cells) also helps keep incompatible



**Figure 4.28** Transverse section of a lipid bilayer membrane, detergent-solubilized integral membrane protein and micelle as they would occur in an aqueous-based solution.

biomolecules physically separated. Lysosomes, for example, house many hydrolytic enzymes including proteases, while in plant cells many chemicals capable of denaturing or modifying proteins are found in vacuoles.

- The intracellular environment is invariably a reducing one, which prevents inactivation of oxygen-sensitive proteins.
- Protein concentration within cells is generally high (up to 400 mg/mL). Proteins are generally more stable when present as concentrated solutions.
- Many chemical metabolites found within cells may specifically or non-specifically stabilize proteins.

Liberation of intracellular proteins by cellular homogenization reverses many of the above conditions. Particular attention to maximizing or maintaining protein stability is thus advisable if working with an intracellular protein.

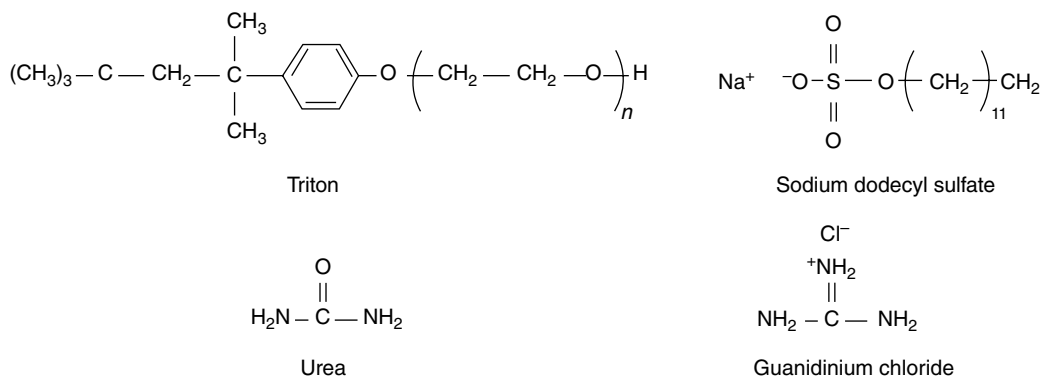
### 4.6.1 Chemical inactivation

A wide range of chemical substances can inactivate proteins. Detergents such as Triton and sodium dodecyl sulfate, chaotropic agents such as urea and guanidinium chloride (Figure 4.29) as well as a wide range of organic solvents can lead to protein denaturation by interrupting the non-covalent forces stabilizing the protein's native structure. Detergents and organic solvents interrupt hydrophobic interactions. Chaotropic agents disrupt both hydrogen bonds and hydrophobic interactions.

Heavy metals can also promote protein modification and hence loss of biological activity. The metal ions can complex directly with selected amino acid residues or enhance the reactivity of additional substances with amino acid residues. Various heavy metals for example can complex directly with cysteine thiol groups, or can enhance oxygen-mediated oxidation of such thiol groups.

**Table 4.9** Various chemical, biological and physical influences that can lead to protein inactivation. The negative impact of many such influences is time and concentration dependent. Refer to text for details.

Inactivating influence	Example	Comments
Chemical influences	Detergents, organic solvents, chaotropic agents, oxidizing agents, heavy metals	Most induce inactivation by interfering with covalent/non-covalent bonds that stabilize protein structure, and hence lead to protein denaturation. Some (e.g. oxidizing agents) can induce loss of activity by covalently modifying an amino acid residue essential to the protein's biological activity
Biological influences	Proteolytic enzymes Carbohydrases (glycoproteins) Phosphatases (phosphoproteins) Microbial contaminants	All induce protein inactivation via hydrolysis of covalent bonds. Microbial contaminants (of for example a stored protein solution) can induce loss of activity via production of degradative enzymes
Physical influences	Extremes of temperature or pH, freeze and thawing, vigorous agitation	Most induce protein inactivation by causing denaturation

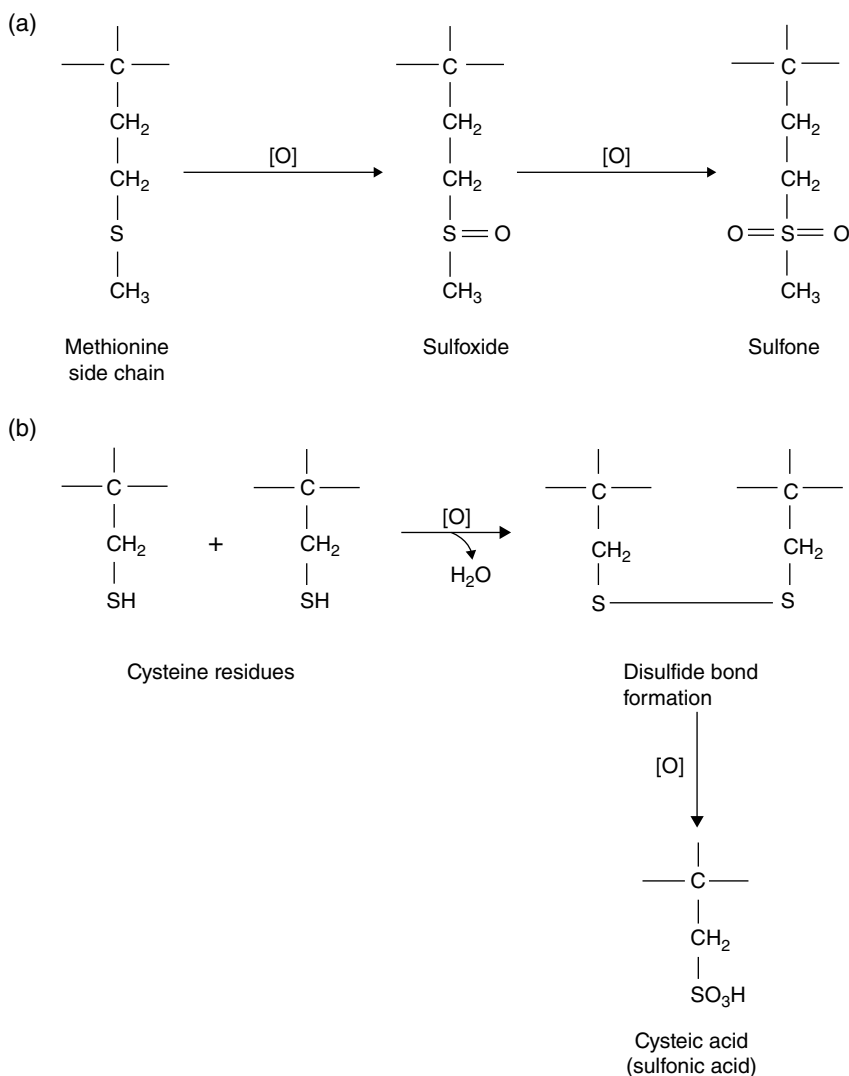


**Figure 4.29** Molecular structure of Triton, sodium dodecyl sulfate (SDS), urea and guanidinium chloride.

Inactivation of proteins by chemical agents is usually concentration and contact time dependent. Inactivation by such means can obviously be avoided by ensuring such substances are not present in the protein solution in the first place. However, sometimes these substances are deliberately added to the protein, for example to achieve solubilization of inclusion bodies or solvent-mediated protein precipitation. Subsequent removal of such substances is therefore advisable. Protein precipitation using organic solvents does not result in protein denaturation at low temperatures (very close to or below 0°C). However, at higher temperatures protein 'flexing' or 'breathing' occurs, which allows entry of organic solvent into the internal protein structure. By interacting with hydrophobic amino acid residues, the solvent molecules disrupt intramolecular

hydrophobic interactions, so favouring protein denaturation.

The side chains of various amino acids are susceptible to oxidation, either by molecular oxygen or various additional oxidizing agents (e.g. peroxide). The sulfur atoms present in the side chain of methionine and cysteine are particularly susceptible to oxidation, and this process is often accelerated in the presence of metal ions. In general, intracellular proteins are more prone to inactivation via oxidation. Oxidation of methionine yields a sulfoxide or a sulfone derivative (Figure 4.30). Oxidation of cysteine usually results in disulfide formation, although cysteic acid can be generated in the presence of strongly oxidizing substances (Figure 4.30). In some instances oxidation of methionine or cysteine residues have little effect

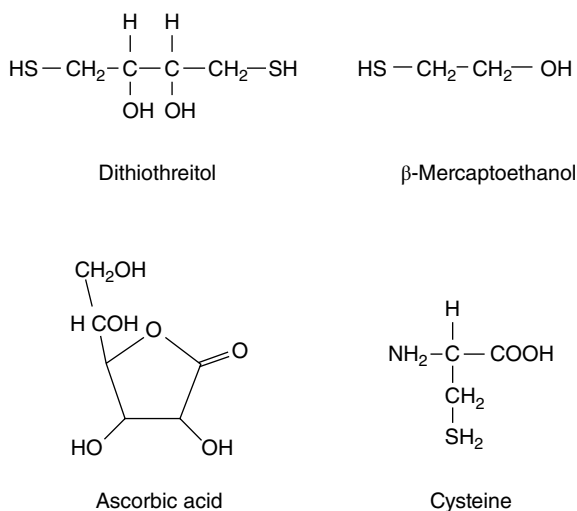


**Figure 4.30** Oxidation of (a) methionine and (b) cysteine side chains, as can occur on exposure to air or more potent oxidizing agents (e.g. peroxide, superoxide, hydroxyl radicals). Refer to text for details.

on a protein's biological activity. However, if the residue plays a central role (either structurally or functionally) its oxidation can result in partial or complete loss of activity. A specific example of such inactivation is considered in the context of detergent proteases (see Chapter 12). Protein oxidation, if a problem, can be minimized by the addition of selected reducing agents to the protein solution. The ones most commonly used include dithiothreitol, as well as  $\beta$ -mercaptoethanol, ascorbic acid and free cysteine (Figure 4.31).

#### 4.6.2 Inactivation by biological or physical influences

By far the most significant biological influence that can lead to protein inactivation is the presence of proteolytic enzymes (proteases, see Chapter 11). Different proteases exhibit different specificities in terms of the exact peptide bond(s) they hydrolyse. If susceptible peptide bonds are present on the protein surface proteolysis likely ensues, a process which



**Figure 4.31** Molecular structure of some reducing agents commonly/potentially used in order to maintain a protein in a reducing environment.

usually leads to protein inactivation (in some instances partial proteolysis can have little effect, or even can have a stimulating effect on protein activity). Most proteins resistant to proteolytic cleavage become susceptible when denatured (susceptible bonds being exposed on protein unfolding). Again, in general, intracellular proteins are likely to be most susceptible to proteolytic degradation.

Proteolysis can be minimized by undertaking the initial stages of protein purification as quickly as possible (thereby quickly separating the proteases from the protein of interest), by maintaining low processing temperatures (the lower the temperature, the lower the proteolytic activity) or by including one or more proteolytic inhibitors in the processing buffers (see Chapter 12).

Carbohydrases and phosphatases can also sometimes influence the biological activity of glycoproteins and phosphoproteins, although this problem is not nearly as widespread as proteolytic inactivation. Modulation or removal of the carbohydrate component of a glycoprotein can influence its solubility and sometimes its biological activity (see Chapter 2). Significant deglycosylation can also potentially render the protein more prone to proteolysis if susceptible peptide bonds are unmasked. Dephosphorylation of phosphoproteins

can potentially seriously affect the latter's biological activity, as the state of phosphorylation can directly influence the activity of these proteins. As in the case of proteases, minimization of unwanted carbohydrate or phosphatase activity can be achieved by rapid processing times, maintaining low temperatures and the use of inhibitors.

Vigorous agitation is one of the most common physical influences leading to loss of protein activity. Agitation usually results in the incorporation of a gaseous phase (usually air) into the protein solution. The presence of air bubbles obviously greatly increases the total liquid-gas interface. Proteins tend to align themselves along such interfaces, a process which often results in their partial or complete denaturation (see Chapter 14). Agitation is usually a feature of cellular disruption, but can also occur during the actual protein purification process (e.g. by over-vigorous stirring during a precipitation step or during mechanical pumping operations characteristic of large-scale protein purification schemes).

Extremes of temperature and pH can also lead to protein inactivation, usually by promoting denaturation. The term 'extreme' must be taken in the context of the 'normal' environment of the protein at which it is usually maximally or near maximally stable. For example, a temperature of 80°C would be extreme in the case of animal proteins, but would be close to the optimal operation temperature of most hyperthermophile-derived proteins (see Chapter 11). Extremes of pH can promote protein unfolding, as it will affect the ionization status of the side chains of (ionizable) amino acid residues. This in turn will affect the range and distribution of ionic attractive/repulsive forces, which play an important role in stabilizing the native conformation of most proteins. Exposure of a protein to extremes of pH can be avoided by keeping the latter in an appropriate buffer at all times during extraction, purification and storage. A buffer is a solution that resists change in its pH when small quantities of either acid or base are added. It consists of a weak acid (H<sup>+</sup> donor) and its conjugate base (H<sup>+</sup> acceptor). Any buffer will maintain its buffering capacity only over a specific pH range (Table 4.10). Extremes of pH can also induce protein destabilization by promoting its

covalent modification. For example, the amide group of asparagine and glutamine residues are labile at extremes of pH (and at high temperatures) and often deamidate under such conditions (Figure 4.32).

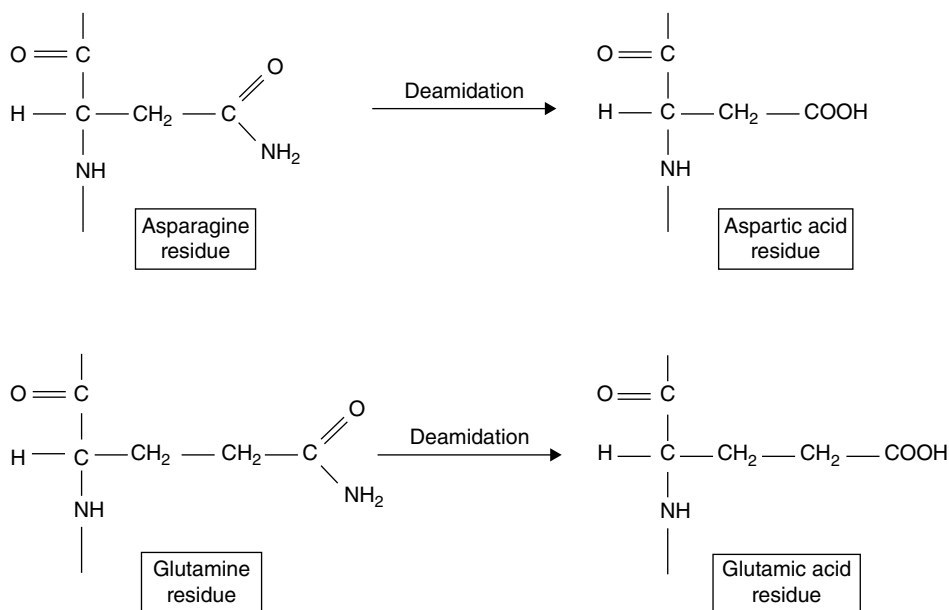
Elevated temperatures can promote protein unfolding, by disrupting the non-covalent forces that stabilize a protein's conformation. Again temperatures can be maintained at low levels by

**Table 4.10** Buffers commonly used to maintain the pH of a protein solution at a pre-specified value.

Buffer	Effective pH range
Clark and Lubs	1.0–2.2
Glycine HCl	2.2–3.6
Citric acid/sodium citrate	3.0–6.2
Sodium acetate/acetic acid	3.7–5.6
Tris/maleate/NaOH	5.4–8.4
Na <sub>2</sub> HPO <sub>4</sub> /NaH <sub>2</sub> PO <sub>4</sub>	5.8–8.0
Tris HCl	7.1–8.9
HEPES/NaOH	7.2–8.2
Glycine/NaOH	8.6–10.6
Carbonate	9.7–10.9
Hydroxide/chloride	12.0–13.0

refrigeration or by keeping the protein solution on ice. Although maintaining a protein solution at low temperatures is generally stabilizing, low-temperature operation can slow down the purification process (e.g. low temperatures slow down flow rates through chromatographic columns if the protein is present in a viscous buffer). This in turn can be detrimental by, for example, prolonging exposure times of the protein with any potential denaturing influences present.

While the process of freezing and subsequent thawing has no detrimental effect on most proteins, it can inactivate some proteins. Inactivation is often dependent on the exact solute composition of the protein solution. As described later, freezing effectively concentrates all solutes present in the aqueous solution. If any 'contaminating' solutes are potential denaturants, this concentration effect could result in protein inactivation. Also as the temperature continues to decrease during the freezing process, some solutes will selectively crystallize out of solution. This can be harmful if one buffer component crystallizes before the other. Such an event would lead to a huge swing in the pH of the remaining uncrystallized liquid containing the protein.



**Figure 4.32** Deamidation of asparagine and glutamine residues, yielding aspartic acid and glutamic acid, respectively.

### 4.6.3 Approaches to protein stabilization

For some proteins no specific stabilization steps need be undertaken during extraction, purification or storage. However, a number of general steps can be undertaken in order to maximize protein stability.

- Ensure the protein is always maintained in a buffered solution set at a pH value where the protein is maximally stable.
- Use only high-purity chemical reagents when making working buffers.
- Ensure extraction/processing temperatures are controlled such that the protein is not exposed to elevated temperatures.
- Minimize processing times to ensure protein contact time with any denaturing influence encountered is minimized.
- Avoid processes such as vigorous agitation or the addition of chemicals known to promote denaturation of the target protein.
- Include substances in all processing buffers that might counteract or inactivate known inactivators of the target protein (e.g. proteolytic inhibitors for protease-sensitive proteins and reducing agents in the case of oxygen-sensitive proteins).
- Include stabilizing agents of the target protein (if known) in all extraction and processing buffers.

Protein stabilizing agents can be of two types, specific or general. As the name suggests, specific stabilizers achieve their effect by interacting biospecifically with the target protein. Examples include substrates (or competitive inhibitors) for many enzymes and, in some cases, antibodies that specifically bind to the protein of interest. General stabilizers act in a non-biospecific manner. Examples include glycerol, various carbohydrates, polymers such as PEG, amino acids such as glycine, and bulk proteins such as bovine serum albumin (BSA) (Table 4.11).

Substances such as glycerol, sugars and PEG are often used as general stabilization agents, particularly for intracellular-derived proteins. These substances likely achieve their intended effect mainly

**Table 4.11** Substances often added to protein solutions in order to enhance protein stability.

Serum albumin
Various amino acids (glycine, alanine, lysine, threonine)
Various carbohydrates (glucose, sucrose, trehalose, maltose)
Alcohols and polyols (glycerol, mannitol, sorbitol, PEG)
Surfactants (usually at low concentrations)

by reducing water activity. Most of the water within intact cells is not freely mobile, but is bound/loosely associated with proteins and other cellular molecules, i.e. 'free water' activity is low. Cellular homogenization (usually using several volumes of buffer per unit weight of cells) liberates intracellular proteins into a dilute solution, displaying greatly increased free water levels. Glycerol, sugars and various polymers can reduce free water levels by hydrogen bonding with bulk water. This more closely mimics normal intracellular conditions, under which proteins are generally more stable.

Various amino acids also stabilize some proteins in solution. Glycine is most commonly used, although alanine, lysine and threonine have also found application in this regard. The molecular mechanism of stabilization is not fully understood. Direct interaction with some proteins is probably a factor but they may also effectively stabilize the protein by reducing its adsorption to internal walls of containers.

Even when stable at higher concentrations, some proteins become considerably more labile when diluted. Dilute proteins may be stabilized in solution by the addition of 'bulking' proteins such as BSA. In some cases this may exert a stabilizing influence by direct interaction with the protein, although they may also function by providing alternative 'target' for any inactivating agents present, and by decreasing levels of surface adsorption of the target protein to the container's surface. Addition of any stabilizing agent is appropriate only if it does not interfere with the subsequent purpose(s) for which the protein is being purified.

Once purified to an acceptable level, most proteins are stored for a period of time before being used for their intended academic or applied purpose.

The storage stability of proteins can vary widely, and depends on (i) the inherent stability of the protein and (ii) the storage conditions chosen. Optimization of storage conditions is invariably a trial and error process, but generally the following options are considered.

- The protein must be stored under the conditions of temperature and pH at which it is maximally stable. Many proteins are stable for months when stored at room temperature, while others are only stable when stored at 4°C or frozen.
- If stored in liquid format, the addition of stabilizing agents (as previously discussed) should be considered. The protein solution should also ideally be filter-sterilized and preservatives added before storage.
- If stored frozen the protein solution should be quickly frozen, and subsequently maintained at -20°C or lower.
- The protein may be more stable if stored in a dry format.

#### 4.6.4 Protein drying

The majority of chemical and biological influences that can negatively impact on protein stability modify proteins when in solution. Removal of the solvent (water) can therefore represent an effective approach for stabilizing many proteins against such influences during storage. Protein drying can be undertaken by a number of means. Freeze drying (lyophilization) is the most gentle and commonly used approach, certainly at laboratory scale. Some additional approaches used at industrial scale are discussed in Chapter 5).

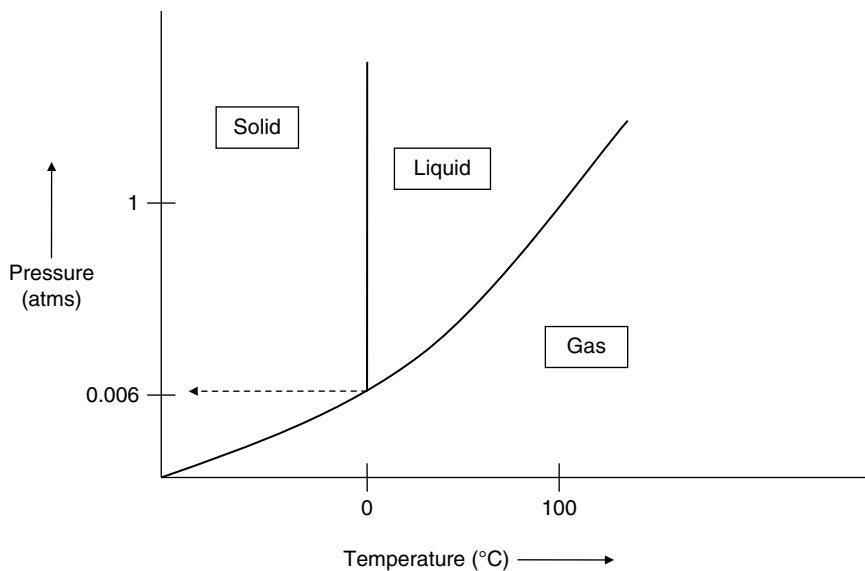
Lyophilization involves the drying of protein (or other materials) directly from the frozen state. This is achieved by firstly freezing the protein solution in a suitable, unstoppered, container. A vacuum is then applied and the temperature is increased in order to promote sublimation of the ice, which occurs under conditions of reduced pressure (Figure 4.33). The ice is drawn off directly as water vapour. The containers can be sealed following completion of the freeze-drying process.

Many freeze-dried proteins may be stored at room temperature for prolonged periods with little or no loss of biological activity. However, some freeze-dried products exhibit significant loss of activity if stored under such conditions, and thus must be stored at lower temperatures. The average moisture content of a freeze-dried protein preparation is in the order of 3%. However, domains often exist within the product that contain a much higher moisture content. This can contribute to product inconsistency.

The first step in the freeze-drying process involves freezing the protein solution in suitable containers, generally glass vials or flasks. As the temperature decreases, ice crystals begin to form. Such crystals contain only pure water molecules. As the ice crystals grow, the protein concentration, and the concentration of all other solutes present in the remaining liquid phase, steadily increases. Any solute species such as salts, buffer components, other chemical additives or proteases present in the product are concentrated many fold. The greater the solute concentration, the greater the reaction rate between such solutes. Proteins damaged by high concentrations of these solutes may be inactivated at this stage. Such inactivation may be due to chemical or biological modification of the protein, or may be caused by protein aggregation.

As cooling continues, some of the solutes present in the concentrated solution may also crystallize and hence are removed from the solution. As the temperature decreases still further, the viscosity of the unfrozen solution increases dramatically, and becomes more and more rubber-like. Eventually, the unfrozen solution will change from the consistency of rubber to that of glass. The temperature at which this occurs is known as the glass transition temperature ( $T_g'$ ). The glass consists of all the uncrystallized solute molecules, including the protein, as well as all uncrystallized water molecules present in association with these solutes. Structurally, this glass is not a solid but actually a liquid which exhibits a very slow flow rate, of the order of micrometers per year. The same is true of traditional glass (e.g. window glass or spectacle glass). Mechanically, however, it may be regarded as a solid. Molecular mobility of all solutes within the





**Figure 4.33** Phase diagram of water, which describes the various phases in which water can exist as a function of pressure and temperature. Thus, at normal atmospheric pressure (1 atm, 101 kPa) it exists as a solid phase (ice) at temperatures below 0°C, as a gas above 100°C and as a liquid in between. However, at reduced atmospheric pressures (below 0.006 atm, 0.6 kPa) the water will sublime directly from solid to gaseous phase, without existing in an intervening liquid phase.

glass, which contains up to 50% water, is to all intents and purposes non-existent. Chemical mobility, and hence reactivity, within the glass phase all but ceases. On initial formation of ice crystals it is therefore desirable to reduce the solution temperature below  $T'_g$  as quickly as possible, in order to minimize protein inactivation.

The  $T'_g$  of any given solution depends on its composition and may be determined experimentally by a technique known as differential scanning calorimetry. This involves heating the glassy product and subsequently plotting temperature versus its specific heat value. A sharp increase in heat flow is observed at the  $T'_g$ . Determination of the  $T'_g$  of a protein solution facilitates the development of a more rational freeze-drying protocol for that particular protein preparation.

During the freeze-drying process a vacuum is applied to the system once a temperature below the  $T'_g$  has been attained. For most protein solutions this involves decreasing the temperature to between -40 and -60°C. The temperature may then be allowed to increase, in order to promote sublimation

of the crystalline water. This requires an input of energy. A laboratory-scale freeze-dryer is shown in Figure 4.34.

## 4.7 Protein characterization

Once purified most proteins are subjected to a battery of characterization studies. The exact range of studies undertaken will very much depend on the ultimate project aim. However, extensive protein characterization studies normally incorporate multiple analyses aimed at investigating:

- protein quantification, identification and purity;
- structural studies;
- physicochemical studies;
- functional studies.

Many of the studies undertaken on an individually purified protein are exactly the same studies



**Figure 4.34** A laboratory-scale freeze drier.

undertaken in high-throughput mode on a set of proteins during proteomic studies. The theory behind such studies as well as the analytical techniques used to undertake these analyses have already been considered in Chapter 1, to which the reader is referred. Moreover, the primary analytical approaches used to determine protein structure have been considered in Chapter 2, again to which the reader is referred. Therefore, this protein characterization section aims to provide a summary integrated overview of the battery of (sometimes overlapping) analytical approaches underpinning protein characterization, rather than including repetitive detail of the techniques themselves. The approaches/techniques most commonly applied to the characterization of individually purified proteins are summarized in Table 4.12.

Characterization of recombinant proteins is undertaken using the same techniques and procedures as used for non-recombinant proteins. However, depending on the expression system and

**Table 4.12** Major studies typically undertaken in order to characterize a protein, along with the associated analytical techniques/approaches by which these are generally achieved.

#### **Protein quantification, identification and purity**

Quantification: various, overviewed in Table 4.3

Identification: immunoassays (Chapter 10), Western blot

(Box 4.1), bioassay (Table 4.4)

Purity: SDS-PAGE, two-dimensional electrophoresis, isoelectric focusing, HPLC analysis, mass spectrometry, capillary electrophoresis

#### **Structural studies**

Primary structure: amino acid composition, peptide mapping, N-terminal sequencing, complete amino acid sequencing

Higher-order structure: circular dichroism, fluorescence spectroscopy

NMR, X-ray crystallography, analytical ultracentrifugation

Post-translational modifications: various, depending on the exact modification (Chapter 2)

#### **Physicochemical studies**

Molecular mass determination: SDS-PAGE, non-denaturing electrophoresis (Ferguson plots), gel filtration, analytical ultracentrifugation, mass spectrometry

pI determination: isoelectric focusing

#### **Functional characterization**

Depends on the biological activity of the protein. Examples include determination of specific activity, the effects of temperature and pH on biological activity, ligand interaction studies

purification procedures used, some characterization studies may assume special significance:

- conformational studies in the case of proteins produced as inclusion bodies that required denaturation and refolding steps;
- N- or C-terminal sequencing in the case of proteins produced as fusion products and whose tag was removed during purification;
- post-translational modification analysis in the case of proteins which are glycosylated in the native state for example.

The advent of bioinformatics and associated software program resources (see Chapters 1 and 2) render available or allow the generation of significant *in silico*-based predictive information about virtually any protein whose gene has been sequenced. Examples include:

- predicted amino acid sequence from genomic data;
- potential post-translational modification sites (e.g. based on the occurrence of specific amino acid sequences within the protein);

- likely final protein cellular/extracellular location (due to presence of specific signal sequences);
- predicted mass and pI values;
- predicted structural information.

In such instances direct analytical characterization of the purified protein will serve to verify (or otherwise) the accuracy of such *in silico*-derived information.

Finally, many of these characterization steps form the basis of not only academic studies but also quality control tests routinely undertaken for commercialized proteins, as outlined in Chapter 5.

### 4.7.1 Functional studies

Functional studies, as the name suggests, aim to investigate the biological function/activities of the purified proteins and how these activities are affected by various pertinent influences. The type and range of studies undertaken very much depend on the protein type and its intended application. For enzymes, basic functional studies usually encompass issues such as:

- determination of specific activity (number of activity units per mg protein);
- determination of substrate range/specificity;
- kinetic characteristics (e.g. determination of  $K_m$ ,  $V_{max}$  and  $K_{cat}$  values);
- effects of various influences (e.g. temperature, pH, inhibitors) on enzyme activity.

If the enzyme is to be used for a specific applied purpose, functional studies of particular significance in the context of that purpose will be investigated in great detail (e.g. temperature vs. activity profile and thermal stability of enzymes destined for application in high-temperature industrial processes).

Likewise, appropriate academic and applied functional studies will be undertaken on non-catalytic proteins. In most such instances underscoring the biological activity of such proteins will be their ability to interact with some specific ligand in a biospecific manner (e.g. cytokines or hormones interacting with their receptors, antibodies binding

to the antigen against which they were raised). A prominent functional property investigated in such instances will be the protein's ligand-binding characteristics. Surface plasmon resonance (SPR) spectroscopy represents a standard method for the investigation of binding properties between a protein and its ligand. SPR is considered in Chapter 10 (Figure 10.11). Biacore™ systems are commercial equipment systems most commonly used to undertake such analyses. These systems characterize molecules in terms of:

- the specificity of their interactions;
- on-off rates (interaction kinetics);
- binding affinity (strength).

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# Chapter 5

## Large-scale protein production

The various sources from which proteins may be obtained have been discussed in Chapter 3. The approaches that can be adopted to purify and characterize these proteins have been outlined in Chapter 4. The general principles provided in these two chapters are, normally, equally applicable to laboratory- and industrial-scale operations. However, industrial-scale operations, as subsequently described, must take into account various additional considerations, including:

- scale-up and process optimization issues;
- economic issues;
- regulatory issues.

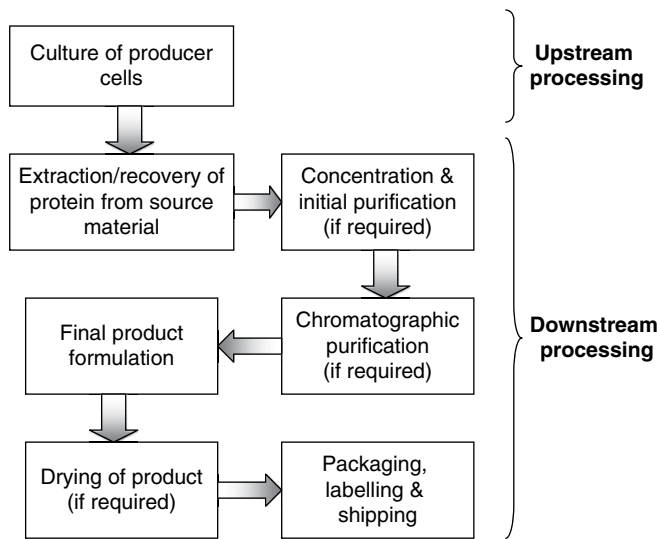
At an industrial scale, the manufacture of a protein product is often divided into upstream and downstream processing (Figure 5.1). Upstream processing refers to the phases of production in which biosynthesis of the protein takes place. For most proteins, therefore, this refers to microbial cell fermentation or animal cell culture. Downstream processing refers to the extraction of the protein from the producer source, and its subsequent purification. It also encompasses additional manufacturing activities such as quality control evaluation, end-product stabilization, adjustment of product potency to within specified

limits, filling of product into suitable final-product containers, and labelling of containers.

### 5.1 Upstream processing

While a relatively small number of commercialized proteins are extracted from animal or plant tissues, the vast majority of such products are sourced from either native or recombinant microorganisms (mainly bacteria and fungi), or from recombinant mammalian cell lines (see Chapters 3 and 6–14). In summary, commercialized proteins (segregated by application) are most commonly produced as follows.

- Therapeutic proteins (i.e. biopharmaceuticals): mammalian cell lines, *Escherichia coli*, and (in a few cases) yeast.
- Diagnostic/analytical proteins: mainly microorganisms but also mammalian cell culture in the case of some analytical antibodies.
- Industrial (bulk) proteins (mainly enzymes): various microorganisms including bacteria (e.g. *Bacillus*), fungi (e.g. *Aspergillus*, *Trichoderma*) and yeasts (e.g. *Kluyveromyces*) in a limited number of cases.



**Figure 5.1** Flow diagram outlining the major steps constituting upstream and downstream processing of protein products.

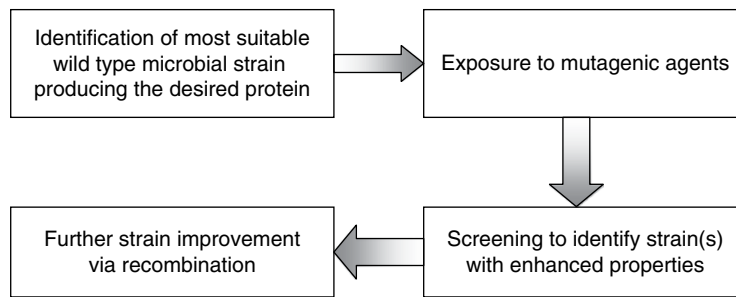
As such, I therefore concentrate on microbial cell fermentation and mammalian cell culture systems in this section.

### 5.1.1 Cell line development

Cells used to produce proteins for any commercial application are usually developed/chosen on the basis of a number of traits or characteristics:

- ability to produce the desired protein at high levels, and ideally extracellularly (extracellular protein production greatly simplifies subsequent downstream processing as there is no requirement to disrupt the microbial cells in order to effect protein release);
- ability to undertake appropriate post-translational modifications, as required;
- will not subsequently modify the desired protein further (e.g. via proteolytic degradation);
- ability to grow quickly and to high cell densities on technically straightforward media;
- be non-pathogenic/generally regarded as safe;
- be genetically stable and straightforward to store as a cell bank;
- be free of any intellectual property constraints.

The development of producer cell lines for recombinant protein production normally entails standard molecular biology techniques, as already described in Chapter 3. However, some products, in particular traditional industrial enzymes, many of which are still on the market, are produced using microbial cell lines initially developed in the pre-recombinant era. The majority are synthesized by a limited number of microorganisms classified as GRAS (generally recognized as safe). GRAS microorganisms include bacteria such as *Bacillus subtilis*, *Bacillus amyloliquefaciens* and various other bacilli, lactobacilli and *Streptomyces* species. GRAS-listed fungi include members of *Aspergillus*, *Penicillium*, *Mucor* and *Rhizopus*. Yeast such as *Saccharomyces cerevisiae* are also generally recognized as safe. GRAS-listed microbes are non-pathogenic, non-toxic and generally should not produce antibiotics. Many of these cell lines produce the desired proteins at levels comparable to or surpassing those achieved by high-level recombinant production systems. Indeed some of these cell lines themselves formed the starting point for further recombinant modification. Non-recombinant protein production can, in some contexts, still be more desirable than recombinant-based production (see Chapter 3). Thus it is worth



**Figure 5.2** Overview of the traditional approach to strain improvement classically pursued when developing microbial strains for the production of commercial proteins such as industrial enzymes. Refer to text for further detail.

briefly considering the non-recombinant production cell optimization approaches that are traditionally (and to a limited extent still) used when developing some commercial proteins, most notably industrial enzymes.

A typical optimization process is outlined in Figure 5.2. Initial screening studies aim to identify one or more wild-type microorganisms producing an enzyme with the desired biochemical characteristics, and which also display acceptable additional characteristics (see bullet list in preceding paragraph). However, such studies rarely identify the 'perfect' enzyme being produced by the 'perfect' cell. The microorganism(s) identified are therefore usually subjected to one (or more usually several) rounds of exposure to mutagenic agents, which introduce random alterations into the genome. These mutated strains are then re-screened in order to identify any which have improved characteristics relative to the wild type. In essence, the approach is a 'blind' shotgun approach as compared with the knowledge-based modern genetic engineering approach to cell development/optimization. Mutagenic agents include physical agents such as ultraviolet (UV), X-ray or  $\gamma$  radiation, and chemical agents such as nitrosoguanidine or methane sulfonate.

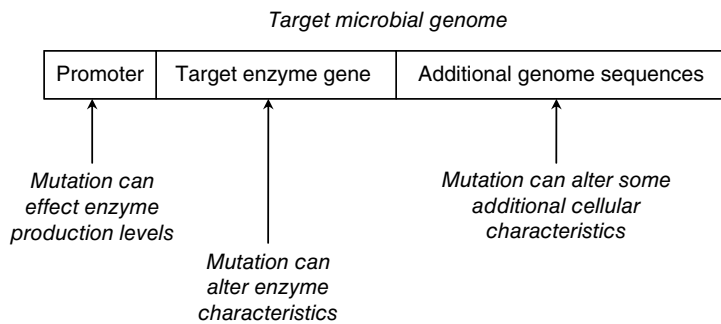
The random genome mutations induced usually include a combination of point mutations and more extensive deletions, rearrangements or insertions, and will typically affect a combination of various genome locations in the microorganism (Figure 5.3). The consequences can be positive or negative in terms of identifying improved producer

microorganisms, with screening studies seeking to identify:

- mutants producing increased levels of the target enzyme (e.g. a mutation may enhance the enzyme promoter region);
- mutants producing an enzyme with improved functional characteristics (e.g. a better pH or temperature-vs.-activity profile in the context of the intended application, as a result of a mutation in the structural gene);
- mutants which display some other improved characteristic, such as mutations in other genes that prevent the enzyme from producing some undesirable contaminant (e.g. a proteolytic enzyme capable of degrading the target protein once it is synthesized) or cell product (e.g. a toxin) or which can improve cell growth on a cheaper fermentation substrate.

Initial rounds of mutations may yield several mutant strains, each with a specific improved property. Attempts may therefore be pursued to develop a single strain which combines two or more of the improved characteristics. This is undertaken via recombination, i.e. by various means which combine various elements of two genomes into one. Recombination can be achieved in various ways:

- transformation (where portions of DNA enter 'competent' recipient cells);
- transduction (transfer of DNA into a recipient cell using a virus);
- conjugation (DNA transfer via cell-cell contact, i.e. mating).



**Figure 5.3** Genome locations potentially altered by random mutagenesis and the possible consequences thereof. Refer to text for further detail.

#### Box 5.1 Case study: the development of *Trichoderma reesei* RUT-C30

A mutant strain of the filamentous fungi *Trichoderma reesei* (*T. reesei* RUT-C30), which is available to purchase from microbial culture collections such as the American Type Culture Collection (ATCC, number 56765), is one of the best-studied and most widely used filamentous fungi for the production of cellulolytic enzymes (see also Chapter 12). The strain was generated by three rounds of random mutagenesis of a wild-type strain (QM6a), originally isolated in the Solomon Islands. The first round of mutagenesis entailed irradiation by UV light, and led to the isolation of a mutant strain displaying catabolite derepression (i.e. the presence of easily metabolizable carbon sources, most notably glucose and glycerol, no longer repressed cellulase synthesis, at least to the same extent as evident with the wild type). A further two rounds of mutagenesis using both UV and chemical mutagens resulted in the isolation of RUT-C30, which displays very significant catabolite derepression and higher general extracellular protein secretion levels, including an approximately 20-fold greater cellulase production compared with the original strain.

The random mutagenesis programme extensively altered many characteristics of the fungus. Early electron microscopic analysis, for example, revealed RUT-C30 to contain sixfold to sevenfold greater endoplasmic

reticulum (ER) content than wild type, when grown on cellulosic media. Genome sequencing and comparative studies have more recently revealed very significant genomic differences. For example, compared with wild type, the genome of RUT-C30 contains 18 large deletions, several smaller deletions and insertions, in addition to point mutations affecting dozens of genes. Overall, the RUT-C30 genome is smaller (by approximately 100 kb) than that of QM6a. Only a subset of the genetic changes are likely to relate to enhanced cellulase production, and many of these seem to be indirect (e.g. mutations enhancing ER structure, vesicle trafficking and protein export from the cell). The exact genetic basis underpinning increased cellulase production remains to be fully elucidated.

The information presented here is largely derived from Peterson, R. and Nevalainen, H. (2012) *Trichoderma reesei* RUT-C30: thirty years of strain improvement. *Microbiology* **158**, 58–68, to which the reader is referred.

A strain improvement case study involving a well-known fungus is presented in Box 5.1.

### 5.1.2 Cell banking systems

Initial product development ultimately results in the isolation/construction of the cell production line (microbial, mammalian or other) that serves



to produce the product. The cells are generally then aliquoted into small amounts, which are placed in ampoules and subsequently immersed in liquid nitrogen. The content of all the ampoules is therefore identical, and the cells are effectively preserved for indefinite periods. This batch of cryopreserved ampoules form a 'cell bank' system, whereby one ampoule is thawed and the cells therein cultured in order to seed a single production run for example.

The cell bank's construction design is normally two-tiered, consisting of a 'master' cell bank and a 'working' cell bank (Figure 5.4). The master cell bank is constructed first, directly from a culture of the newly constructed production cell line. It can consist of several hundred individually stored ampoules. These ampoules are not used to directly seed a production batch. Instead, they are used, as required, to generate a working cell bank. The generation of a single working cell bank normally entails thawing a single master cell bank ampoule, culturing of the cells therein, with their subsequent aliquoting into multiple ampoules. These ampoules are then cryopreserved and form the working cell bank. When a single batch of new product is required, one ampoule from the working cell bank is thawed and used to seed that batch. When all the vials which compose the first working cell bank are exhausted, a second vial of the master cell bank is used to generate a second working cell bank, and so on.

The rationale behind this master/working cell bank system is to ensure an essentially indefinite supply of the originally developed production cells for manufacturing purposes. This is more easily understood by example. If only a single-tier cell bank system existed, containing 250 ampoules, and 10 ampoules were used per year to manufacture 10 batches of product, the cell bank would be exhausted after 25 years. However, if a two-tier system exists, where a single master cell bank ampoule is expanded as required to generate a further 250-ampoule working cell bank, the entire master cell bank would not be exhausted for 6250 years.

The upstream processing element of the manufacture of a batch of a commercial protein product begins with the removal of a single ampoule of the working cell bank. This vial is used to inoculate a

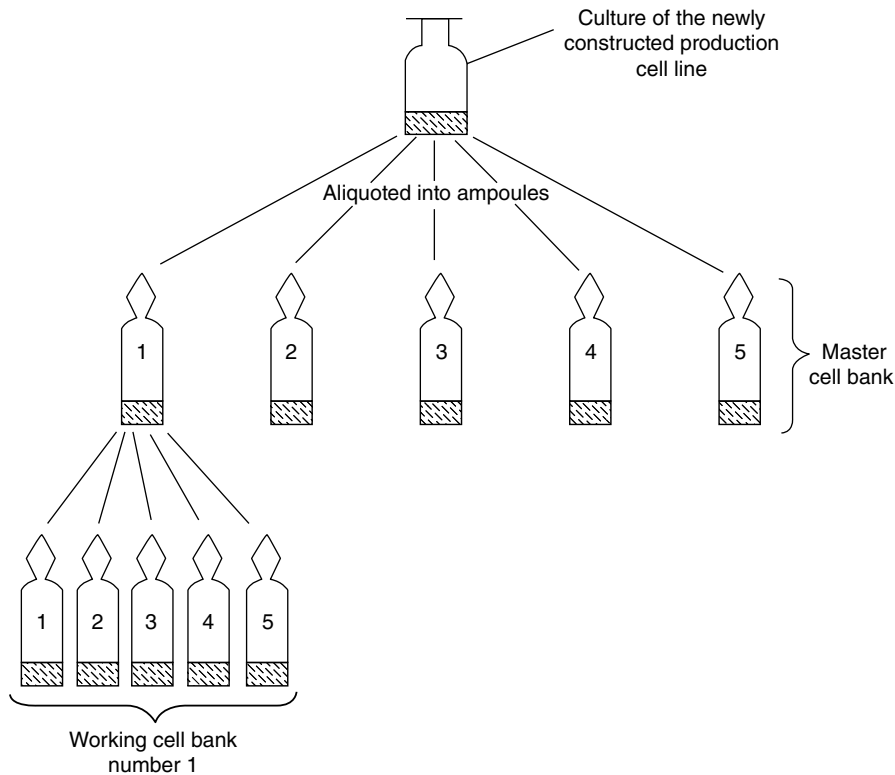
small volume of sterile media, with subsequent incubation under appropriate conditions, a process that represents the growth of laboratory-scale starter cultures of the producer cell line (see next section). This starter culture is in turn used to inoculate a production-scale starter culture which is used to inoculate the production-scale bioreactor (Figure 5.5). The media composition and fermentation conditions required to promote optimal cell growth/product production will have been established during initial product development, and routine batch production is a highly repetitive, highly automated process. Bioreactors are generally manufactured from high-grade stainless steel and can vary in size from a few tens of litres to several tens of thousands of litres. At the end of the production-scale fermentation process, the crude product is harvested, which signals commencement of downstream processing.

### 5.1.3 *Microbial cell fermentation*

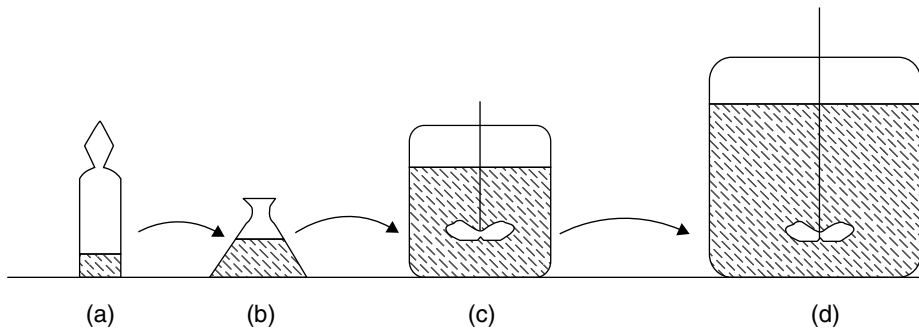
Microbial cell fermentation has a long history in the production of various biological products of commercial significance, including ethanol, antibiotics, organic acids and proteins. As a result, a wealth of technical data and experience has accumulated in the area. In this chapter I first consider aspects of microbial cell culture at laboratory level, before going on to consider industrial-scale culture.

At laboratory scale, most microorganisms may be grown on agar plates or via submerged fermentation, with some also suited to growth via solid-state fermentation (Box 5.2). Microbial culture on agar plates is usually undertaken for purposes such as:

- initial isolation of individual microbial strains from microbial communities within environmental samples;
- in order to screen microorganisms for some functional attribute, such as antibiotic resistance, the ability to produce a specific product such as an enzyme (Box 5.2), or the screening of recombinant cell libraries;
- for routine (and usually short-term) microbial culture maintenance.



**Figure 5.4** The master cell bank/working cell bank system. For simplicity each bank contains only five ampoules. In reality, each bank would likely consist of several hundred ampoules. Working cell bank number 2 will be generated from master cell bank vial number 2 only when working cell bank number 1 is exhausted, and so on.



**Figure 5.5** Outline of typical upstream processing stages involved in the production of a single batch of product. Initially, the contents of a single ampoule of the working cell bank (a) are used to inoculate a few hundred millilitres of media (b). After growth, this laboratory-scale starter culture is used to inoculate several litres to tens of litres of media present in a small bioreactor (c). This production-scale starter culture is used to inoculate the production-scale bioreactor (d). This process is equally applicable to prokaryotic or eukaryotic-based producer cell lines, although the bioreactor design, conditions of growth, etc. will differ in these two instances.

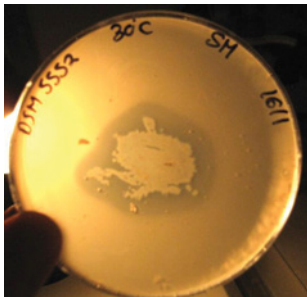
If the aim is to produce either the cells themselves or some cell product for further study or use, culture via submerged fermentation is most commonly applied. This entails microbial growth in

liquid-based culture media under environmentally controlled conditions.

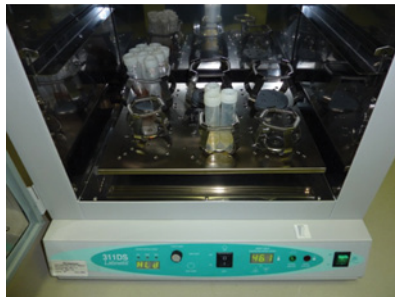
In terms of laboratory-scale systems, low-volume culture (e.g. a few millilitres) is often

**Box 5.2** Various formats under which laboratory-scale microbial cell cultivation are typically undertaken

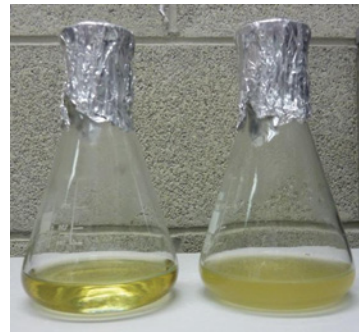
(a)



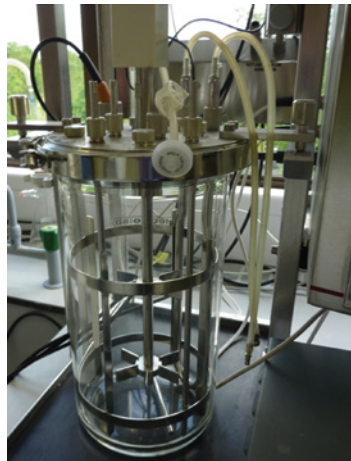
(b)



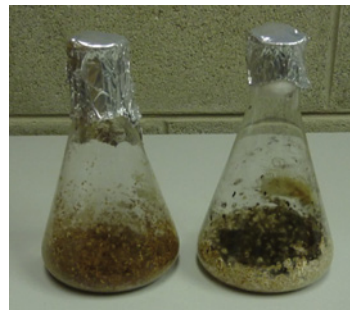
(c)



(d)



(e)



Photograph (a) shows a strain of *Bacillus subtilis* growing on a milk agar plate. The milk proteins give the agar an opaque white appearance. As such this system can be used for the screening of microorganisms capable of producing extracellular proteolytic enzymes. Extracellular protease production by the strain growing is indicated by the clear zone surrounding the microorganism, generated by proteolytic-mediated milk protein hydrolysis. Photograph (b) represents 5-mL overnight cultures of *E. coli* growing in 30-mL sterile tubes. Photograph (c) represents 50 mL nutrient media present in 250-mL conical flasks. The flask on the left was uninoculated (control), whereas the flask on the right represents an

overnight culture of *E. coli*. Photograph (d) shows a laboratory-scale benchtop fermenter. Photograph (e) shows growth of a strain of *Aspergillus niger* via solid-state fermentation. In this case the fermentation substrate is wheat bran (see later in main text). The flask on the left is uninoculated (control), whereas the one on the right represents 6-day-old fungal growth, which has sporulated. The growing fungal mycelia are white in appearance, the spores are black. Spores form when culture conditions become unfavourable (e.g. an essential nutrient becomes limiting), and can germinate subsequently if favourable culture conditions are re-established.

undertaken in tubes. Somewhat larger-volume fermentations (tens to hundreds of millilitres) are often undertaken in conical flasks, while laboratory-scale commercial fermentation systems with capacities typically ranging from 1 to 15 L are also available (Box 5.2).

Some microorganisms may also be cultured using solid-state fermentation. Solid-state (substrate) fermentation is a fermentation process occurring in the absence or near-absence of free water (Box 5.2). It is most commonly applied in the case of filamentous fungi producing an extracellular product of interest. Such fungi are best studied using solid-state fermentation due to their hyphal growth, which have the capability to not only grow on the surface of the substrate particles but also penetrate through them.

The exact constituents of fermentation media will of course be tailored to suit optimal growth of, or product production by, the microorganism of interest. The fermentation media must incorporate all macronutrients (e.g. a source of carbon and nitrogen) and micronutrients (e.g. appropriate minerals and vitamins) required by the microorganisms. Submerged media, whatever their exact composition, are often described as defined, semi-defined or complex.

- *Defined media*: consist of a chemically defined cocktail of individual nutrients dissolved in water (e.g. glucose,  $\text{NH}_4\text{Cl}$ ,  $\text{NaCl}$ ,  $\text{KH}_2\text{PO}_4$ ,  $\text{CaCl}_2$ ).
- *Semi-defined media*: contain a combination of defined media constituents, along with one or more ingredients whose exact composition is not fully characterized (e.g. yeast extract, which is the water-soluble portion of autolysed yeast, and will therefore contain a rich and varied array of biomolecules that effectively serve as macronutrients and micronutrients).
- *Complex media*: generally contain some defined components but quantitatively the majority of added constituents are complex in nature, such as yeast extract and tryptone (an enzymatic digest of casein).

Examples of defined, semi-defined and complex media which can be used to culture *E. coli* are provided in Table 5.1.

**Table 5.1** Quantitative composition of the various constituents in a complex media (Terrific broth, TB), a fully defined media (Minimal media 9, M9) and a semi-defined media (M9-yeast extract, M9/YE).

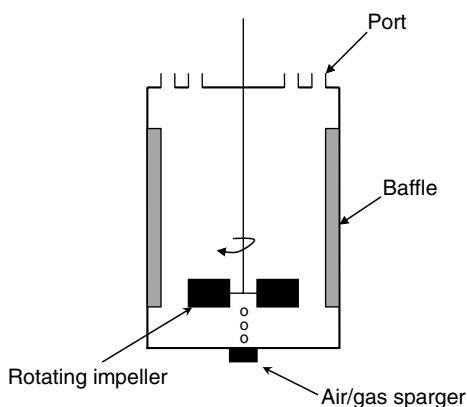
Component (g/L)	TB	M9	M9/YE
Tryptone	12.00	–	–
Yeast extract	24.00	–	2.00
NaCl	–	0.50	0.50
Glycerol	4% (v/v)	–	–
Glucose	–	4.00	4.00
$\text{KH}_2\text{PO}_4$	2.31	3.00	3.00
$\text{K}_2\text{HPO}_4$	12.54	–	–
$\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$	–	12.80	12.80
$\text{NH}_4\text{Cl}$	–	1.00	1.00
$\text{MgSO}_4$	–	0.20	0.20
$\text{CaCl}_2$	–	0.01	0.01

In some such instances, the media constituents will contain appropriate inducer molecules (those that will induce the production of some desired cellular product). Examples include:

- the use of specific inducers to induce the expression of a recombinant protein by an engineered cell line;
- the use of a specific inducer to induce the expression/production of specific biomolecules by non-engineered cell lines.

Submerged microbial fermentation can also be operated in batch, feed-batch or continuous mode.

- *Batch operations* (usually the most common formats at laboratory scale) are closed systems in that additional media constituents are not added to the system during the fermentation process, and full harvest occurs at the end of the fermentation period.
- *Feed-batch operations* differ in that they are not closed systems, with additional nutrients, inducer molecules, etc. being introduced as considered desirable during fermentation operation.
- *Continuous systems* are characterized by the addition of fresh nutrients (and removal of a proportion of the media) on a continuous ongoing basis.



**Figure 5.6** Schematic diagram of a generalized microbial fermenter.

Solid-state fermentation systems are generally operated in batch mode using solid, complex media constituents such as wheat bran (a fibrous byproduct of wheat flour processing, containing cellulose, hemicellulose as well as various minerals and vitamins).

Microbial fermentation at an industrial (bioprocess) scale is based on the same general principles as described above in the context of laboratory-based systems. Submerged fermentation formats are employed in the overwhelming majority of cases and almost always on a batch or feed-batch basis. A generalized microbial fermenter design is presented in Figure 5.6, although different design formats are also available. The impeller, driven by an external motor, serves to ensure even distribution of nutrients and cells in the tank. The baffles (stainless steel plates attached to the side walls) serve to enhance impeller mixing by preventing vortex formation. Various ports are also present through which probes are inserted that monitor pH, temperature and sometimes the concentration of a critical metabolite (e.g. the carbon source). Additional ports serve to facilitate addition of acid or base (pH adjustment) or, if required, addition of nutrients or media withdrawal during the fermentation process. Process-scale fermenters are shown in Figure 5.7.

Typically, the manufacture of a batch of microbial-produced protein product involves filling the production vessel with the appropriate quantity of purified water. Heat-stable nutrients required for producer cell growth are then added and the resultant

media is sterilized *in situ*. This can be achieved by heat and many fermenters have in-built heating elements or, alternatively, outer jackets through which steam can be passed in order to heat the vessel contents. Heat-labile ingredients can be sterilized by filtration and added to the fermenter after the heat step.

Fermentation follows subsequent to inoculation with the production-scale starter culture (Figure 5.5). Exact fermentation duration will depend on cell growth rate. In the case of *E. coli* this may be less than 24 hours, while it may be several days in the case of many fungi. During fermentation, air (sterilized by filtration) is sparged into the tank to supply oxygen and the fermenter is also operated at a temperature appropriate to optimal cell growth (usually 25–35°C, depending on producer cell type). In order to maintain this temperature, cooling rather than heating is required in some cases. Large-scale fermentations, in which cells grow rapidly and to a high cell density, can generate considerable heat as a result of microbial metabolism and mechanical activity (e.g. stirring). Cooling is achieved by passing the coolant (cold water or glycol) through a circulating system associated with the vessel jacket or sometimes via internal vessel coils.

The composition of fermentation media depends on factors such as:

- exact nutrient requirements of the producer cell line to maximize cell growth and product production;
- economics (total media cost);
- regulatory considerations.

The use of a defined media, free from animal-derived product constituents, is preferred and often a regulatory necessity when producing healthcare products, particularly therapeutic proteins. This ensures maximal batch-to-batch fermentation consistency, and dispels any safety worries concerning the introduction of pathogens from animal-derived media ingredients into the product stream. Although high-purity defined media constituents are relatively expensive, they are not prohibitively so in the context of manufacturing high-value therapeutic/diagnostic products.

(a)



(b)



**Figure 5.7** Typical bioprocess-scale fermentation equipment. Photo of a 20 litre pilot scale/small bioprocess-scale fermentation unit (a). Photo of a process scale mammalian cell culture facility, housing a 1000 litre bioreactor (on the left) and a 200 litre scale bioreactor (on the right), (b). Photo (b) copyright FUJIFILM Diosynth Biotechnologies UK Ltd, [www.fujifilmdiosynth.com](http://www.fujifilmdiosynth.com)

In contrast, complex media are more commonly used in the microbial production of bulk industrial enzymes, and this is far more sensitive to process economics. In this context, the use of bulk media ingredients (especially carbon and nitrogen sources) that constitute byproducts from other processes

(and hence are usually inexpensive) can be particularly attractive. Attractive potential carbon sources include:

- molasses (residual byproduct of sugar production), which typically contains up to 50% sucrose,

as well as lower levels of nitrogenous compounds and some vitamins and minerals;

- malt extract, an aqueous extract from malted barley, up to 90% of which consists of a carbohydrate mix (mainly glucose, maltose and oligosaccharides), as well as some nitrogen-based biomolecules and vitamins);
- whey (lactose-containing byproduct of cheese manufacture).

Similarly, potential nitrogen sources based on byproducts from other processes include:

- corn steep liquor (a byproduct of starch extraction from corn, containing amino acids, vitamins and minerals);
- soya bean meal (residual byproduct from the extraction of oil from soya, containing proteins, some carbohydrates and some oil).

Media composition is also influenced by any requirements to achieve effective induction of synthesis of the required protein. In the case of recombinant proteins this is often straightforward, in that the expression vector dictates what specific inducer is required, and the said inducer is then usually added to the media after an initial cell growth to high biomass levels (see Chapter 3, Box 3.1).

In the case of non-recombinant systems, the target protein is often one which is not constitutively expressed by the cell, in other words an inducing substance must be included in the medium. In practice this most often occurs in the context of the production of many traditional industrial enzyme products. In these circumstances a natural inducer is often used as the main carbon or nitrogen source in the fermentation media. Specific examples include:

- use of starch as carbon source for microbial production of industrial amylases;
- use of wheat bran as carbon source for the production of lignocellulosic enzymes;
- use of proteins such as milk proteins as carbon/nitrogen source for the production of proteolytic enzymes.

Also, in many cases, synthesis of the desired enzyme is subject to catabolite repression. Under

such circumstances media composition and fermentation conditions must be carefully controlled to ensure the continued absence of the repressor or the producer cell must be mutated/engineered in order to inactivate catabolite repression (Box 5.1).

Scale-up of fermenter vessels (and indeed animal cell culture vessels; see section 5.1.4) often introduces process challenges that must be tightly monitored and controlled. Among the most prominent such issues is reduced mixing quality, which can stress the producer cells. Media in process-scale vessels can potentially display both substrate and oxygen gradients along the vessel length, particularly in feed-batch reactors, as typically substrate is added from the top and aeration occurs from the bottom. These effects are more pronounced in larger vessels due to (i) their longer height; (ii) the larger volume of media to be stirred, resulting in longer mixing times; and (iii) stronger pressure gradients, affecting oxygen transfer. Therefore, unless carefully controlled, cells at the top of the fermenter can for example be exposed to excess substrate (e.g. glucose) while simultaneously experiencing oxygen limitations, while cells near the bottom of the vessel can experience the opposite.

Such conditions can place considerable metabolic stress on the cells, with follow-on cellular growth and protein production consequences. For example, exposure of *E. coli* to excess glucose concentrations (typically above about 30 mg/L) results in acetate overproduction, while simultaneous oxygen limitation induces the formation of ethanol as well as lactate and succinate. Acid production can lead to local acidification, with consequent negative effects on cell growth rates. Process-scale mixing and general cellular metabolic activity can also result in zonal overheating, and hence conditions of cellular stress.

Such issues can be minimized by careful process up-scaling development, and tight monitoring of individual batches of product. Close monitoring is facilitated by the use of multiple in-line fermenter sensors, continuously measuring real-time critical parameters (e.g. concentration of glucose, dissolved oxygen), as well as pH and temperature.

### 5.1.4 Mammalian cell culture

Mammalian cell culture is more technically complex and more expensive than microbial cell fermentation. It is therefore usually used only in the manufacture of high-value healthcare proteins that show post-translational modifications (see Chapter 2) essential to their intended function, and when a microbial system cannot adequately reproduce the post-translational modifications in question. In practice, this usually refers to glycosylation and the use of animal cell culture would be appropriate where the carbohydrate content and pattern is essential to the protein's biological activity, stability or serum half-life. Therapeutic proteins falling into this category include some blood factors (Chapter 6), full-length antibodies (Chapter 7), erythropoietin (Chapter 8), the gonadotrophins (Chapter 8), and some cytokines (Chapter 9).

The culture of animal cells differs from that of microbial cells in several generalized respects, including:

- it requires more complex media;
- the duration of culture is extended due to slow growth of animal cells (Chapter 3);
- animal cells are more fragile than microbial cells due to the absence of an outer cell wall.

Basic animal cell culture media generally contain:

- most L-amino acids;
- many or most vitamins;
- salts (e.g. NaCl, KCl, CaCl<sub>2</sub>);
- carbon source (often glucose);
- antibiotics (e.g. penicillin or streptomycin);
- buffering agent (often CO<sub>2</sub> based);
- sometimes supplemental serum.

Antibiotics are usually required to prevent microbial growth consequent to accidental microbial contamination. Supplemental serum (often bovine or fetal calf serum, or synthetic serum composed of a mixture of growth factors, hormones and metabolites typically found in serum) can be required as a source of often ill-defined growth factors required by some animal cell lines. Much research and cell

line development work has been undertaken (very largely successfully) in order to develop fully synthetic media devoid of animal-derived products (most notably serum) for use in culturing cells producing therapeutic proteins (see Chapter 3). This is significant in that it reduces the potential for product contamination by animal pathogens.

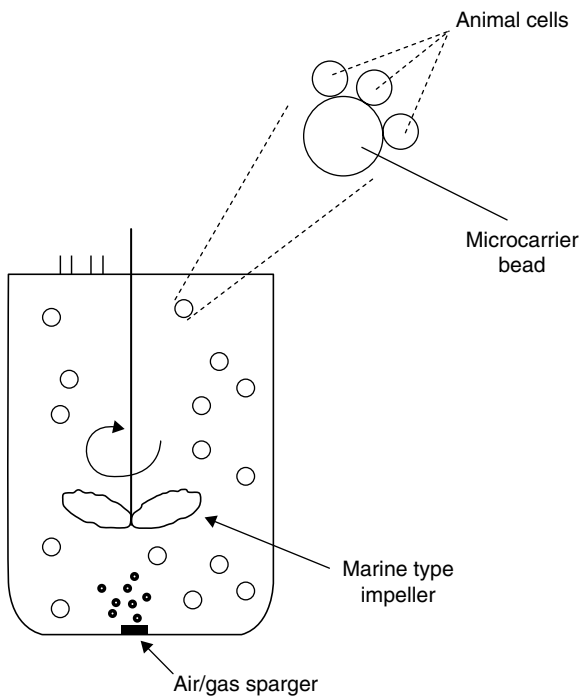
The media constituents, several of which are heat-labile, are generally dissolved in purified water and filter-sterilized into the pre-sterile animal cell reactor. Reactor design (and operation) differs somewhat from microbial fermentations, mainly with a view to minimizing damage to the more fragile cells during cell culture (Figure 5.8).

Although the generalized reactor design presented in Figure 5.8 is commonly employed on industrial scales, alternative reactor configurations are also available. These include hollow fibre systems as well as the classical roller bottle systems. Roller bottles are still used in the industrial production of a few vaccines. Roller bottles are cylindrical bottles partially filled with media, placed on their sides and mechanically rolled during cell culture. This system is gentle on the cells and the rolling action ensures homogeneity in the culture media and efficient oxygen transfer. The major disadvantage associated with applying roller bottle technology on an industrial scale is that many thousands of bottles are required to produce a single batch of product. More recently, the use of one-off disposable systems has become increasingly popular.

Traditional bioprocess-scale bioreactors are made from stainless steel, display high capital costs, complex engineering and set-up, along with demanding cleaning-in-place/sterilizing-in-place (CIP/SIP) requirements between individual batch manufacturing runs. Cited potential advantages of single-use (disposable) systems include low capital costs, quick set-up, no CIP/SIP requirements (saving time and money) and no potential for cross-contamination between batches.

In essence, disposable bioreactors are large plastic bags made from biocompatible materials (e.g. ethylene vinyl acetate/polyethylene copolymers, similar to those approved for collection and storage of blood donations) that have integrated vents for





**Figure 5.8** Conventional design of an animal cell bioreactor. The bioreactor displays several structural differences compared with microbial fermentation vessels (see Figure 5.6). Note in particular (i) the use of a marine-type impeller (some animal cell bioreactors – air lift fermenters – are devoid of impellers, and use sparging of air/gas as the only means of media agitation); (ii) the absence of baffles; and (iii) curved internal surfaces at the bioreactor base. These modifications aim to minimize damage to the fragile animal cells during culture. Many (mainly transformed) cells can grow in free submerged suspension in such vessels. However, other cell lines grow only when attached to a solid substratum. In such cases microcarrier beads (described later in the text) can be used.

media entry/recovery, probes for bioprocess monitoring, and which come pre-sterilized (by  $\gamma$ -irradiation). However, such disposable systems are not without their own limitations. For example, they are not as mechanically strong as stainless steel, hence are limited in capacity, often to 500–2000 L. Conventional stainless steel bioreactors used for the production of high-volume animal cell-derived products would more typically display 10,000 L or greater capacity.

Different animal cell types display different properties pertinent to their successful culture. Those used to manufacture biopharmaceuticals are invariably continuous (transformed) cell lines. Such cells will grow relatively vigorously and easily in submerged culture systems. Unlike most transformed cell lines, non-continuous cell lines generally:

- display anchorage dependence (i.e. will only grow and divide when attached to a solid substratum – most continuous cell lines will grow in free suspension);
- grow as a monolayer;
- exhibit contact inhibition (i.e. physical contact between individual cells inhibits further division);
- display a finite lifespan, i.e. die, generally after 50–100 cell divisions, even when cultured under ideal conditions;
- display longer population doubling times and grow to lower cell densities compared with continuous cell lines;
- usually have more complex media requirements.

Many of these properties would obviously limit the applicability of non-continuous cell lines in the industrial-scale production of recombinant proteins. However, such cell types are routinely cultured for research purposes and toxicity testing for example

The anchorage-dependent growth properties of some cell lines impact on how they are cultured, both on a laboratory and industrial scale. If grown in roller bottles or other low-volume containers, cells grow attached to the internal walls of the vessel. Large-scale culture can be undertaken in conventional bioreactors in conjunction with the use of microcarrier beads (Figure 5.8). Microcarriers are solid or sometimes porous spherical particles approximately 200  $\mu\text{m}$  in diameter manufactured from such materials as collagen, dextran or plastic. They display densities slightly greater than water, such that gentle mixing within the animal cell bioreactor is sufficient to maintain the beads in suspension and evenly distributed throughout the media. Anchorage-dependent cells attach to and grow on the beads' outer surface/outer pores.

## 5.2 Downstream processing

As already outlined, downstream processing incorporates all post-fermentation/cell culture processing steps. Various guidelines are generally applied when developing a downstream processing procedure.

- Initial characterization of the starting material should be undertaken in detail, such that a rational purification scheme may be developed. The final-product purity required should be clearly defined.
- The purification scheme should be kept as simple and as straightforward as possible, ensuring that the highest end-product yield is obtained within the shortest time period practicable.
- Fractionation techniques (and the conditions under which the techniques are used) should be selected such that they exploit the greatest differences in physicochemical properties between the protein of interest and other impurities.
- A highly selective step should be used as early as possible in the purification scheme. This may reduce the number of subsequent steps that must be employed to achieve the desired level of purity.

When purifying a protein for research purposes, technical issues are usually the sole issues considered during design of the purification procedure. In general the protein is purified to homogeneity, and less emphasis is placed on factors such as percentage yield obtained or the duration/cost of the purification scheme. In the case of commercially produced proteins, economic and often regulatory issues are also important considerations. Commercial logic dictates that the product be produced to the required specification as inexpensively as possible. Manufacturing guidelines issued by regulatory authorities such as the Food and Drug Administration (FDA) must also be taken into account in the manufacture of proteins destined for therapeutic, diagnostic or food use.

The degree of purity required is an overriding factor in the design of any downstream processing procedure. This is largely dependent on the intended

application of the final product. As a general principle, the extent of downstream processing of any protein is maintained at the minimum required to produce an acceptable final product. Unnecessary steps in the purification protocol often reduce the product's biological stability, and the yield of product decreases as the number of purification steps increases. Most proteins of industrial interest may be grouped into one of two broad categories.

1. Proteins produced in bulk, often as relatively crude preparations. Such proteins are usually enzymes that have a wide variety of applications in the food and beverage processing industries (see Chapters 11–13) or are milk-derived proteins used for food application (see Chapter 14).
2. Proteins destined for therapeutic and/or diagnostic applications (see Chapters 6–10). These proteins are generally produced in quantities which are orders of magnitude lower than bulk protein preparations, and to a very high degree of purity.

Proteins used for therapeutic or *in vivo* diagnostic purposes are subjected to the most stringent purification procedures, as the presence of molecular species other than the intended product may have an adverse clinical impact. Proteins used for *in vitro* diagnostic and analytical purposes are also usually highly purified. In such cases, however, the level of purification required is generally not quite as high as for those proteins intended for *in vivo* administration. In some instances, design of a downstream purification procedure that removes specific contaminating proteins is more important than purification of the protein to homogeneity.

### 5.2.1 Scale-up of protein purification

Industrial-scale protein purification protocols are initially designed at laboratory level. Scale-up studies are then undertaken in order to produce sufficient quantities of the protein to meet market demands as economically as possible. Generally speaking, the costs associated with the production

of a unit quantity of any protein decline with increasing scale of production:

- most chemical and other raw materials required may be purchased more cheaply in bulk;
- many overhead costs remain largely independent of scale of production;
- labour costs (per unit product produced) decreases with increased scale of production.

Some techniques/procedures used for laboratory-scale purifications are not amenable to scale-up. For example, disruption of the bacterial cell wall by sonication, while feasible on a small scale, is inefficient when applied to large-scale situations. Treatment with lysozyme on a large scale would be uneconomic.

Other techniques routinely used in laboratory-scale purification procedures must be modified before they can be successfully used on a large scale. For example, laboratory-scale centrifugation is generally carried out in a batch (fixed volume) centrifuge, whereas continuous-flow centrifuges are more typically employed in industrial-scale purification systems. Continuous-flow centrifuges allow homogenate to be pumped continuously and the clarified solution continually collected. The deposited solids can be removed from the centrifuge bowl by periodically stopping the centrifuge and manually removing the pelleted material. However, most modern continuous-flow centrifuges are designed to allow continuous discharge of collected solids through a peripheral nozzle, or alternatively facilitate intermittent discharge of pelleted material via a suitable discharge valve.

Rational design of a protein purification procedure, with all stages being amenable to direct scale-up, is especially desirable when working with a protein of therapeutic interest. Such proteins are initially produced in small quantities that are then subjected to animal trials. If encouraging results are obtained, limited clinical studies may then be initiated. Such trials, while requiring relatively little protein, could take several years to complete and are expensive to carry out. If they prove the protein is safe and therapeutically effective, scale-up of production is normally initiated, in line with anticipated market demands. Any but the most minor of

changes to the original purification process during such scale-up may invalidate the earlier clinical studies. In such cases, therefore, it is critically important to ensure that the purification system initially developed in the laboratory can be scaled up without difficulty.

It is difficult to define what exactly constitutes a small- or large-scale protein purification process. Laboratory-scale purification procedures generally yield microgram to milligram quantities of the protein product. Pilot-scale production often yields gram quantities while large-scale purification yields quantities in the order of kilograms.

### ***5.2.2 Downstream processing scale-up: bioprocess engineering aspects***

Scale-up of protein purification systems poses many difficulties and pitfalls, not only for scientific personnel but also for the process engineer. The process engineer is charged with designing and installing all the equipment required to successfully process the protein product on a preparative scale and there are many parallels between scale-up issues relating to upstream processing.

Most large-scale process equipment such as holding vessels and transfer pumps are constructed from stainless steel or plastics such as polypropylene. Glass, so commonly used on a laboratory scale, is seldom used for large-scale preparative work, mainly due to its lack of structural strength. The grade of stainless steel or the type of plastic used in the manufacture of process vessels must be selected with care. Materials must be inert and resistant to the corrosive action of any chemical used during the process. They should not allow any leaching of potentially toxic metals or chemicals into the product stream.

Liquid transfer, so easily achieved on a laboratory scale by physically pouring the liquid from one vessel into another, is achieved on a preparative scale by pumping the liquid via a series of stainless steel or plastic pipes. Only certain pump designs are suitable for transfer of liquid protein solutions.

Some pump systems tend to entrap air during their pumping action, which can lead to protein denaturation.

Preparative-scale chromatographic equipment also often differs in design and appearance from laboratory-scale systems. Most small-scale chromatographic columns are manufactured from glass or transparent plastics and range in capacity from 1 to 500 mL or so. Preparative-scale chromatographic columns range in capacity from hundreds of millilitres to several litres and, in some cases, up to several hundred litres. While some such columns are manufactured from glass, the majority are manufactured from reinforced plastic or stainless steel. Scale-up is often achieved by increasing the column diameter as opposed to column height. Columns several metres in diameter are available commercially. In the case of some chromatographic systems (e.g. gel filtration), the degree of separation achieved is proportional to the column length. Scale-up of such systems is made more difficult by the compressible nature of many chromatographic beads. Such difficulties may be overcome by employing more recently developed bead types that exhibit enhanced structural characteristics, or by altering column design.

Complete large-scale chromatographic systems are designed, manufactured and marketed by several companies. Most such purification systems consist not only of the process equipment required but also of a computerized control unit, which facilitates a high degree of system automation. In general, innovations in downstream processing techniques are introduced infrequently, largely for two reasons. Firstly, regulatory authorities such as the FDA review licence applications to produce and market proteins designed for use in the food or healthcare industries. Such applications are more likely to be looked upon favourably if proposed downstream processing procedures are based on established validated methodologies. Secondly, many protein products produced in a highly purified form command a high sale price. Many manufacturers have therefore tended simply to add on the cost of downstream processing to the final-product price. As the level of competition between rival product manufacturers increases,

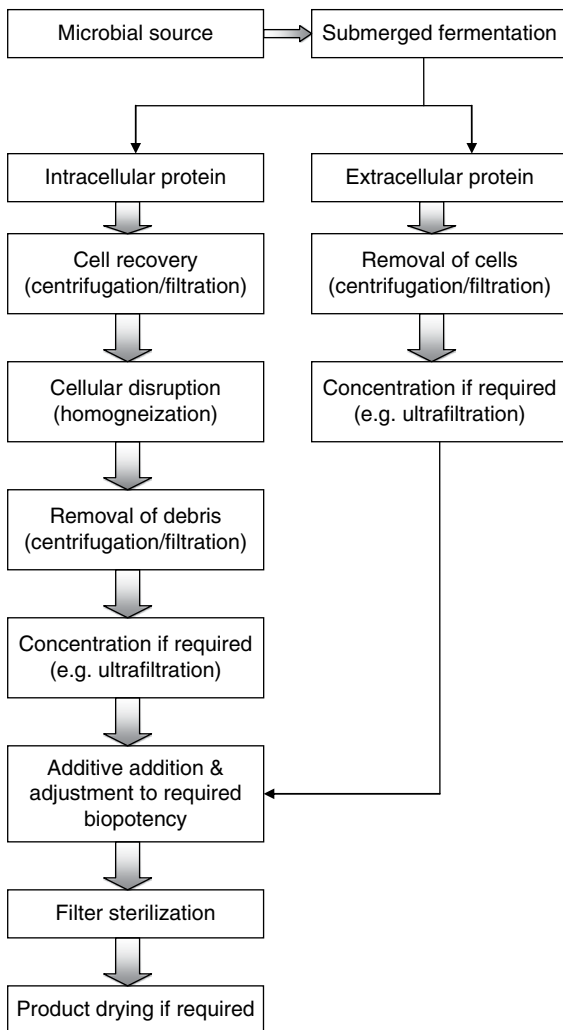
downstream processing costs, which can account for up to 80% of the overall production costs, will become more and more critical in determining product competitiveness.

### 5.2.3 Bulk protein production

Industrial-scale enzymes are produced in bulk, and are generally subjected to little downstream processing. The vast majority of such 'bulk' enzymes are biopolymer-degrading enzymes such as amylases, proteases and pectinases (see Chapter 12). These are produced extracellularly by submerged fermentation of various (recombinant and non-recombinant) microbial species, particularly species of *Bacillus* or selected species of *Aspergillus* and several specific case studies are presented in Chapters 12 and 13. Many such bulk enzyme preparations simply consist of concentrated cell-free fermentation products.

A generalized downstream processing scheme utilized in the production of bulk enzyme preparations from microbial sources is outlined in Figure 5.9. Once maximum yield of the protein has been achieved in the fermentation process the fermentation media is cooled to under 5°C. This stabilizes the protein product and discourages further microbial growth. The microbial cells must then be removed from the culture medium, typically by centrifugation or filtration. The collected cells are usually washed several times in order to recover any enzyme trapped in the cell paste, and the wash is added to the cell-free media, which contains the bulk of the desired enzyme. If the enzyme is extracellular, as is usual, then filtration tends to be the method of choice.

As outlined in Chapter 4, depth and/or membrane filters may be used. For example, a fermentation broth or a homogenate may first be passed through a depth filter, the eluate from which is then passed through a membrane filter. The depth filter removes most of the microorganisms, and invariably increases effective lifespan of the more expensive membrane filter. Membrane filter configuration may be flat disc type, although (particularly in the case of industrial systems) the filter is usually shaped



**Figure 5.9** A generalized outline of bulk protein production from microbial sources. Solid-state fermentation can be used to produce some extracellular proteins. Under such circumstances, the product is extracted from the solid culture media in an aqueous media, followed by its clarification (filtration or centrifugation). It is then concentrated if required and further processed as shown.

into a cartridge configuration (Figure 5.10). This is achieved by placing the rectangular membrane filter sheet on a supporting mesh of the same size and subsequently folding it into a pleated structure. The two ends are then sealed together to form a cylinder, which is placed between a plastic core and outer structure that physically protects and supports the filter material itself. Pleating allows a large filtration

surface area to be accommodated in a compact area. Such filters are normally housed in stainless steel filter housing systems.

Many filtration systems incorporate several filters of different pore sizes into a single cartridge system (Figure 5.10). For example, sheets of a pre-filter, two membrane filters of differing pore size and a supporting mesh may be placed on top of each other in series, folded into pleats and formed into a cylindrical cartridge by joining both ends. Most cartridge filters can be repeatedly sterilized by autoclaving or on-line steaming and they may also be operated for considerable periods of time at elevated temperatures (70–75°C).

If the enzyme is intracellular, cell harvesting by continuous centrifugation is usually undertaken. After harvesting the cells are resuspended in water/buffer and are disrupted in order to release their intracellular contents. The cell homogenate must then be centrifuged in order to remove any intact cells, in addition to cellular debris. The protein of interest should now be present in the supernatant. If the enzyme is produced by recombinant DNA technology, regulatory laws generally require the inclusion of a specific processing step that kills all the producer (genetically modified) organisms before their disposal.

In both cases a concentrating step is generally the next undertaken. This is necessary to reduce the process volume to more manageable levels, especially in the case of extracellular enzymes present in large initial volumes of fermentation medium. Concentration is often achieved by ultrafiltration. Large-scale ultrafiltration systems invariably employ cartridge-type filters (Figure 5.11). In some cases, further concentration is undertaken by vacuum evaporators. As the name suggests this entails applying a vacuum to the enzyme-containing liquid, with subsequent heating to around 40°C.

If the crude enzyme preparation is presented in liquid form, the final steps of the downstream processing procedure normally involves the addition of various stabilizers and preservatives. This is often followed by a filter sterilization step. These activities help ensure an extended product storage shelf-life and guarantee that no production microorganism is present in the final product

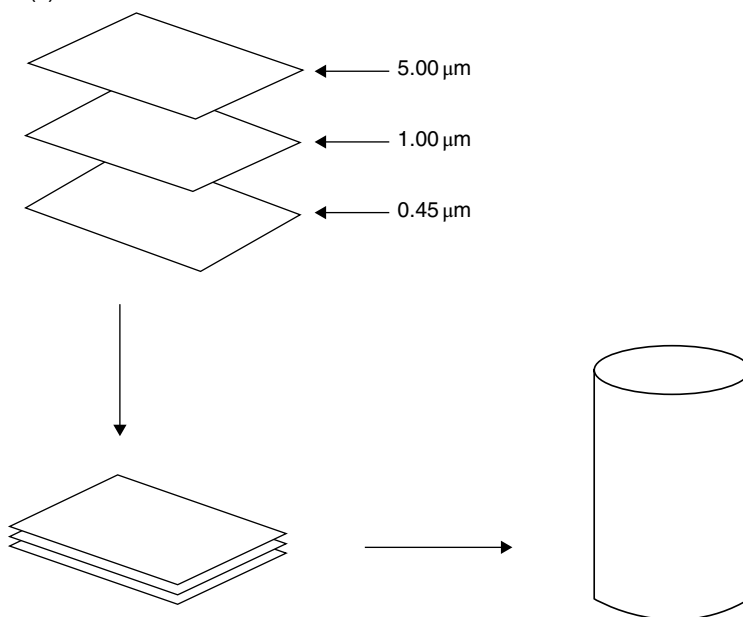
(a)



(b)



(c)



**Figure 5.10** (a) A range of cartridge filters and (b) a range of filters and their stainless steel housings. In each case the Pleated filter is protected by an outer plastic supporting mesh. Photographs courtesy of Pall Corporation. Schematic (c) represents a cartridge filter in which filter sheets of three different pore sizes are housed.



**Figure 5.11** Example of an ultrafiltration system used on an industrial scale. This system (SPM 180) comprises 16.7 m<sup>2</sup> of spiral wound membrane. It can be used in ultrafiltration or diafiltration mode. Photograph courtesy of Amicon Ltd.

(production organisms are usually considered to be highly confidential by the company). The most common preservatives/ stabilizers used include propylene glycol, glycerol and sorbitol. The final-product volume is also adjusted as appropriate, in order to ensure that enzyme activity falls within its specified limits. The product is then placed in drums and stored at low temperatures. Some enzymes are also formulated as slurries. This involves addition of agents such as ammonium sulfate, sodium sulfate or sodium chloride to the enzyme concentrate. This promotes enzyme precipitation/crystallization. Various substances such as silicon dioxide can be added to the slurry in order to keep the enzyme in suspension. In many cases enzyme slurries exhibit longer shelf-lives than enzymes stored fully in solution.

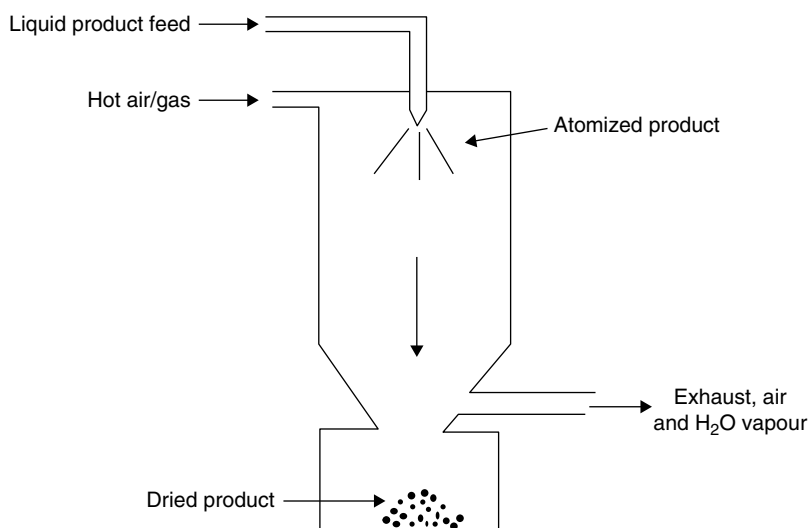
Many bulk enzymes are sold in solid form. Proteins may be dried via various techniques. The most important techniques used industrially are freeze-drying (lyophilization; discussed in Chapter 4), spray-drying and drum-drying. The latter two methods are faster and cheaper than lyophilization, but are also relatively harsh, leading to thermal inactivation of heat-labile proteins. Fortunately, most industrial-scale proteins are

relatively heat-stable and can withstand drying by such techniques.

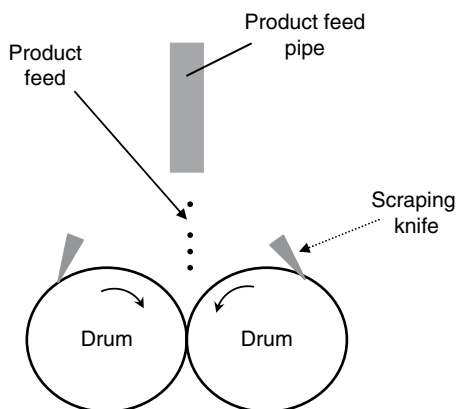
Spray-drying essentially involves the generation of an aerosol of tiny droplets from the enzyme-containing liquid/slurry and these are directed into a stream of hot gas. This results in evaporation of the water content of the droplets, leaving behind solid enzyme-containing particles (Figure 5.12). Spray-dried enzyme preparations may subsequently be further dried in vacuum ovens if required. The dried preparation may then be ground into a fine powder and the activity adjusted to the required bioactivity by the addition of suitable powder diluents. An alternative drying technique used in some processes is that of drum-drying (Figure 5.13). In general, drum-drying is harsher than spray-drying, leading to increased protein denaturation, and hence it is not as commonly used.

Powdered enzyme preparations can suffer from the disadvantage of a high level of dust formation during handling. Exposure of downstream processing personnel and/or end-product users to such enzyme-containing dust may cause severe allergic reactions. The problem can be overcome by encapsulating the enzymes. Initially, encapsulation of enzymes involved their mixing with a liquefied wax and subsequent spray-drying. This process, known as prilling, generated granules in which both the wax and enzyme were homogeneously distributed. An alternative process involved granulation of a pure enzyme-containing paste. These granules are subsequently coated with wax. The coating process ensures retention of granule integrity and thus minimizes subsequent dust formation.

Bulk enzyme preparations marketed in dried form include a variety of microbial amylases used in the starch processing industry, pectinases used in the clarification of fruit juice, and proteases incorporated into detergent preparations (see Chapter 12). Milk-derived caseins and whey proteins as well as animal proteins such as gelatin are also produced commercially in large quantities in dried form. The approaches undertaken in the industrial-scale manufacture of these proteins are generally protein specific, and are outlined in Chapter 14.



**Figure 5.12** Schematic representation of the spray-drying process. The protein solution is atomized, forming tiny product droplets. Hot air/gas is fed into the system as shown, and travels concurrently with the atomized product down the length of the drying chamber. In this way the hottest driest air comes into contact with the wettest material. The product is dried by the air. The dried product collects at the base of the drying chamber, while the wet air/gas is removed as shown.



**Figure 5.13** Schematic representation of the drum-drying process. The protein solution is fed into the system such that a thin layer coats the surface of the heated revolving drums. The heat evaporates off the solvent water, leaving a dried film of product which is scraped off by knives.

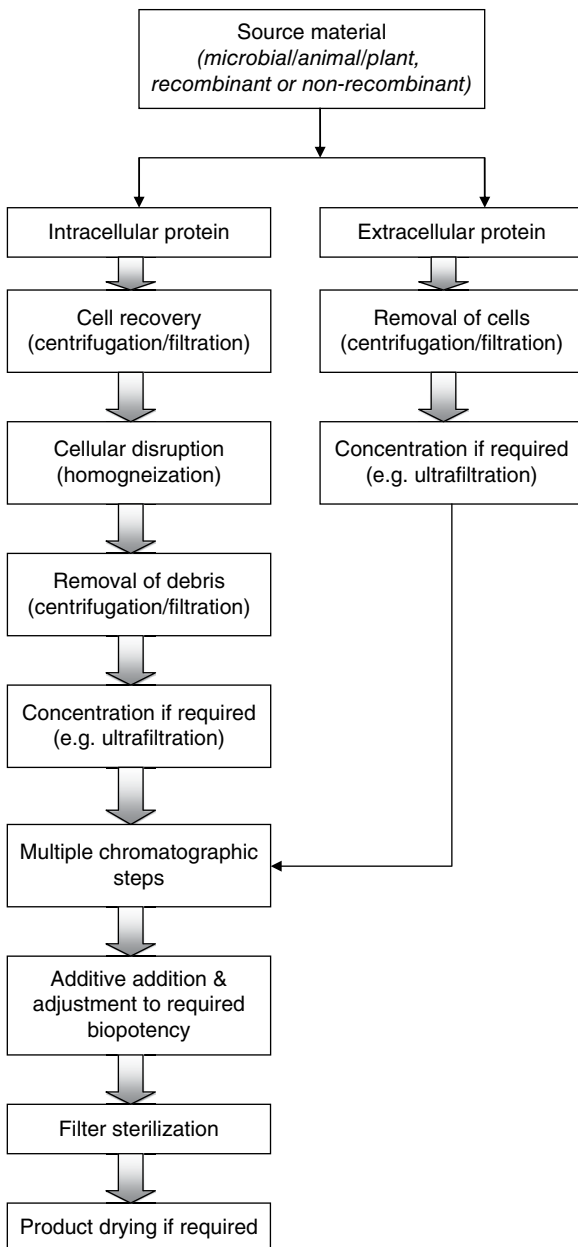
#### 5.2.4 Purification of proteins used for therapeutic or analytical purposes

Proteins destined for analytical (including diagnostic) or therapeutic applications must be purified to a very high degree. This is particularly true in the

case of any protein preparation which is to be administered parenterally (biopharmaceuticals, i.e. proteins used for therapeutic or *in vivo* diagnostic use). Parenteral preparations are defined as sterile products intended for administration by injection, infusion or implantation into the body. The high level of purity demanded is necessary in order to minimize or eliminate the occurrence of adverse clinical reactions against unwanted trace contaminants in the product. This is discussed in detail in section 5.4. Many proteins used for *in vitro* diagnostic or general analytical purposes (e.g. antibodies or enzymes) must also be partially purified in order to remove any contaminating substances that would otherwise interfere with their diagnostic applications.

The downstream processing of such proteins invariably involves not only preliminary treatments but also several high-resolution chromatography steps (see Chapter 4). A typical overall purification process is outlined in Figure 5.14. If the protein of interest is secreted into the extracellular medium, the initial purification step involves the removal of whole cells by centrifugation or ultrafiltration. If the protein of interest is intracellular, cell harvesting is followed by homogenization to achieve cell disruption.





**Figure 5.14** Generalized outline of the production of protein products destined for therapeutic or diagnostic application. Additional steps may be required to ensure complete removal of specific non-protein contaminants. Other chromatography types sometimes used include affinity chromatography or chromatofocusing. Final protein products are generally greater than 99% pure if destined for therapeutic application. Numerous quality control steps are also undertaken.

If the intracellular protein has accumulated in soluble form, the next step involves the removal of any remaining whole cells and cellular debris, generally by centrifugation. If the protein has accumulated in the form of an inclusion body, homogenization is followed by a low-speed centrifugation step that collects the inclusion bodies. After a washing step, the inclusion bodies are solubilized by a suitable method (Chapter 3).

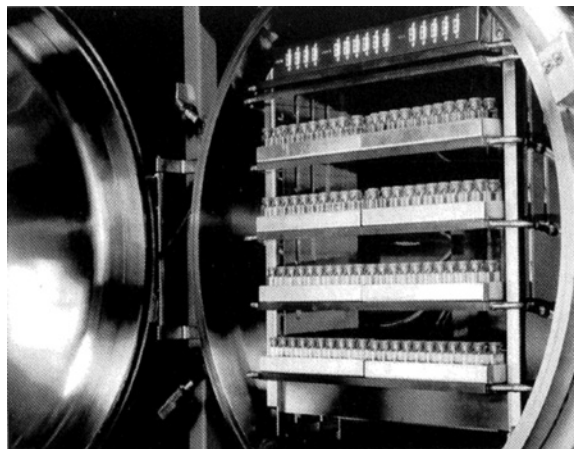
A minimum of three (and more usually five or six) chromatographic steps are usually utilized during the purification of a protein intended for parenteral administration. Although there are many different chromatographic techniques available, by far the most common techniques used industrially are those of ion-exchange chromatography (most common), affinity chromatography, hydrophobic interaction chromatography and gel filtration chromatography. The use of affinity tag systems coupled to immobilized metal ion affinity chromatography (IMAC) purification, so commonly used for laboratory-scale recombinant protein purifications (see Chapter 4 and also Box 3.1), are far less frequently used at industrial scale. This may be partly due to difficulties in achieving 100% removal of affinity tags, which could cause therapeutic complications.

Gel filtration chromatography is often the last or 'polishing' step undertaken, for a number of reasons. It effectively fractionates the required protein molecules from dimeric or higher molecular mass aggregates, which may form during the earlier purification steps. It may also effectively remove molecules such as ligands that may have leached from affinity columns. However, gel filtration columns have a low loading capacity. They must also be run at relatively low flow rates, and hence are often quite expensive in terms of their operation. The old maxim that 'time is money' is particularly true when applied to downstream processing. By employing the relevant irrigation buffer, gel filtration may also be used to incorporate the final purified protein into the buffer system most suitable for its final processing or storage. Chromatographic systems are run mainly under low or intermediate pressure conditions, although preparative high-pressure systems (HPLC, see Chapter 1) can be applied to some smaller, very stable proteins.

Unlike traditional pharmaceutical preparations most therapeutic proteins are heat-labile. They are inactivated at temperatures significantly above 40–50°C. Therefore terminal sterilization of product by heat (autoclaving) is not an option. In many cases, sterilization by irradiation is also impractical due to lack of appropriate irradiation facilities, in addition to the fact that many proteins are adversely affected by irradiation. Therapeutic/diagnostic proteins are sterilized by filtration and subsequently introduced into their final (pre-sterilized) containers using strict aseptic techniques. Sterilization by filtration is achieved using a filter of 0.22 µm pore size or smaller. Such filters will remove bacteria and fungi, but are not guaranteed to remove all viruses or mycoplasmas. In-line 1.0-µm or 0.45-µm filters are usually utilized immediately prior to the sterilizing (0.22-µm) filter.

The level of risk of contamination in relation to aseptically prepared products depends on three parameters: (i) the concentration of airborne microorganisms; (ii) the neck diameter of the container to be filled with product; and (iii) the time period that the open container is exposed to the environment. Reduction of any one of these parameters decreases the likelihood of accidental microbial contamination during an aseptic fill. The concentration of airborne microorganisms is minimized by carrying out all manipulations within a clean area environment (see below), and minimizing the number of personnel present during the aseptic operation. In this regard, design and installation of highly automated aseptic filling systems should greatly reduce the dangers of product contamination. A number of automated filling systems are available commercially and some may be amenable to CIP and subsequently sterilized by steaming-in-place. The neck diameter of the container to be filled should be minimized and the filling operation should be operated at maximum speed in order to further reduce the likelihood of accidental microbial contamination. Sterile aqueous preparations filled aseptically may contain suitable antimicrobial preservatives added to an appropriate final concentration. Insulin injections, for example, generally contains 0.1–0.25% phenol or cresol as preservative.

If the final product is sold in a powdered format, it is freeze-dried; this is because therapeutic proteins



**Figure 5.15** The drying chamber of an industrial-scale freeze-dryer. VirTis image is reproduced by permission of SP Scientific, [www.SPScientific.com](http://www.SPScientific.com).

are invariably mammalian and are almost always more heat-labile compared with extracellular microbial enzymes used for industrial purposes for example. Industrial-scale freeze-dryers (Figure 5.15) usually cater for thousands of product vials during each drying cycle. On completion of the product fill, rubber stoppers are partially inserted into the mouth of each vial in such a way as to allow water vapour to flow freely from within the vial during the drying process (see Chapter 4). The vials are placed on a series of trays which are loaded onto shelves in the drying chamber. Each shelf may be electrically heated or cooled. After loading is completed, the drying chamber door is closed. The shelf temperature is then decreased to temperatures in the order of –40 to –60°C in order to freeze the product. Various probes are inserted into test vials such that the actual product temperature may be accurately monitored.

On reaching the predetermined temperature, which should be below the product's glass transition temperature (see Chapter 4), chamber evacuation is initiated. Appropriate increases in shelf temperature promote efficient primary and secondary drying under vacuum. On completion of the freeze-drying cycle, the vacuum is released and the vials are sealed *in situ*. Shelf design in modern freeze-drying systems allows hydraulic upward, or downward, movement of the individual shelves. As any one particular shelf

**Table 5.2** Some advantages and disadvantages associated with the lyophilization (freeze-drying) of proteins.

#### Advantages

Freeze-drying represents one of the least harsh methods of protein drying

Yields a lightweight product which reduces shipping and distribution costs

Freeze-dried proteins can be rapidly reconstituted (rehydrated) prior to use

Freeze-drying is accepted by regulatory authorities as a technique suitable for the preservation of finished products destined for parenteral administration

#### Disadvantages

Equipment required at an industrial scale is extremely expensive

Running costs are high

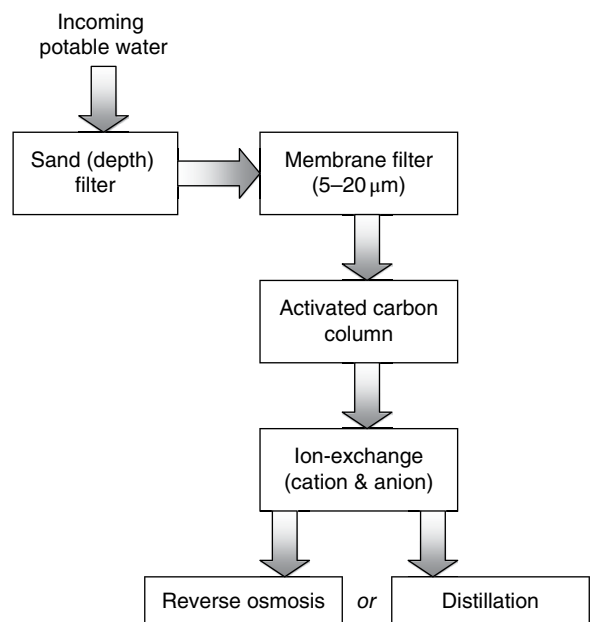
Freeze-drying entails long processing times (typically 3–5 days)

Some proteins exhibit an irreversible decrease in biological activity on freeze-drying

moves upwards, the partially inserted rubber seals will be inserted fully into the vials when they come in contact with the shelf immediately above. All variable parameters, such as temperature and reduced pressure levels, may be pre-programmed and thus the freeze-drying cycle may be fully automated. Parameters such as individual shelf temperature, product temperature and vacuum level are continually monitored and recorded during the process. Most lyophilization systems also allow *in situ* heat sterilization of the drying chamber. This greatly facilitates its subsequent use when freeze-drying proteins that must be processed under sterile conditions. Some characteristics of lyophilization as a process-scale drying technique are listed in Table 5.2.

## 5.3 Therapeutic protein production: some special issues

Certain precautions must be observed when producing therapeutic proteins destined for parenteral administration. Such modes of administration can obviously introduce the protein or drug directly into the bloodstream. In this way the defence

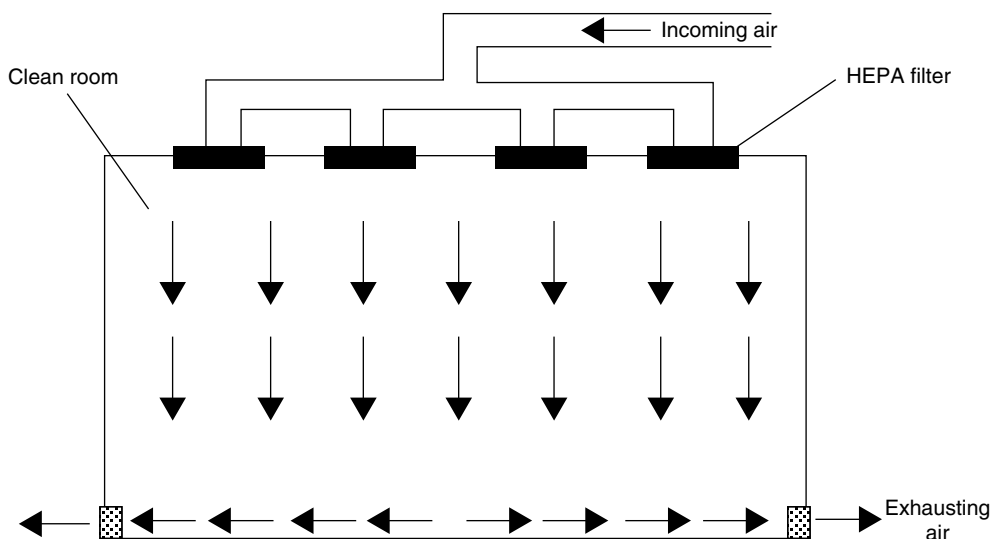


**Figure 5.16** Generalized system by which potable water is purified in order to facilitate its use in biopharmaceutical processing. Water passed through cation and anion exchange columns is termed 'purified' or 'deionized' water. The generation of water for injection (WFI) entails a subsequent distillation or reverse osmosis step.

mechanisms associated with natural assimilation of molecular species through the skin or gastrointestinal tract are bypassed. For this reason, every aspect of the manufacturing protocol employed in the production of such therapeutic proteins must attain high standards of safety. Manufacturing processes are undertaken in environmentally controlled areas (clean areas) and cleaning, decontamination and sanitization of equipment coming into direct contact with the product stream is particularly important. The solvent/diluent used during the manufacturing process is almost always highly purified water, termed water for injection (WFI). WFI is usually manufactured in-house in the biopharmaceutical plant by subjecting deionized water to distillation or reverse osmosis (Figure 5.16).

### 5.3.1 Clean areas

Clean areas are specially designed rooms in which general environmental conditions are tightly controlled.



**Figure 5.17** Schematic representation of HEPA-filtered air generation and flow through a clean room.

High-efficiency particulate air (HEPA) filters are installed in the ceilings of such rooms. All air entering the clean room is filtered through the HEPA units. Filters may be classified according to their ability to remove particulate matter. Air normally exits a clean room via specialized outlets incorporated into the walls just above floor level. HEPA filters are evenly spaced over the ceiling area in order to generate a relatively uniform downward current of filtered air throughout the room (Figure 5.17).

### 5.3.2 Cleaning, decontamination and sanitation

Cleaning may be defined as the removal of 'dirt'. Dirt, as defined here, includes all organic and inorganic material that may accumulate in process equipment and processing areas during routine downstream processing. In this regard any protein or other molecules retained in a chromatographic column subsequent to elution of the protein of interest may be regarded as dirt. Decontamination generally refers to the inactivation and removal of undesirable substances such as endotoxins and other pyrogenic compounds (see section 5.4.2), in addition to other harmful substances such as viruses. Sanitation refers to the removal and

**Table 5.3** The range of substances that cleaning, decontamination and sanitation of therapeutic protein production equipment aims to remove.

Cellular debris
Precipitated/aggregated materials
General particulate material
Pyrogenic substances
Viral particles
Microorganisms
Traces of protein or other molecules from previous production runs
Lipids and related substances

inactivation of viable organisms. In many but not all instances cleaning procedures are also effective decontamination and sanitation methods. Such procedures are applied to both process equipment and to the area in which the manufacturing process is undertaken. Cleaning, decontamination and sanitation of process equipment forms an integral part of any manufacturing procedure (Table 5.3).

Process equipment that may be detached and/or dismantled can be cleaned and sanitized relatively easily. Processing vessels may therefore be cleaned simply by rinsing or scrubbing thoroughly with high-purity water. Detergents may be required but, if used, all traces must be subsequently washed away with WFI. Detachable tubing may be cleaned in a somewhat similar fashion. Pumps and other

equipment such as continuous-flow centrifuges should be dismantled if possible to ensure a thorough cleaning of the constituent parts. Such cleaning procedures will remove virtually all 'dirt' and will significantly reduce the level of viable organisms (bioburden) present. It will not, however, guarantee sterility. Components may be sterilized by a number of methods, including moist or dry heat, irradiation or chemicals. Sterilization of any process equipment with chemical agents such as formaldehyde, hypochlorite or peroxides must be undertaken with caution, due to the possibility that traces of such chemicals would remain in the sanitized equipment. This would contaminate products during subsequent production runs. Most processing equipment may be effectively sterilized by autoclaving, as can ancillary equipment such as flexible tubing and other plastic materials. Prior to their use, most filters are assembled in their final housings (usually stainless steel modules) and are also sterilized by autoclaving.

The internal surfaces of process fixtures such as metal pipework or some vessels may be sterilized by passing 'live' steam for an appropriate period of time. CIP involves the cleaning and sanitation of fixtures without their disassembly or removal from their normal functioning positions. While all process equipment should be cleaned at regular intervals, only those items of equipment which come into direct contact with products are generally sterilized.

Chromatographic systems require regular cleaning, decontamination and sanitation. Such systems are generally cleaned in place. Prior to pouring or repouring a column, the column and chromatographic beads may be cleaned or sterilized separately. Before sterilization, the chromatographic column should be disassembled and scrupulously cleaned, firstly with a detergent, followed by rinsing thoroughly with high-purity water. Depending on the material from which it is manufactured, the column may subsequently be sterilized by autoclaving or by treatment with chemicals such as sodium hydroxide (NaOH). Most chromatographic media may be sterilized by autoclaving, though chemicals such as hypochlorite or peroxides may also be used under certain circumstances.

On packing columns with relevant bead materials it becomes necessary to devise a suitable protocol to

achieve effective CIP of the chromatographic system. Several cleaning/sanitizing agents may be employed. On completion of a production run a thorough flushing of the column with several column volumes of water or suitable process buffer may flush out many column contaminants. Rinsing of the beads with a concentrated solution of neutral salts such as KCl or NaCl is often effective in removing precipitated or aggregated proteins, or other molecules which may be weakly bound. Inclusion of a chelating agent such as EDTA in the buffer may help remove any metal ions. Subsequent washing with a solution of NaOH, or in certain cases with detergents, may be required in order to remove tightly bound material. In some instances, where column fouling by lipids is particularly problematic, increasing the column temperature to 40–60°C may be effective. Most lipid material is solubilized at such temperatures. This approach may be adopted only if it has been shown that subjecting the chromatographic system to such changes in temperature will not adversely effect its fractionation characteristics.

NaOH is extensively used as a CIP agent. It is effective in removing many contaminants that bind tightly to the chromatographic beads, in addition to removing/destroying pyrogenic material such as bacterial endotoxins, viral particles and bacteria. NaOH is popular due to not only its efficiency as a cleaning agent, but also its ready availability and the fact that it is inexpensive.

The real possibility of the presence of pathogenic viral particles in biological source material is a matter of concern. Many viruses harbour oncogenes (cancer-causing genes) while others represent the causative agents of serious illnesses such as hepatitis or AIDS. NaOH will inactivate human immunodeficiency virus (HIV) within minutes.

The effectiveness of NaOH as a cleaning and sanitizing agent is both time and concentration dependent. Generally, NaOH is applied at concentrations of 0.5–1.0 mol/L, and should have a bead contact time of 1 hour or more. NaOH may be used only if it is compatible with both the chromatographic gel and all the column components. Most chromatographic media may be exposed to NaOH for reasonable time periods without adverse effect. Silica gel is an exception as it quickly dissolves at pH

values greater than 8.0. Following exposure to the NaOH solution, the chromatographic system should immediately be thoroughly rinsed with sterile pyrogen-free water or an appropriate buffer. Efficient removal of NaOH subsequent to completing a CIP protocol is necessary in order to prevent column deterioration due to prolonged exposure to alkaline conditions and to ensure that residual NaOH does not contaminate the product during the next production run.

Routine application of an effective CIP procedure is greatly simplified if the overall chromatographic system has been designed with process hygiene in mind. The choice of beads and column type will determine what CIP agents may be utilized. Design of the chromatographic column and ancillary equipment will determine the susceptibility to microbial contamination. Valves and pipe connections represent danger points when considering the risk of introducing microbial contaminants. Pipe connections in particular should be designed such that no 'dead-leg' zones are present.

## 5.4 Range and medical significance of impurities potentially present in protein-based therapeutic products

Proteins destined for parenteral administration must be free from all impurities that might have an adverse effect on the well-being of the patients to whom they are administered. Impurities most commonly encountered are outlined in Table 5.4.

The majority of techniques utilized during downstream processing are designed to separate protein molecules from each other, i.e. to purify the protein of interest from the hundreds, or in many cases thousands, of other proteins present in the starting material. In many instances non-proteinaceous impurities may be efficiently removed by one or more of the protein fractionation steps of downstream processing. In other instances it may

**Table 5.4** The range and medical significance of potential impurities present in therapeutic protein products.

Impurity	Medical consequence
Microorganisms	Potential establishment of severe microbial infections (septicaemia)
Viral particles	Potential establishment of a severe viral infection
Pyrogenic substances	Fever response which in serious cases culminates in death
DNA	Significance is unclear; could bring about an immunological response
Contaminating proteins	Immunological reactions. Potential adverse effects if the contaminant exhibits unwanted biological activity

be necessary to include specific steps in order to remove certain impurities.

Details of the various potential product impurities, their medical significance and methods to minimize or eliminate these from the final protein product form the subject matter of the remainder of this chapter.

### 5.4.1 Microbial and viral contaminants

Pharmaceutical products intended for parenteral administration must be sterile, the one exception being live bacterial vaccines. The presence of microorganisms in the final product is unacceptable for a number of reasons.

- Parenteral administration of contaminated product would likely lead to the establishment of a severe infection in the recipient patient.
- Microorganisms may be capable of metabolizing the product itself, thus reducing its potency. This is particularly true of protein-based biopharmaceuticals, as most microbes produce an array of extracellular proteases.
- Microbial-derived substances secreted into the product could adversely affect the recipient's health. Examples include endotoxin secreted

from Gram-negative bacteria, or microbial proteins that would stimulate an immune response.

Terminal sterilization by autoclaving guarantees product sterility. However, heat sterilization is not a viable option in the case of proteins. Sterilization by filtration, followed by aseptic filling into a sterile final-product container, carries an inherently greater risk of product contamination. Finished-product sterility testing of such preparations thus represents one of the most critical product tests undertaken by quality control.

Biopharmaceutical products are also subjected to screening for the presence of viral particles prior to final-product release. Although viruses could be introduced via infected personnel for example during downstream processing, proper implementation of good manufacturing practice (GMP) minimizes such risk. Any viral particles found in the finished product are most likely derived from raw material sources. Examples could include HIV or hepatitis viruses present in blood used in the manufacture of blood products. Such raw materials must be screened for the presence of likely viral contaminants before processing.

A variety of murine (mouse) and other mammalian cell lines have become popular host systems for the production of recombinant human therapeutic proteins. These cell lines are sensitive to infection by various viral particles. Producer cell lines are screened during product development studies to ensure freedom from a variety of pathogenic adventitious agents, including various species of bacteria, fungi, yeast, mycoplasma, protozoa, parasites, viruses and prions. Suitable microbiological precautions must subsequently be undertaken to prevent producer cell banks from becoming contaminated with such pathogens.

Removal of viruses from the product stream can be achieved in a number of ways. The physico-chemical properties of viral particles differ greatly from those of most proteins, ensuring that effective fractionation is automatically achieved by most chromatographic techniques. Gel filtration chromatography, for example, effectively separates viral particles from most proteins on the basis of differences in size.

In addition to chromatographic separation, specific downstream processing steps may be undertaken which are aimed at removal or inactivation of viral particles potentially present in the product stream. Significantly, many are 'blanket' procedures, equally capable of removing known or potentially likely viral contaminants and any uncharacterized/undetected viruses. Filtration through a 0.22- $\mu\text{m}$  filter effectively removes microbial agents from the product stream, but fails to remove most viral types. Repeat filtration through a 0.1- $\mu\text{m}$  filter is more effective in this regard. Alternatively, incorporation of an ultrafiltration step (preferably at the terminal stages of downstream processing) also proves effective.

Incorporation of downstream processing steps known to inactivate a wide variety of viral types provides further assurance that the final product is unlikely to harbour active virus. Heating and irradiation are among the two most popular such approaches. Heating the product to 40–60°C for several hours inactivates a broad range of viruses. Many therapeutic proteins can be heated to such temperatures without being denatured themselves. Such an approach has been used extensively to inactivate blood-borne viruses in blood products. Exposure of product to controlled levels of UV radiation can also be quite effective, while having no adverse effect on the product itself.

A range of assay techniques may be used to detect and quantify viral contaminants in both raw materials and finished therapeutic products. No generic assay exists that is capable of detecting all viral types potentially present in a given sample. Viral assays currently available will detect only a specific virus, or at best a family of closely related viruses. The strategy adopted therefore usually entails screening product for viral particles known to be capable of infecting the protein source material. Such assays will not normally detect newly evolved viral strains or uncharacterized/unknown viral contaminants. This fact underlines the importance of including at least one step in downstream processing which is likely to indiscriminately inactivate or remove viruses from the product. This acts as a safety net.

Current viral assays fall into one of three categories: immunoassays (see also Chapter 10), assays

based on viral DNA probes, and bioassays (see also Chapter 4).

1. *Immunoassays*. Generation of antibodies that can recognize and bind to specific viruses is straightforward. A sample of live or attenuated virus, or a purified component of the viral capsid, can be injected into animals to stimulate polyclonal antibody production (or to facilitate monoclonal antibody production by hybridoma technology). Harvested antibodies are then employed to develop specific immunoassays that can be used to routinely screen test samples for the presence of that specific virus. Immunoassays capable of detecting a wide range of viruses are available commercially. The sensitivity, ease, speed and relative inexpensiveness of these assays render them particularly attractive.
2. *Viral DNA probes*. An alternative assay format entails the use of virus-specific DNA probes. These can be used to screen the therapeutic product for the presence of viral DNA.
3. *Bioassays*. Viral bioassays of various different formats have also been developed. One format involves incubation of the final product with cell lines sensitive to a range of viruses. The cells are subsequently monitored for cytopathic effects or other obvious signs of viral infection. A range of mouse, rabbit or hamster antibody production tests may also be undertaken. These bioassays entail administration of the product to a test animal. Any viral agents present will elicit production of antiviral antibodies in that animal. Serum samples (withdrawn from the animal approximately 4 weeks after product administration) are screened for the presence of antibodies recognizing a range of viral antigens. This can be achieved by enzyme immunoassay in which immobilized antigen is used to screen for the virus-specific antibodies. These assay systems are extremely sensitive, as minute quantities of viral antigen will elicit strong antibody production. A single serum sample can also be screened for antibodies specific to a wide range of viral particles. However, time and expense militate against this particular assay format.

## 5.4.2 Pyrogenic contaminants

Pyrogens are substances that, when they enter the bloodstream, influence hypothalamic regulation of body temperature, usually resulting in fever. Medical control of pyrogen-induced fever proves very difficult, and in severe cases results in patient death.

Pyrogens represent a diverse group of substances, including various chemicals, particulate matter, and endotoxin (lipopolysaccharide, LPS), a molecule derived from the outer membrane of Gram-negative bacteria. Such Gram-negative organisms harbour 3–4 million LPS molecules on their surface, representing in the region of 75% of their outer membrane surface area. Gram-negative bacteria clinically significant in human medicine include *E. coli*, *Haemophilus influenzae*, *Salmonella enterica*, *Klebsiella pneumoniae*, *Bordetella pertussis*, *Pseudomonas aeruginosa*, *Chlamydia psittaci* and *Legionella pneumophila*.

In many instances the influence of pyrogens on body temperature is indirect. For example, entry of endotoxin into the bloodstream stimulates the production of interleukin (IL)-1 (see Chapter 9) by macrophages. It is the IL-1 that directly initiates the fever response (hence its alternative name 'endogenous pyrogen').

While entry of any pyrogenic substance into the bloodstream can have serious medical consequences, endotoxin receives most attention because of its ubiquitous nature. It is therefore the pyrogen most likely to contaminate parenteral products. Effective implementation of GMP minimizes the likelihood of product contamination by pyrogens. For example, GMP dictates that chemical reagents used in the manufacture of process buffers are extremely pure. Such raw materials are therefore unlikely to contain chemical contaminants displaying pyrogenic activity. Furthermore, GMP encourages filtration of virtually all parenteral products through a 0.45 or 0.22  $\mu\text{m}$  filter at points during processing and prior to filling in final-product containers (even if the product can subsequently be sterilized by autoclaving). Filtration ensures removal of all particulate matter from the product. In addition, most final-product containers are rendered particle-free immediately prior to filling by an

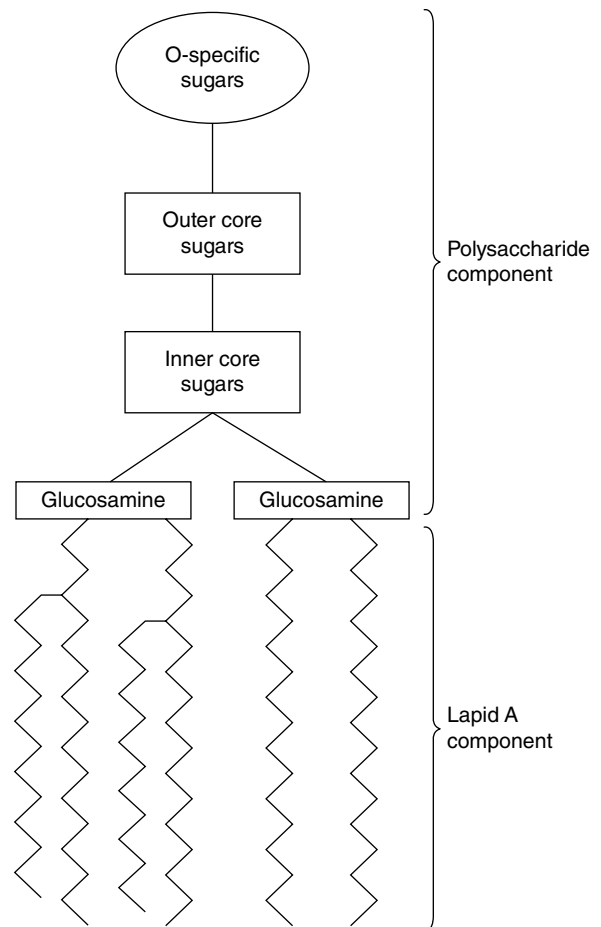


automatic pre-rinse using WFI. As an additional safeguard, the final product will usually be subject to a particulate matter test by quality control before final-product release. The simplest format for such a test could involve visual inspection of vial contents, although specific particle detecting and counting equipment is more routinely used.

Contamination of the final product with endotoxin is more difficult to control for the following reasons.

- Many recombinant therapeutic proteins are produced in Gram-negative bacterial systems, and thus the product source is also a source of endotoxin.
- Despite rigorous implementation of GMP, most protein preparations will be contaminated with low levels of Gram-negative bacteria at some stage of manufacture. These bacteria shed endotoxin into the product stream that is not removed during subsequent bacterial filtration steps. This is one of many reasons why GMP dictates that the level of bioburden in the product stream should be minimized at all stages of manufacture.
- The heat stability exhibited by endotoxin (see later) means that autoclaving of process equipment will not destroy endotoxin present on such equipment.
- Adverse medical reactions caused by endotoxin are seen in humans at dosage rates as low as 0.5 ng/kg body weight.

The structural detail of a generalized endotoxin (LPS) molecule is presented in Figure 5.18. As its name suggests, LPS consists of a complex polysaccharide component linked to a lipid A moiety. The polysaccharide moiety is generally composed of 50 or more monosaccharide units linked by glycosidic bonds. Sugar moieties often found in LPS include glucose, glucosamine, mannose and galactose, as well as more extensive structures such as L-glyceromannoheptose. The polysaccharide component of LPS may be divided into several structural domains. The inner (core) domains vary relatively little between LPS molecules isolated from different Gram-negative bacteria. The outer (O-specific) domain is usually bacterial strain-specific.



**Figure 5.18** Structure of a generalized lipopolysaccharide (LPS) molecule. LPS constitutes the major structural component of the outer membrane of Gram-negative bacteria. Although LPS of different Gram-negative organisms differ in their chemical structure, each consists of a complex polysaccharide component linked to a lipid component. Refer to text for specific details.

Most of the LPS biological activity (pyrogenicity) is associated with its lipid A moiety. This usually consists of six or more fatty acids attached directly to sugars such as glucosamine. Again, as is the case in relation to the carbohydrate component, lipid A moieties of LPS isolated from different bacteria can vary somewhat. The structure of *E. coli*'s lipid A has been studied in greatest detail. Its exact structure has been elucidated, and it can be chemically synthesized.

Pyrogens may be detected in parenteral preparations (or other substances) by a number of methods.

Two such methods are widely employed in the pharmaceutical industry. Historically, the rabbit pyrogen test constituted the most widely used method. This involves parenteral administration of the product to a group of healthy rabbits, with subsequent monitoring of rabbit temperature using rectal probes. Increased rabbit temperature above a certain point suggests the presence of pyrogenic substances. The basic rabbit method, as outlined in the European Pharmacopoeia, entails initial administration of the product to three rabbits. The product is considered to have passed the test if the total (summed) increase in temperature of all three animals is less than 1.15°C. If the total increase recorded is greater than 2.65°C, the product has failed. However, if the response observed falls between these two limits, the result is considered inconclusive and the test must be repeated using a further batch of animals.

This test is popular because it detects a wide spectrum of pyrogenic substances. However, it is also subject to a number of disadvantages, including:

- it is expensive (there is a requirement for animals, animal facilities and animal technicians);
- excitation/poor handling of the rabbits can affect the results obtained, usually prompting a false-positive result;
- subclinical infection/poor overall animal health can also lead to false-positive results;
- use of different rabbit colonies/breeds can yield variable results.

Another issue of relevance is that certain biopharmaceuticals, for example cytokines such as IL-1 and tumour necrosis factor (see Chapter 9), themselves induce a natural pyrogenic response. This rules out use of the rabbit-based assay for detection of exogenous pyrogens in such products. Such difficulties have led to the increased use of an *in-vitro* assay, the *Limulus* amoebocyte lysate (LAL) test. This is based on endotoxin-stimulated coagulation of amoebocyte lysate obtained from horseshoe crabs. This test is now the most widely used assay for the detection of endotoxins in biopharmaceutical and other pharmaceutical preparations.

Development of the LAL assay was based on the observation that the presence of Gram-negative

bacteria in the vascular system of the American horseshoe crab (*Limulus polyphemus*) resulted in the clotting of its blood. Tests on fractionated blood showed the factor responsible for coagulation resided within the crab's circulating blood cells, the amoebocytes. Further research revealed that the bacterial agent responsible initiation of clot formation was endotoxin.

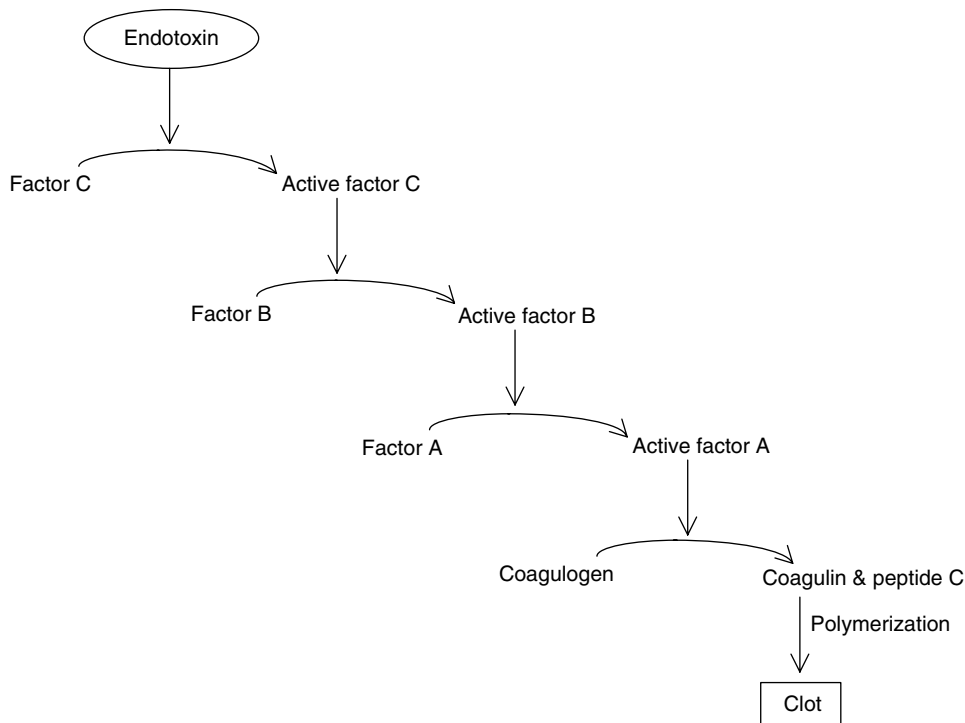
The endotoxin molecule activates a coagulation cascade quite similar in design to the mammalian blood coagulation cascade (Figure 5.19). Activation of the cascade also requires the presence of divalent cations such as calcium or magnesium. The final steps of this pathway entail the proteolytic cleavage of the polypeptide coagulogen, forming coagulin, and a smaller peptide fragment. Coagulin molecules then interact non-covalently, forming a 'clot' or 'gel'.

The LAL-based assay for endotoxin became commercially available in the 1970s. The LAL reagent is prepared by extraction of blood from the horseshoe crab, followed by isolation of its amoebocytes by centrifugation. After a washing step, the amoebocytes are lysed, and the lysate dispensed into pyrogen-free vials. The assay is normally performed by making a series of 1 : 2 dilutions of the test sample using (pyrogen-free) WFI (and pyrogen-free test tubes). A reference standard endotoxin preparation is treated similarly. LAL reagent is added to all tubes, incubated for 1 hour, and these tubes are then inverted to test for gel (i.e. clot) formation, which would indicate presence of endotoxin.

More recently a colorimetric-based LAL procedure has been devised. This involves addition to the LAL reagent of a short peptide, susceptible to hydrolysis by the LAL clotting enzyme. This synthetic peptide contains a chromogenic tag (usually *para*-nitroaniline), which is released free into solution by the clotting enzyme. This allows spectrophotometric analysis of the test sample, facilitating more accurate end-point determination.

The LAL system displays several advantages when compared with the rabbit test, most notably:

- sensitivity (endotoxin levels as low as a few picograms per millilitre of sample assayed will be detected);



**Figure 5.19** Activation of clot formation by endotoxin. The presence of endotoxin causes stepwise sequential activation of various clotting factors present naturally within the amoebocytes of the American horseshoe crab. The net result is the generation of the polypeptide fragment coagulin, which polymerizes thus forming a gel or clot.

- cost (the assay is far less expensive than the rabbit assay);
- speed (depending on the format used, the LAL assay may be conducted within 15–60 minutes).

Its major disadvantage is its selectivity – it only detects endotoxin-based pyrogens. In practice, however, endotoxin represents the pyrogen by far the most likely to be present in pharmaceutical products. The LAL method is used extensively within the industry. It is used not only to detect endotoxin in finished parenteral preparations, but also in WFI and in biological fluids such as serum or cerebrospinal fluid.

Before the LAL assay is routinely used to detect/quantify endotoxin in any product, its effective functioning in the presence of that product must be demonstrated by validation studies. Such studies are required to prove that the product (or, more likely, excipients present in the product) do not interfere with the rate/extent of clot formation (i.e. are neither

inhibitors nor activators of the LAL-based enzymes). LAL enzyme inhibition could facilitate false-negative results on sample assay. Validation studies involve observation of the effect of spiking endotoxin-negative product with known quantities of endotoxin, or spiking endotoxin with varying quantities of product, before assay with the LAL reagents.

All ancillary reagents used in the LAL assay system (e.g. WFI, test tubes, pipette tips for liquid transfer) must obviously be endotoxin-free. Such items can be rendered endotoxin-free by heat. However, its heat-stable nature renders necessary very vigorous heating in order to destroy contaminant endotoxin. A single autoclave cycle is insufficient, with total destruction requiring three consecutive autoclave cycles. Dry heat may also be used (180°C for 3 hours or 240°C for 1 hour).

GMP requires that, where practicable, process equipment coming into direct contact with the protein product stream should be rendered endotoxin-free (depyrogenated) before use. Autoclaving, steam,

or dry heat can effectively be used on many process vessels, pipework, etc., which are usually manufactured from stainless steel or other heat-resistant material. Such an approach is not routinely practicable in the case of some items of process equipment such as chromatographic systems. Fortunately, endotoxin is sensitive to strongly alkaline conditions, and thus routine CIP of chromatographic systems using NaOH (1 mol/L) represents an effective depyrogenation step. More gentle approaches such as exhaustive rinsing with WFI (until an LAL test shows the eluate to be endotoxin-free) can also be effective.

It is generally unnecessary to introduce specific measures aimed at endotoxin removal from the product during downstream processing. Endotoxin present in the earlier stages of production are often effectively removed from the product during chromatographic fractionation. The endotoxin molecule's highly negative charge often facilitates its effective removal from the product stream by ion-exchange chromatography. Gel filtration chromatography also serves to remove endotoxin from the product. While individual LPS molecules exhibit an average molecular mass of less than 20 kDa, these molecules aggregate in aqueous environments generating supramolecular structures of molecular mass 100–1000 kDa.

The molecular mass of most therapeutic proteins is considerably less than 100 kDa (Table 5.5). The proteins would thus elute from gel filtration columns much later than contaminating endotoxin aggregates. Should the biopharmaceutical exhibit a molecular mass approaching or exceeding 100 kDa, effective separation can still be achieved by inclusion of a chelating agent such as EDTA in the running buffer. This promotes depolymerization of the endotoxin aggregates into monomeric (20 kDa) form.

Additional techniques capable of separating biomolecules on the basis of molecular mass (e.g. ultrafiltration) may also be used to remove endotoxin from the product stream.

### 5.4.3 DNA contaminants

The significance of DNA-based contaminants in parenteral products remains unclear. Theoretically,

**Table 5.5** Molecular mass of some therapeutic proteins. Many are glycosylated, thereby exhibiting a range of molecular masses due to differential glycosylation.

Protein	Molecular mass (kDa)
Interferon $\alpha$	20–27
Interferon $\beta$	20
Interferon $\gamma$	20–25
Interleukin 2	15–20
Interleukin 1	17.5
Interleukin 12	30–35
Tumour necrosis factor $\alpha$	52*
GM-CSF	22
G-CSF	21
Erythropoietin	36
Thrombopoietin	60
Insulin-like growth factor 1	7.6
Epidermal growth factor	6
Nerve growth factor	26
Insulin	5.7
Human growth hormone	22
Follicle-stimulating hormone	34
Luteinizing hormone	28.5

\* Biologically active, trimeric form.

GM-CSF, granulocyte–macrophage colony-stimulating factor;

G-CSF, granulocyte colony-stimulating factor.

entry of contaminant DNA into the genome of recipient cells, if such a process were to occur, could have serious clinical implications. These could include alteration of the level of expression of cellular genes, or expression of a foreign gene product. Many cell lines employed to produce certain therapeutic proteins (e.g. hybridomas, recombinant CHO and BHK cell lines) are known to contain active oncogenes. Although health risks associated with the presence of naked DNA in parenteral preparations is considered to be minimal, the potential presence of oncogenes in an injectable product is deemed inappropriate.

In many cases there is little need to incorporate specific DNA removal steps in downstream processing procedures. Nucleic acids present in crude cell extracts are often degraded by endogenous

nucleases, although exogenous nucleases can be added if considered necessary. Nuclease treatment will result in degradation of nucleic acids, yielding nucleotides. Nucleotides are a much less potential hazard compared with DNA segments corresponding to intact genes. Most protein purification steps subsequently remove nucleotides from product-containing fractions. Nucleic acid molecules may also be precipitated from solution by addition of positively charged polymers such as polyethylenimine.

Because of major differences in their physicochemical properties, most chromatographic steps will result in effective separation of DNA from proteins. As in the case of endotoxin, DNA's highly negative charge ensures that an ion-exchange step is particularly effective in its removal from the product stream. Levels of contaminating DNA are normally measured using a species-specific DNA hybridization assay. If the protein of interest is purified from *E. coli*, total *E. coli* DNA is radiolabelled and used as a probe to detect any *E. coli* DNA contaminating the product.

DNA validation studies are normally carried out in order to illustrate that the downstream purification process used is capable of reducing the level of DNA in the final product to within acceptable limits. The definition of what constitutes a suitable upper limit is somewhat arbitrary, but quantities of up to 10 pg of residual DNA per therapeutic dose are generally considered acceptable.

DNA validation studies are carried out by methods very similar to those of viral validation studies. This involves spiking a raw material sample with a known amount of DNA and applying this to a scaled-down version of the proposed purification system. The DNA distribution profile may be monitored after each purification step, together with the fractionation behaviour of the protein of interest. In this way, a DNA reduction factor may be calculated. DNA obtained from the same source as the protein of interest is normally used in validation studies. The DNA employed to spike samples may itself be radiolabelled, allowing direct detection. Alternatively, the DNA applied may be unlabelled, and in such circumstances a specific labelled DNA probe must be used to detect the spiking material.

The actual quantity of DNA used must be carefully considered as excess DNA may itself adversely affect the purification procedure. Large quantities of exogenous DNA added to the product sample could, for example, bind anion exchange resins and therefore significantly reduce their capacity to bind other molecules. The physical characteristics of the DNA employed should also receive some thought as DNA molecules present in any given protein starting sample may vary widely in terms of molecular mass. It may thus be prudent to carry out validation studies using DNA spikes that exhibit a wide range of molecular masses.

#### 5.4.4 Protein contaminants

The majority of purification steps included in downstream processing protocols are designed to specifically fractionate differing protein molecules from one another. Despite the availability of a wide range of fractionation methods, it remains a difficult task to purify a specific protein to homogeneity while obtaining an economically viable yield. The range of potential protein contaminants depends on the source of the protein, its method of production and the downstream processing procedures used.

Modified forms of the protein product itself may also be considered as impurities. All proteins are susceptible to a variety of structural modifications that may alter their biological activity or immunological characteristics (see also Chapter 4):

- aggregation;
- oxidation and deamidation;
- incorrect disulfide bond formation;
- proteolysis;
- alteration of post-translational modifications.

In many instances, such altered products are generated during downstream processing and their effective separation from the intact parental molecules can prove quite difficult. Aggregated or extensively degraded molecules may be removed from the intact product by gel filtration chromatography, as can some molecules with extensively altered glycosylation patterns. Oxidized or deaminated proteins

may be separated by techniques such as ion-exchange chromatography or isoelectric focusing.

A major clinical significance of protein impurities relates to their antigenicity. Some protein contaminants may also display biological activity deleterious to either the protein product or the recipient patient. The potential for adverse immunological reactions depends on not only the immunogenicity of the product or contaminants but also the route of administration and in particular the frequency of administration. Contaminating proteins also act as adjuvant-like materials, thereby further increasing the immunogenicity of the protein. Immunological complications are less likely to feature if a particular therapeutic product is administered on a one-off basis, in contrast to a product which must be repeatedly administered to the recipient patient.

On administration, all injected proteins run the risk of eliciting an immune response. Some protein vaccines or toxoids are administered specifically for this purpose. Many therapeutically important proteins are derived from human sources and, as such, are non-immunogenic. Other proteins often share extensive structural homology with the natural human product. Porcine insulin for example differs from human insulin by only one amino acid residue, while bovine insulin differs from human insulin by three amino acids. Therefore, administration of bovine, and in particular porcine, insulin normally do not elicit a strong immunological response.

Different protein molecules differ in their intrinsic ability to stimulate an immune response. It is not yet possible to predetermine if any particular protein will initiate a strong immunological reaction. In general, however, the less homology exhibited between the product and the analogous human protein, the greater the possibility that an immunological reaction will be observed. Larger polypeptides or proteins also tend to exhibit increased immunogenicity as do proteins exhibiting extensive post-translational modifications that differ from modifications observed in their human counterparts.

Immunological responses to administered proteins are clinically undesirable for a number of reasons (unless the product is used specifically as an

immunogen). In cases where repeat administration is required, antibodies may be raised against the 'foreign' protein. These antigen-specific antibodies may decrease or nullify the potency of the product. This effectively means that the patient develops clinical resistance to the protein drug or that increasing quantities of the protein must be administered in order to sustain a particular level of clinical responsiveness. Binding of antibody to the therapeutic protein may also distort the dose-response curve. While initial binding of antigen by antibody may decrease the product's apparent potency, the reversibility of antibody-antigen binding usually results in a slow sustained release of free protein in the bloodstream. Binding of the therapeutic protein by antibodies may also affect normal degradation of the product. Many hormones such as insulin are removed from general circulation by a process of receptor-mediated endocytosis, and binding of antibody may severely restrict this uptake mechanism. Patients may also develop allergic responses to the particular product or to impurities consistently found in association with the products. Such allergic reactions or hypersensitivity generally result from the action of IgE antibodies or from T cell-mediated cellular toxicity.

The various approaches to detecting protein-based impurities in a purified protein product have been reviewed in Chapters 1 and 4. One point sometimes overlooked is that many protein contaminants, be they modified versions of the protein of interest or different protein molecules, may be completely innocuous.

### **5.4.5 Chemical and miscellaneous contaminants**

A variety of miscellaneous contaminants are often found in the product stream at various stages of purification. Some such contaminants, such as minor levels of lipid or polysaccharide, may be derived from the producing cells. However, the majority of miscellaneous contaminants are introduced from exogenous sources. The nature of the purification procedure largely dictates which if any such contaminants might be present in the product

stream. In some instances contaminants introduced during the initial stages of purification may subsequently be removed by one or more of the later purification steps. In other cases contaminants may co-purify with the protein of interest. Under such circumstances it may be necessary to incorporate an additional purification step in order to achieve effective removal of the contaminant(s) concerned. It is particularly important to ensure that all traces of potentially toxic, carcinogenic or otherwise unsafe contaminants are removed from the finished product. Final-product containers of suitable quality should be used in order to reduce or eliminate the risk of leaching of chemical or other substances into the product during storage.

Many chemical contaminants (e.g. buffer components and ligands) are low-molecular-mass compounds. In such instances, gel filtration or ultrafiltration may effectively remove this material. Higher-molecular-mass contaminants are often more difficult to separate from the product. Methods utilized to remove such contaminants may be logically chosen if the physicochemical properties of both contaminant and protein product are known.

In some instances (e.g. if a particular likely contaminant is known to be toxic) it may become necessary to validate the purification system with regard to its ability to effectively remove that contaminant. This is best achieved by spiking a sample of the material at the stage of purification where the putative contaminant is introduced, and subjecting this spike sample to the remainder of the purification steps. An appropriate assay capable of detecting the contaminant in question must be available.

As an additional safety measure finished products are often subjected to 'abnormal toxicity' or 'general safety' tests. Safety tests normally entail the intravenous administration of a dose of up to 0.5 mL of product to at least five healthy mice. The animals are then observed for a period of 48 hours, and should exhibit no adverse symptoms other than the symptoms expected. Death or illness of one or more of the test animals signals further analysis and in such instances the test may be repeated using a larger number of animals. Safety tests are undertaken in order to detect any unexpected or

unacceptable biological activities associated with the product concerned.

### 5.4.6 Labelling and packing of finished products

On filling and sealing in their final containers, all protein products are subsequently labelled and packed. Such operations are generally highly automated and do not require significant technical input. However, labelling is a critical operation in its own right. Mislabelling remains one of the most frequent causes of product recall. Information present on a product label should include the name and strength or potency of the product, batch number, date of manufacture and expiry date, in addition to the storage conditions to be employed. Information detailing the presence of any preservatives or other excipients may also be included, in addition to a brief summary of the correct mode of product usage.

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## Chapter 6

# Therapeutic proteins: blood products, vaccines and enzymes

The human vascular system contains between 5 and 6 L of blood. On average, this accounts for 8.5–9.0% of total body weight. Whole blood consists of red blood cells (which constitute 99% of all blood cells), white blood cells and platelets. These are suspended in a fluid termed plasma. Plasma has a characteristic straw colour, due largely to the presence of bilirubin, a breakdown product of haemoglobin. It may be obtained by centrifugation of whole blood, following the addition of a suitable anticoagulant to freshly drawn blood. The anticoagulant prevents clotting of the blood while the centrifugation step removes the cells suspended in the plasma. If blood is allowed to clot, the clot exudes a fluid termed serum. The clot consists of suspended cellular elements and platelets entrapped or enmeshed in an extensive cross-linked network of fibrin molecules. Fibrin is derived from fibrinogen, a plasma protein. Plasma is essentially serum in which fibrinogen is present.

The various components of blood serve a wide range of physiological functions within the body. Blood is ideally suited to play a transportational role due to the extensive network formed by the vascular system. Blood functions to transport a wide range of substances within the body, such as nutrients, waste products, gases, antibodies, enzymes, parenterally

administered substances, hormones and other regulatory factors. Blood also plays a vital role in several additional physiological processes, such as maintenance of tissue hydration levels and regulation of body temperature.

Many specific functions of blood are carried out by proteins found in plasma. Electrophoretic separation of plasma proteins reveals five major bands, referred to as albumin,  $\alpha_1$ -globulins,  $\alpha_2$ -globulins,  $\beta$ -globulins and  $\gamma$ -globulins. Serum albumin represents the most abundant protein, accounting for more than 50% of total plasma protein. All globulin protein fractions contain a variety of different protein molecules.

## 6.1 Blood products

### 6.1.1 Whole blood and blood plasma

Whole blood is aseptically collected from human donors and is immediately mixed with an anticoagulant to prevent clotting. Suitable anticoagulants include heparin and sodium citrate. Whole blood

may be used as the source of a variety of blood constituents:

- red blood cells;
- platelets;
- various clotting factors;
- immunoglobulins;
- additional plasma constituents;

The use of whole blood for such purposes would be wasteful if the specific purified component required is already available. Furthermore, fractionation procedures used to produce specific purified blood products considerably reduce the risks of accidental transmission of disease from contaminated blood donations.

Blood obtained from donors must be screened for the presence of a variety of likely pathogenic contaminants, particularly hepatitis B and C viruses and human immunodeficiency virus (HIV). Whole blood is normally administered to patients following severe blood loss. Concentrated red blood cell fraction and plasma-reduced blood is also available, and may be administered for certain clinical conditions.

Blood plasma is prepared from whole blood by centrifugation and is then normally stored frozen until use. Plasma is normally employed clinically as a source of therapeutically important plasma proteins such as clotting factors, when the purified products are unavailable. Yet another widely employed derivative of whole blood is termed plasma protein solution or plasma protein fraction. This aqueous solution is normally prepared by limited fractionation of serum or plasma and consists predominantly of serum albumin with minor quantities of globulin proteins. Plasma protein solution is administered in cases of shock caused by a large decrease in the volume of blood. Such sudden blood loss may be a result of internal or external bleeding, extensive burns or dehydration.

### **6.1.2 Blood-derived proteins**

Specific blood proteins used therapeutically include a range of factors involved in the blood clotting

process, fibrinolytic agents that degrade clots, serum albumin and immunoglobulin preparations. Most such proteins have been commercially available for many years. While these products have traditionally been extracted or purified from blood donated by human volunteers, most are now produced by recombinant DNA technology.

#### **6.1.2.1 Blood coagulation factors: biochemistry and function**

A variety of plasma proteins form an integral part of the blood clotting process. A genetic or induced deficiency in any one blood factor results in severely impaired coagulation ability, with serious medical consequences. The vast majority of hereditary diseases characterized by poor coagulation responses result from a deficiency of blood factors VIII or IX. A variety of other non-hereditary clinical conditions, such as vitamin K deficiency, may also result in impairment of the blood coagulation process.

When a blood vessel is damaged or cut, specific elements in blood initiate the process of haemostasis – the curtailment and eventual cessation of blood loss. Haemostasis ultimately depends on two interdependent physiological processes: (i) the formation of a platelet plug and (ii) the blood coagulation process. Haemostasis is characterized by the rapid attachment of platelets to the damaged area. Platelets also adhere to each other, and in this way often stem blood flow. The congregated platelets also secrete a variety of amines such as adrenaline, which stimulate localized constriction of the blood vessels. The process of blood coagulation is also initiated, resulting in the formation of a blood clot (thrombus) at the site of damage.

Coagulation depends on a number of clotting factors found naturally in the blood. Such factors are designated by roman numerals, although each is also known by a common name. Blood factors are listed in Table 6.1. With the exception of factor VI (calcium ions), all other factors are protein-based. Many of the blood factors (e.g. factors II, VII, IX, X, XI and XII) exhibit proteolytic activity on activation. The unactivated factors are therefore protease zymogens. Activated factors catalyse the proteolytic cleavage of another factor in the clotting sequence,

**Table 6.1** Blood clotting factors.

Factor number	Factor name
Factor I	Fibrinogen
Factor II	Prothrombin
Factor III	Thromboplastin (tissue factor)
Factor IV	Calcium
Factor V	Labile factor (proaccelerin)
Factor VI	*
Factor VII	Proconvertin
Factor VIII	Antihaemophilic factor
Factor IX	Christmas factor (plasma thromboplastin component)
Factor X	Stuart factor
Factor XI	Plasma thromboplastin antecedent
Factor XII	Hageman factor
Factor XIII	Fibrin stabilizing factor

Activated forms of the above factors are designated by the addition of the letter 'a' to the factor number, e.g. factor VIIa represents activated factor VII. \* The protein originally termed factor VI was later discovered to be factor Va, thus factor VI is now unassigned.

thus resulting in activation of this next factor. The clotting sequence is therefore a molecular cascade in which sequential activation of clotting factors is observed. Each single activated molecule will in turn catalyse the activation of numerous molecules of the next factor in the sequence. This results in considerable stepwise amplification of the initial signal.

Not all the clotting factors listed in Table 6.1 exhibit proteolytic activity. However, these non-proteolytic accessory factors (factors III, IV, V and VIII) form essential components of the coagulation system. They are generally activated by one of the proteolytic factors, and on activation serve to enhance the rate of activation of other blood factor zymogens. The presence of certain phospholipid components released from damaged tissue or from platelets also serves to accelerate the rate of coagulation.

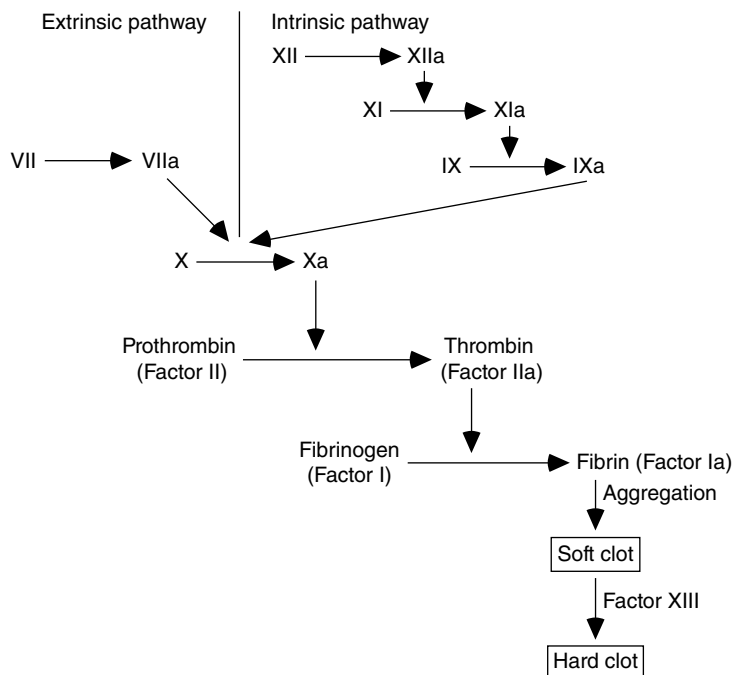
Two distinct blood coagulation pathways exist: the intrinsic and extrinsic pathways. The extrinsic pathway relies on factors normally present in plasma. This pathway is initiated when factor VII is activated through contact with injured surfaces.

Functioning of the extrinsic pathway requires, in addition to blood factors, the presence of tissue factor (factor III). Tissue factor, along with calcium (factor IV), factor VII and phospholipid, greatly stimulates activation of factor X. Tissue factor is an accessory protein present in a wide range of tissue types. It is particularly abundant in saliva, in addition to lung and brain, and is an integral membrane glycoprotein. Tissue factor is released on tissue damage, along with phospholipid components of the membranes. Hence it can initiate the extrinsic coagulation cascade at the site of damage. The clotting process occurs most rapidly if initiated via this pathway. While the initial activation sequence of these pathways differ, the terminal sequence of events is identical in both cases (Figure 6.1). These final (common) steps of the coagulation cascade involve the conversion of prothrombin (factor II) to thrombin (factor IIa). This proteolytic reaction is catalysed by activated Stuart factor (Xa).

Thrombin catalyses the proteolytic cleavage of soluble fibrinogen yielding insoluble fibrin. Fibrin monomers subsequently interact forming a fibrin clot. Initially this interaction is of a non-covalent nature, yielding a large aggregated 'soft' clot. This soft clot is subsequently converted into a hard clot by factor XIII, also known as fibrin stabilizing factor. Activated factor XIII catalyses the formation of covalent cross-links between a lysine residue of one fibrin monomer and a glutaminyl residue of an adjacent fibrin molecule. Factor XIII is itself activated by thrombin in the presence of calcium.

### 6.1.2.2 Haemophilia A and B

Genetic defects that significantly decrease the level of production, or alter the amino acid sequence, of any blood factor may result in serious illness. Such illness is characterized by poor coagulation, with resulting prolonged haemorrhage. Defects in all clotting factors with the exception of tissue factor, calcium and phospholipid have been documented. Well in excess of 90% of all such defects relate to a deficiency of factor VIII. Many of the remaining cases are due to a deficiency of factor IX. The clinical disorders associated with deficiencies of factors



**Figure 6.1** Simplified version of the intrinsic and extrinsic blood coagulation pathways.

VIII or IX include haemophilia A, von Willebrand disease and haemophilia B.

Factor VIII complex consists of two separate gene products. The smaller (170 kDa) polypeptide exhibits coagulant activity and is often designated VIII:C. This polypeptide is coded for by the *FVIII* gene. The larger polypeptide, designated von Willebrand factor (VIII:vWB), is predominantly associated with platelet adhesion. This factor is coded for by the *VWF* gene. On synthesis, individual von Willebrand factor polypeptides polymerize forming large multimeric structures. The product of the *FVIII* gene (VIII:C polypeptide) then associates with the multimeric VIII:vWB protein, forming the overall complex structure VIII:C–VIII:vWB, which may be co-purified from plasma. This overall structure displays a molecular mass in excess of 1 MDa, approximately 15% of which is carbohydrate.

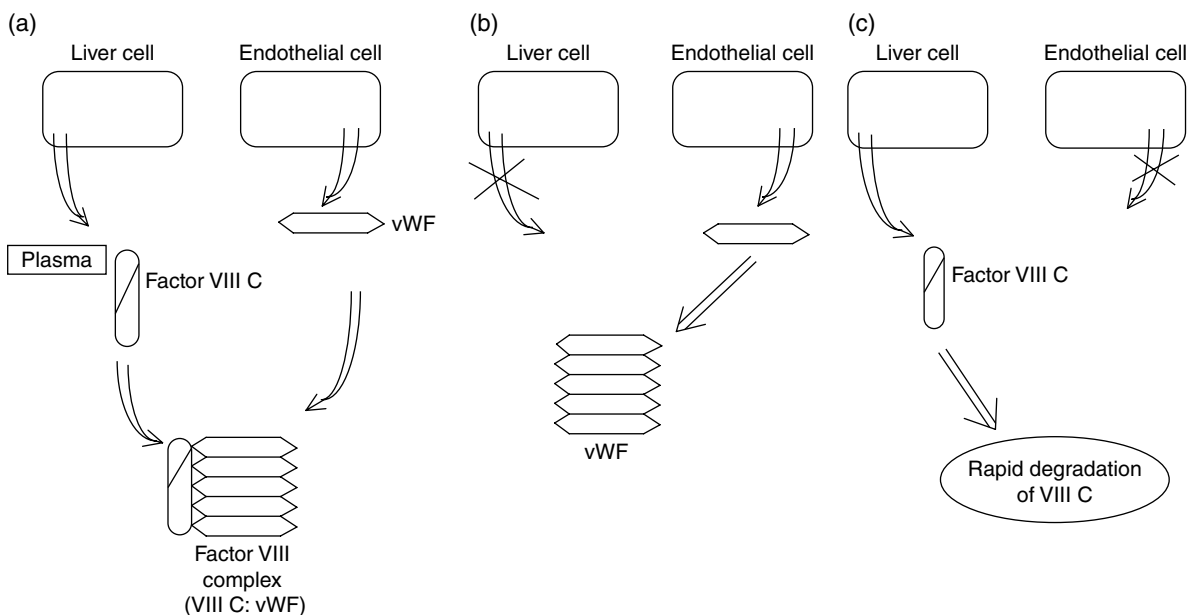
Failure to synthesize VIII:C results in classical haemophilia (haemophilia A), while failure to synthesize VIII:vWB results in von Willebrand disease. In the case of haemophilia A, the VIII:vWB gene product is synthesized as usual, although von Willebrand disease is characterized by absence of

both factors VIII:C and VIII:vWB. Patients suffering from von Willebrand disease actually synthesize normal factor VIII:C, although this polypeptide is rapidly degraded as its stabilization requires association with the VIII:vWB polypeptide (Figure 6.2). Haemophilia B, also known as Christmas disease, results from a deficiency of factor IX. Its clinical consequences are identical to those of classical haemophilia but it does not occur as frequently as the latter disease (see also Box 6.1).

The nature and severity of the clinical features of haemophilia depend on the level of the factor in the plasma. Patients with very low levels (<1% of normal quantity) of factor VIII:C or factor IX are likely to experience frequent and spontaneous bouts of bleeding. Persons with higher levels of active factor (3–5% or above) experience less severe clinical symptoms.

### 6.1.2.3 Production of clotting factors for medical use

Management of the bleeding disorders is normally attained by administration of concentrates of the relevant deficient factor. Factors VIII and IX were



**Figure 6.2** (a) Synthesis of factor VIII complex as occurs in healthy individuals. In the case of persons suffering from haemophilia A, synthesis of factor VIII:C is blocked (b), preventing constitution of an active factor VIII complex in plasma. Persons suffering from von Willebrand disease fail to synthesize vWF (c). Although they can synthesize VIII:C, this is rapidly degraded on entering the blood due to lack of its vWF stabilizing factor.

traditionally purified by suitable fractionation techniques from plasma obtained from healthy human donors. Factor IX, when purified by traditional fractionation procedures (mainly precipitation steps), usually contains appreciable quantities of factors II, VII, X and XI. This preparation may therefore also be used in clinical cases where deficiency of one of these additional factors is observed.

More recently, plasma-derived factor VIII preparations have been purified to a greater extent using high-resolution chromatographic techniques, including immunoaffinity chromatography. However produced, the final product is normally sterilized by filtration, filled into final containers and freeze-dried. The containers are sealed under vacuum or under an oxygen-free nitrogen atmosphere in order to minimize the possibility of oxidative deterioration of the product. Anticoagulants such as heparin or sodium citrate are usually present in the final preparation, although such preparations do not contain antibacterial agents or other preservatives.

The importance of utilizing plasma free of viral contaminants in the preparation of blood products cannot be overemphasized. In addition to screening blood donations, several other approaches may be adopted in order to further reduce the likelihood of accidental transmission of infectious agents. Such approaches include addition of an antiviral substance to the final-product preparation or heat inactivation treatment. Purified/partially purified preparations are also less likely to contain potential pathogens as compared to whole plasma due to chromatographic resolution.

#### *Recombinant blood factors*

Recombinant DNA techniques facilitate the production of recombinant blood factors. To date several such products have gained regulatory approval for medical use (Table 6.2). Recombinant production overcomes any potential problems of source availability (due to inadequate supplies of blood donations). However, the major advantage of recombinant production is the virtual elimination of the risk of accidental transmission of blood-borne diseases. As most

**Box 6.1 Case study: production of BeneFix, a recombinant coagulation factor IX**

Haemophilia B is caused by a mutation in the factor IX gene that very significantly reduces expression of biologically active factor IX protein. The condition occurs almost exclusively in males as the factor IX gene is located on the X chromosome. Its incidence ranges between 1 in 25,000 and 1 in 30,000 live male births. Factor IX is a single-chain, 415 amino acid, 55-kDa glycoprotein composed of five structural domains: a 'γ-carboxyglutamate' (Gla) domain, two 'epidermal growth factor-like' domains, an 'activation peptide' (AP) domain and a 'serine protease' domain.

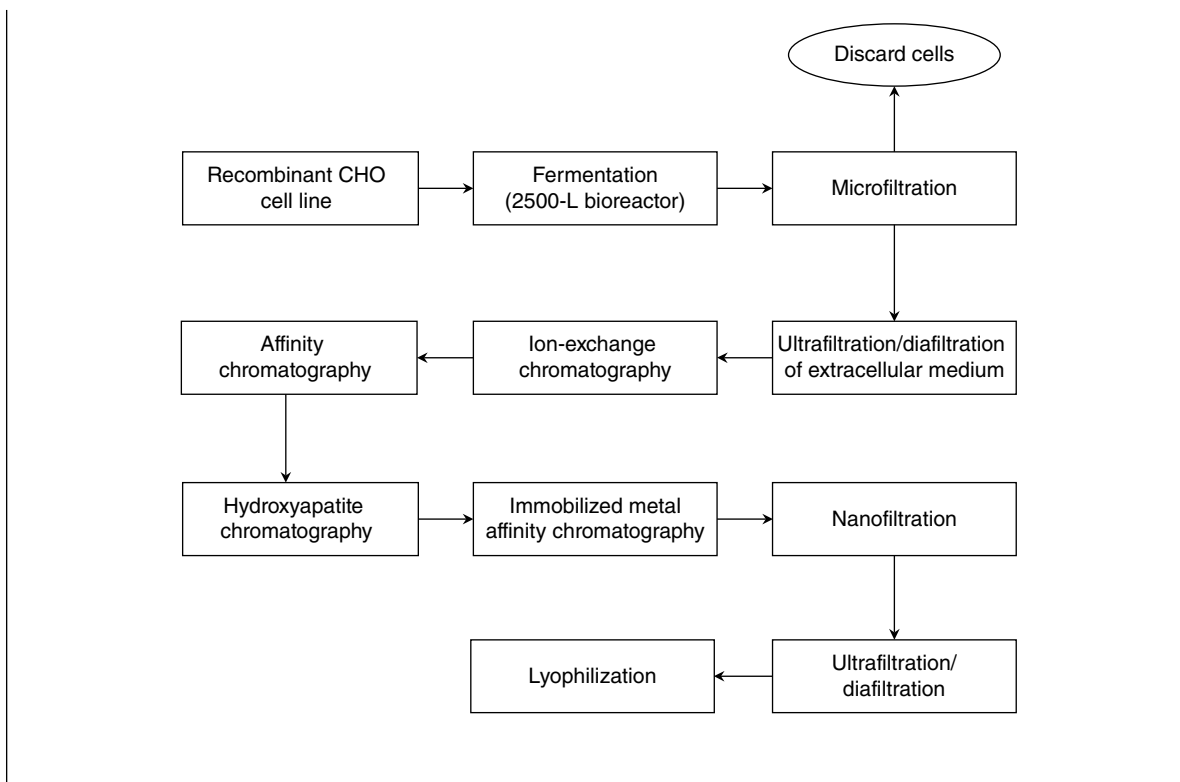
The mature protein displays multiple post-translational modifications. The Gla domain houses 12 glutamate residues which are normally γ-carboxylated, and the protein also houses a total of seven potential glycosylation sites. Factor IX is a zymogen, converted *in vivo* into its enzymatically active form by proteolytic cleavage at two sites (Arg<sup>145</sup> and Arg<sup>180</sup>). This cleavage yields activated factor IX (IXa), a heterodimer held together by a disulfide linkage, and a 35-residue peptide.

BeneFix is a recombinant factor IX product marketed by Wyeth. It is produced in an engineered CHO cell line containing multiple copies of an expression vector that houses a nucleotide sequence coding for the human protein. The manufacturing process (summarized schematically below) traditionally involves culture of the CHO cells in a 2500-L bioreactor for 3 days (until high cell densities are attained). Intact cells are then removed from the (product-containing) extracellular media by microfiltration. The media is next subjected to an ultrafiltration (concentration) step, followed by diafiltration (to ensure the product's presence in a suitable processing buffer). High-resolution purification is achieved using a combination of four successive chromatographic steps:

1. ion exchange (Q Sepharose);
2. an affinity step using Matrex cellulose sulfate (a heparin analogue used for affinity purification of proteins with a heparin-binding domain and which also partially acts via an ion-exchange mechanism, due to the negatively charged sulfate groups);
3. a hydroxyapatite chromatographic step;
4. an immobilized metal affinity step, using copper (II) as the immobilized metal ion.

A nanofiltration step is also undertaken using a membrane filter displaying a 70-kDa cut-off point. This step is included largely as an added layer of viral safety. The 55-kDa product passes through the membrane, which would retain any potential viruses present in the product stream. A final ultrafiltration/diafiltration step is undertaken in order to concentrate the purified product and achieve buffer exchange (i.e. place the product in its final formulation buffer) before product fill. Lyophilization is then undertaken. The formulation buffer contains as excipients: histidine (as a buffer), polysorbate-80 (protects the protein from damage during freezing), sucrose (stabilizes/protects the protein in the freeze-dried state), and glycine (helps produce a high-quality freeze-dried 'cake'). The final product displays a shelf-life of at least 2 years when stored at 2–8°C.

Final-product purity is assessed (confirmed) by SDS-PAGE, size exclusion HPLC, reverse-phase HPLC and N-terminal sequencing. Product identity is confirmed by bioassay, degree of electrophoretic mobility, peptide mapping, carbohydrate fingerprinting and determination of the molecule's Gla content. The bioassay, which is also used to determine final product potency, is based on a clotting assay using factor IX-deficient plasma. In total over 150 tests are performed on each batch of product before its release for sale.



**Table 6.2** Recombinant blood clotting factors that have gained approval for general medical use in the EU and/or the USA. Most are also approved for use in various other world regions.

Product	Company	Therapeutic indication	Approved
Benefix (recombinant human factor IX produced in CHO cells)	Wyeth	Haemophilia B	1997 (USA and EU)
Kogenate FS (recombinant human factor VIII produced in BHK cells. Sold as Helixate NexGen in EU)	Bayer	Haemophilia A	2000 (USA and EU)
NovoSeven (recombinant human factor VIIa, produced in BHK cells)	Novo-Nordisk	Some forms of haemophilia	1995 (EU) 1999 (USA)
NovoThirteen (Catridecog; recombinant blood factor XIII, a subunit dimer, produced in <i>S. cerevisiae</i> )	Novo	Bleeding in patients with factor XIII deficiency	2012 (EU)
Recombinate (recombinant human factor VIII, produced in a CHO cell line)	Baxter Healthcare	Haemophilia A	1992 (USA)
ReFacto (moroctocog-alfa, i.e. B-domain deleted recombinant human factor VIII, produced in CHO cells)	Pfizer	Haemophilia A	1999 (EU) 2000 (USA)
Advate (octocog alfa; recombinant human factor VIII, produced in CHO cells)	Baxter AG	Haemophilia A	2004 (EU)
Recothrom (thrombin, topical, recombinant; recombinant human thrombin expressed in CHO cells)	Zymogenetics	Control of minor bleeding during surgery	2008 (USA)
Xyntha (antihæmophilic factor; recombinant human factor VIII expressed in CHO cells)	Wyeth	Hemophilia A	2008 (USA)

blood factors display post-translational modifications (glycosylation, proteolytic processing and, in some cases,  $\gamma$ -carboxylation), eukaryotic expression systems are used. Products thus far approved are produced in engineered CHO cells (e.g. BeneFix and ReFacto) or BHK cells (e.g. NovoSeven). After initial recovery from the production system, recombinant blood factors are subject to a number of chromatographic purification steps in order to yield a purified product, which is usually marketed in lyophilized format. An overview of the production on one such product (BeneFix) is presented in Box 6.1.

Recothrom, a recombinant form of thrombin (blood factor IIa) produced in an engineered CHO cell line, has been approved in more recent times for the control of minor bleeding during surgery (Table 6.2). The product is unusual in that it is applied topically, directly on the surface of the bleeding tissue (with very few exceptions, therapeutic proteins are administered by injection). Thrombin is a serine protease that functions at the terminal stage of the normal blood clotting process (Figure 6.1). Its inactive circulating zymogen (prothrombin, factor II) is a 582 amino acid, 72.5-kDa glycoprotein containing six  $\gamma$ -carboxyglutamate residues towards its N-terminal end. It is proteolytically activated during the blood coagulation cascade by factor Xa and the active thrombin molecule consists of two polypeptides linked via a single disulfide linkage. The smaller polypeptide contains 49 amino acids while the larger contains 259 amino acids.

The recombinant molecule is synthesized and released from the producing CHO cell line in zymogen form. Downstream processing steps include its proteolytic activation, multiple chromatographic purification steps and a solvent-detergent treatment step as well as nanofiltration as viral clearance steps. The finished product is then lyophilized.

## 6.2 Anticoagulants

Anticoagulants function to prevent blood from clotting. They are used clinically in cases where a high risk of blood clot formation is diagnosed and are

also utilized to prevent the formation of further clots. Anticoagulants are thus often administered to patients who have suffered heart attacks, strokes or deep vein thrombosis in an effort to prevent recurrent episodes.

Thrombosis refers to the formation of blood clots. The blood clot itself is termed a thrombus. Thrombosis will most readily occur within diseased blood vessels. The formation of a thrombus in an artery will obstruct blood flow to the tissue it normally supplies. Formation of a thrombus in the coronary artery (coronary thrombosis) will obstruct blood flow to the heart muscle. This results in a heart attack, which is usually characterized by death, or 'infarction', of part of the heart muscle, hence the term myocardial infarction. The presence of thrombi that arrest blood flow to brain tissue usually results in a stroke. Furthermore, on its formation, a thrombus or part of a thrombus may become detached and travel in the blood, only to be lodged in another blood vessel. This obstructs blood flow at that point. This process is termed 'embolism', and may also induce heart attacks or strokes. Deep vein thrombosis refers to the formation of thrombi in the deep veins of the leg. It can occur in normal, apparently healthy veins, but risk factors include surgery, immobility, advanced age and pregnancy. Deep vein thrombi display a tendency to form emboli. Some anticoagulants of therapeutic value are listed in Table 6.3.

### 6.2.1 Traditional anticoagulants

Heparin is a glycosaminoglycan-based anticoagulant. It consists of sulfated polysaccharide chains of varying length, with molecular masses generally ranging between 3000 and 40,000 Da. It is synthesized and stored in many body tissues, especially in lung, liver, intestinal cells and cells lining blood vessels. Heparin preparations available commercially are normally prepared from porcine intestinal mucosa or from beef lung. This glycosaminoglycan exerts its anticoagulant activity by binding and thus activating antithrombin III, an  $\alpha_2$ -globulin of molecular mass 60 kDa which



**Table 6.3** Anticoagulants which are used therapeutically.

Anticoagulant	Structure	Source	Molecular mass (Da)
Heparin	Glycosaminoglycan	Beef lung, pig gastric mucosa	3000–40,000
Dicoumarol	Coumarin-based	Chemical manufacture	336.3
Warfarin	Coumarin-based	Chemical manufacture	308.4
Hirudin	Polypeptide	Recombinant production	7000
Antithrombin	Polypeptide	Recombinant production	58,000

is found in plasma. Activated antithrombin III inhibits a variety of activated blood factors, including IIa, IXa, Xa, XIa and XIIa. Thus heparin, released naturally *in vivo* or administered therapeutically, inhibits the blood coagulation cascade. Administration of inappropriately large doses of heparin may result in severe haemorrhage. The anticoagulant activity of heparin is related to the molecular mass of the polysaccharide molecules. This fact has facilitated the development of low-molecular-mass heparins that retain anticoagulant ability while exhibiting decreased haemorrhagic effects.

Dicoumarol and warfarin are coumarin-based anticoagulants. Both may be administered orally. These vitamin K antimetabolites exert their anticoagulant effect by inhibiting the vitamin K-dependent  $\gamma$ -carboxylation of coagulation factors II, VII, IX and X. The  $\gamma$ -carboxylation of such factors is essential if they are to bind calcium ions, as is required during the normal coagulation process. The major adverse effects of administration of dicoumarol or warfarin is, predictably, the possibility of severe haemorrhage.

### 6.2.2 Hirudin

The buccal secretion of leeches contains an anticoagulant termed hirudin. Components present in the saliva of leeches do not participate in the digestive process but function primarily to interact with, and inhibit, the host animal's haemostatic mechanism. Leech bites are thus characterized by subsequent prolonged bleeding, often lasting several hours.

Leeches have been used medically for centuries as vehicles to promote bloodletting, as well as in instances where localized anticoagulant activity was required. Indeed the leech has enjoyed somewhat of a medical comeback in some regions of the world, where it is sometimes employed to remove blood from inflamed areas and in procedures associated with plastic surgery.

The saliva of leeches contains a variety of peptides. Hirudin is the major anticoagulant present in the saliva of the European leech (*Hirudo medicinalis*). Hirudin was first reported in the 1880s, though its characterization was not undertaken until the 1950s. The hirudin gene was cloned in the mid-1980s and has subsequently been expressed in a number of host systems. The polypeptide consists of 65 amino acids and has a molecular mass of 7 kDa. The molecule contains a sulfated tyrosine residue at position 63 and is also characterized by a high content of acidic amino acids towards the C-terminal end. The overall conformation of the molecule consists of a globular domain stabilized by three intramolecular disulfide bridges, and an elongated C-terminal region.

The anticoagulant activity of hirudin stems from its ability to bind thrombin (factor IIa) tightly. This results in inactivation of the thrombin molecule. Thrombin not only catalyses the proteolytic cleavage of fibrinogen thus forming fibrin and hence promoting clot formation, but also plays a role in activation of factors V, VIII and XIII. Binding of hirudin to thrombin masks both thrombin's fibrinogen-binding site and its catalytic site.

Although the natural anticoagulant activity of hirudin had been recognized for quite some time,

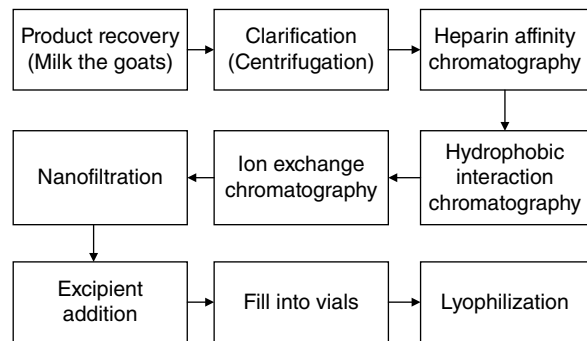
the lack of adequate quantities rendered its widespread clinical use impractical. Hirudin appears to have a number of therapeutic advantages over some other anticoagulants:

- it acts directly on thrombin;
- it does not require a cofactor to exert its inhibitory effect;
- high doses are less likely to promote haemorrhage;
- it is a particularly weak immunogen.

Hirudin produced by recombinant means is now available and two hirudin-based products (trade names Revasc and Repludan) are approved for general medical use. Both products are produced in *Saccharomyces cerevisiae* strains transformed with an expression plasmid housing a synthetic nucleotide sequence coding for hirudin. The products are secreted by the yeasts into the fermentation medium, from where they are recovered and purified. Both products display identical biological activity to that of native hirudin. Their structures differ from the native molecule only by the absence of a sulfate group on Tyr<sup>63</sup>, though they retain anticoagulant activity.

### 6.2.3 Antithrombin

Human antithrombin (AT) is a plasma glycoprotein synthesized and stored in the vascular endothelium (the cells lining the interior surface of blood vessels) and is normally present in blood at levels in the region of 125 µg/mL. AT is one of the most significant natural inhibitors of blood coagulation and exerts its anticlotting effect by complexing with (and hence directly inhibiting) several blood clotting factors, most notably thrombin (IIa) and factor Xa. The complex is then rapidly cleared from circulation via internalization and degradation in liver cells. In addition to a coagulation factor-binding domain, AT displays a heparin-binding domain and binding of heparin greatly accelerates the rate of complex formation (heparin is normally coadministered with the product).



**Figure 6.3** Overview of the likely mode of manufacture of ATryn, a recombinant antithrombin produced in the milk of transgenic goats.

Hereditary AT deficiency is a rare genetic disorder categorized by either a decrease in absolute serum AT levels or by the presence of mutated (dysfunctional) forms of the molecule. The major clinical consequence is an increase in the risk of clot formation in deep veins, pulmonary embolism as well as venous clot formation. Particularly high-risk circumstances include surgery, bed rest and pregnancy.

ATryn (antithrombin alfa) is a recombinant form of human AT produced by transgenic goats in their milk. The 432 amino acid, single-chain protein is, like the native human molecule, glycosylated. It harbours four N-linked glycosylation sites (Asn 96, 135, 155 and 192) as well as three disulfide linkages. ATryn is approved for general medical use in Europe for the treatment of certain thromboembolic events during surgery of patients with congenital AT deficiency. An outline of its likely mode of manufacture is presented in Figure 6.3. At the core of downstream processing are four high-resolution chromatographic steps, as well as nanofiltration, included as a precautionary viral removal step. Glycine, sodium chloride and sodium citrate are added as excipients.

## 6.3 Thrombolytic agents

Thrombolytic agents, as the name suggests, trigger the lysis or degradation of blood clots. They are administered medically as soon as possible

after inappropriate blood clot formation, and are mainly used to minimize the damage caused by heart attacks, strokes, deep vein thrombosis or embolisms.

### 6.3.1 The fibrinolytic system

Fibrinolysis forms an intrinsic part of the natural wound healing process (Figure 6.4). This process refers to the enzymatic degradation and removal of blood clots from the circulatory system. The process is largely mediated by the serine protease plasmin. Plasmin catalyses the enzymatic degradation of the fibrin strands present in the clot. Plasmin is derived from plasminogen, its circulating zymogen. Human plasminogen is a 90-kDa glycoprotein synthesized in the kidney. Plasminogen consists of a single polypeptide chain and contains numerous intrachain disulfide linkages. Two natural forms exist which differ in carbohydrate content.

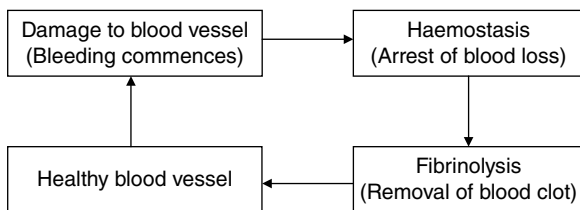
Plasminogen may be activated by a variety of specific serine proteases, yielding active plasmin. Tissue plasminogen activator (tPA) represents the most important physiological activator of plasminogen (Figure 6.5). tPA, also referred to as fibrinokinase, is a

527 amino acid, 70-kDa glycoprotein displaying serine protease activity. It activates plasminogen by cleaving a single Arg–Val bond. tPA found in human plasma is predominantly formed in the vascular endothelium. Two forms of tPA may be purified. Type I tPA is a single-chain polypeptide, whereas type II consists of two polypeptide chains connected by a disulfide linkage. Type II tPA is derived from type I by proteolytic cleavage.

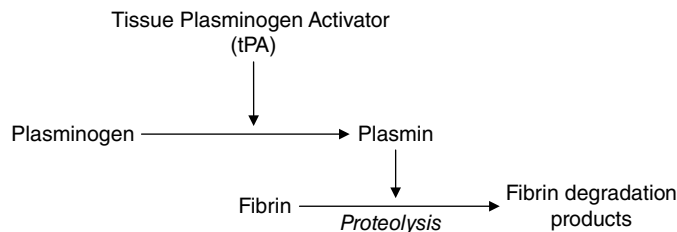
Fibrin contains binding sites for both plasminogen and tPA. Activation of plasminogen by tPA forming plasmin thus occurs most efficiently on the surface of blood clots. Any active plasmin found free in blood is quickly inactivated by another plasma protein termed  $\alpha_2$ -antiplasmin.

### 6.3.2 Tissue plasminogen activator-based products

Human tPA preparations produced by recombinant DNA have been available commercially since the late 1980s (Table 6.4). Activase was the first recombinant tPA product to be approved for therapeutic use. It is produced in engineered CHO cells which harbour a cDNA sequence coding for natural human tPA. Because of its short plasma half-life (about 2 minutes), Activase must be administered via infusion, usually over periods of up to 90 minutes. Subsequent to its approval, several engineered tPAs displaying an extended plasma half-life (thereby allowing administration via direct injection) have been developed and approved. tPA displays five distinct structural domains whose functional attributes have been determined via



**Figure 6.4** Simplified representation of wound healing.



**Figure 6.5** The fibrinolytic system, as triggered naturally by tissue plasminogen activator (tPA).

**Table 6.4** Recombinant tPA-based products that have gained approval for general medical use in the EU and/or the USA. Most are also approved for use in various other world regions.

Product	Company	Therapeutic Indication	Approved
Activase (alteplase, recombinant human tPA produced in CHO cells)	Genentech	Acute myocardial infarction	1987 (USA)
Ecokinase (reteplase, recombinant tPA; differs from human tPA in that three of its five domains have been deleted, produced in <i>E. coli</i> )	Galenus Mannheim	Acute myocardial infarction	1996 (EU), withdrawn in 2000
Retavase (reteplase, recombinant tPA; same as Ecokinase)	Boehringer Mannheim/Centocor	Acute myocardial infarction	1996 (USA)
Rapilysin (reteplase, recombinant tPA; same as Ecokinase)	Actavis	Acute myocardial infarction	1996 (EU)
Tenecteplase (also marketed as Metalyse; TNK-tPA, modified; produced in CHO cells)	Boehringer Ingelheim	Acute myocardial infarction	2001 (EU), withdrawn in 2005
TNKase (modified recombinant tPA; same as tenecteplase)	Genentech	Acute myocardial infarction	2000 (USA)

**Table 6.5** The five structural domains of native tPA and the biological function of each domain.

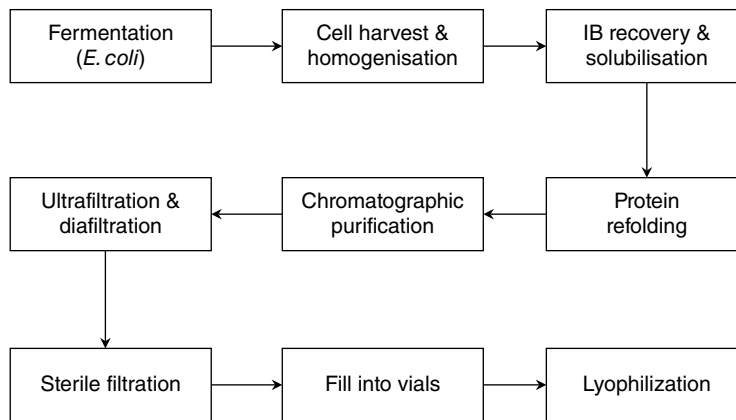
Domain	Function
Protease domain (P)	Displays plasminogen-specific proteolytic activity
Kringle-2 domain ( $K_2$ )	Facilitates fibrin-based stimulation of proteolytic activity
Finger domain (F)	Promotes high-affinity binding to fibrin
Epidermal growth factor domain (EGF)	Mediates hepatic clearance from blood by binding to specific hepatic receptors
Kringle-1 domain ( $K_1$ )	Associated with hepatic binding

mutational studies (Table 6.5). Additionally, native tPA harbours four potential glycosylation sites, three of which are actually glycosylated and the carbohydrate component is associated with rapid clearance from the bloodstream.

Reteplase (sold under the trade names Ecokinase, Retavase and Rapilysin) is an engineered domain-deleted variant of native tPA consisting only of the catalytic (P) and kringle 2 ( $K_2$ ) domains. The engineered nucleotide construct is expressed in *Escherichia coli*, so the product is also unglycosylated. The P and  $K_2$  domains provide the engineered molecule with catalytic activity against fibrin. The absence of the EGF and  $K_1$  domains, along with the glycocomponent, confers a significantly

extended plasma half-life on the molecule (19 minutes), facilitating its convenient clinical administration via a single intravenous injection. The manufacture of Rapilysin is overviewed in Figure 6.6.

TNKase (Tenecteplase/Metalyse) is yet another engineered tPA displaying an extended half-life in blood (Table 6.4). Manufactured in a CHO cell line by Genentech, TNKase differs from native tPA by displaying amino acid substitutions at three positions. The effects of these substitutions are (i) to increase specificity for fibrin binding, which has a knock-on effect of decreasing plasma clearance, and (ii) enhancing resistance to plasminogen activator inhibitor-1, a natural tPA inhibitor.



**Figure 6.6** Overview of the production of Rapilysin, a modified tPA produced in recombinant *E. coli* cells. IB, inclusion body.

## 6.4 Additional blood-related products

In addition to proteins involved in the promotion or dissolution of blood clots, several other serum proteins are of considerable real or potential therapeutic value. Examples include human serum albumin (HSA),  $\alpha_1$ -antitrypsin and, of course, immunoglobulins (antibodies). Antibodies are considered in Chapter 7, while HSA and  $\alpha_1$ -antitrypsin are considered here.

### 6.4.1 Human serum albumin

At typical concentrations of 42 g/L, serum albumin constitutes the most abundant protein present in serum, representing approximately 60% of total plasma protein. It is also one of the smallest known plasma proteins, with a molecular mass of approximately 69 kDa. It is one of a small number of plasma proteins devoid of a carbohydrate moiety. The protein is synthesized in the liver as preproalbumin. Removal of several amino acid residues from its amino-terminus during passage through the endoplasmic reticulum yields mature albumin, which consists of 585 amino acids. The albumin molecule exhibits several intrachain disulfide linkages. It

consists of three similar domains (I, II and III) and is 67%  $\alpha$ -helical in structure.

A major function of albumin is to provide most of the natural osmotic pressure of plasma. It also plays an important transportational function and is especially important in transporting sparingly soluble substances in aqueous media within the body. Most free fatty acids bind tightly to albumin and are transported in plasma in this manner.

Very little albumin is found in the urine of healthy individuals. Certain medical conditions, especially some forms of kidney disease, are characterized by secretion of large quantities of serum albumin into the urine. Some forms of liver disease may also result in significant decreases in hepatic protein synthesis, with a resultant marked reduction in the concentration of several plasma proteins, most notably albumin. Measurement of albumin concentrations in biological fluids can therefore have diagnostic value.

Aqueous solutions of HSA are available commercially. These usually range in concentration from 5 to 25%. HSA preparations are administered to patients suffering from some forms of kidney or liver disease and is also used as a plasma volume expander for patients suffering from shock as a result of a decrease in blood volume. Such decreases are often associated with surgery or occur subsequent to serious injury.

Albumin is normally purified from serum, plasma or placenta obtained from healthy donors. All raw materials are screened for the presence of viral or other potentially pathogenic organisms prior to purification. The methods of purification utilized (precipitation and chromatography) must yield product which is at least 95% pure. Like most proteinaceous preparations the purified albumin is sterilized by filtration and subsequently aseptically filled into sterile containers. Although no preservatives are added, stabilizers such as sodium caprylate that protect the product, particularly against the effects of heat, are normally included in the final product. Subsequent to final filling, the product is subjected to a heat step that promotes inactivation of any pathogens which may be present. This normally involves heating to 60°C for a period of up to 10 hours.

HSA has also been produced as a heterologous product in a number of recombinant systems, including *E. coli*, *Bacillus subtilis*, *Saccharomyces cerevisiae*, *Pichia pastoris* and *Aspergillus niger*. However, HSAs relatively large size, as well as the presence of so many disulfide bonds, can complicate recombinant production of high levels of correctly folded products. However, the main stumbling block in replacing native HSA with a recombinant version is an economic one. Unlike most biopharmaceuticals, HSA can be produced in large quantities and inexpensively by direct extraction from its native source. Native HSA currently sells at \$3–5 per gram. Recombinant HSA products will find it difficult to compete with this price.

### 6.4.2 $\alpha_1$ -Antitrypsin

$\alpha_1$ -Antitrypsin is a 52-kDa serum glycoprotein. The protein consists of 394 amino acid residues and contains three glycosylation sites. The  $\alpha_1$ -antitrypsin gene is located on the distal long arm of chromosome 14.  $\alpha_1$ -Antitrypsin is synthesized in the liver and constitutes over 90% of the  $\alpha_1$ -globulin band observed on electrophoresis of serum. It is normally present in serum at a concentration of 2 g/L. A number of genetic variants of the protein have been described.

$\alpha_1$ -Antitrypsin constitutes the major serine protease inhibitor present in mammalian serum. It

serves as a potent inhibitor of the protease elastase and as such prevents damage to lung tissue by neutrophil elastase. Neutrophils are a particular form of granulocyte, a type of white blood cell. Genetic deficiencies resulting in the absence of  $\alpha_1$ -antitrypsin in human plasma have been described. These deficiencies are particularly prevalent in persons of northern European descent, with the disorder affecting in the region of 1 in 2500 such individuals. Persons suffering from such deficiencies often develop life-threatening emphysema. This results from unchecked damage to lung tissue by neutrophil elastase. Replacement therapy with  $\alpha_1$ -antitrypsin delivered by intravenous infusion may arrest progression of this disease.

$\alpha_1$ -Antitrypsin preparations used medically are normally obtained from pooled plasma fraction. Manufacture normally entails an ethanol precipitation step, followed by heat treatment (60°C for 10 hours) as a pathogen inactivation step. This is sometimes followed by an ultrafiltration step, largely included as a viral removal step. The large quantities of inhibitor required (about 200 g per patient annually) and the possibility of accidental transmission of disease via infected source material has encouraged the development of alternative recombinant sources.  $\alpha_1$ -Antitrypsin has been successfully produced in various recombinant systems, including in the milk of both transgenic mice and sheep, although no recombinant product has thus far been approved for general medical use.

## 6.5 Vaccine technology

Vaccines play a central role in not only human but also veterinary medicine and represent the only commonly employed prophylactic (i.e. preventive) approach undertaken to control many infectious diseases. The current annual global vaccine market stands well in excess of \$3 billion. Moreover, vaccines along with antibody-based products (Chapter 7) represent by far the largest subgroup of biotechnology medicines currently in development.

Vaccination seeks to exploit the natural defence mechanisms conferred on us by our immune system. A vaccine contains a preparation of antigenic

components consisting of, derived from or related to a pathogen. In most instances, on vaccine administration both the humoral and cell-mediated arms of the immune system are activated. The long-term immunological protection induced will normally prevent subsequent establishment of an infection by the same or antigenically related pathogens. While some vaccines are active when administered orally, most are administered parenterally. Normally, an initial dose is followed by subsequent administration of one or more repeat doses. Such booster doses serve to maximize the immunological response.

Traditional vaccine preparations have largely been targeted against viral and bacterial pathogens, as well as some bacterial toxins and, to a lesser extent, parasitic agents such as malaria. Approximately 30 such vaccines remain in medical use. These can largely be categorized into one of several groups:

- live attenuated bacteria, e.g. bacillus Calmette–Guèrin, BCG, used to immunize against tuberculosis;
- dead or inactivated bacteria, e.g. cholera and pertussis vaccines;
- live attenuated viruses, e.g. measles, mumps and yellow fever viral vaccines;
- inactivated viruses, e.g. hepatitis A and polio (Salk) viral vaccines;
- toxoids, e.g. diphtheria and tetanus vaccines;
- pathogen-derived antigens, e.g. hepatitis B, meningococcal, pneumococcal and *Haemophilus influenzae* vaccines.

However, an increased understanding of the molecular mechanisms underlying additional human diseases suggests several novel applications of vaccines to treat or prevent autoimmune conditions and cancer.

### 6.5.1 The impact of genetic engineering on vaccine technology

Recombinant DNA technology has rendered possible the large-scale production of polypeptides normally present on the surface of virtually any pathogen.

These recombinant polypeptides, when purified from the producer organism, can then be used as ‘subunit’ vaccines. This method of vaccine production exhibits several potential advantages over conventional vaccine production methodologies.

- Production of a clinically safe product. The pathogen-derived polypeptide is now expressed in a non-pathogenic recombinant host. This all but precludes the possibility that the final product could harbour undetected pathogen.
- Production of subunit vaccine in an unlimited supply. Previously, production of some vaccines was limited by supply of raw material (e.g. hepatitis B surface antigen, see below).
- Consistent production of a defined product that would thus be less likely to cause unexpected side effects.

A number of such recombinant (subunit) vaccines have now been approved for general medical use (Table 6.6). The first such product was hepatitis B surface antigen (rHBsAg), which gained marketing approval from the Food and Drug Administration in 1986, and several additional products are now on the market. Two billion people are infected with hepatitis B worldwide: 350 million individuals suffer from lifelong chronic infection, and more than 1 million infected patients die each year from the associated complications of liver cirrhosis and/or liver cancer. Prior to approval of the recombinant product, hepatitis B vaccines consisted of HBsAg purified directly from the blood of hepatitis B sufferers. When present in blood, HBsAg exists not in monomeric form but in characteristic polymeric structures that display a diameter of 22 µm. Production of hepatitis B vaccine by direct extraction from blood suffered from two major disadvantages.

- The supply of finished vaccine was restricted by the availability of infected human plasma.
- The starting material will likely be contaminated by intact viable hepatitis B viral particles (and perhaps additional viruses such as HIV). This necessitates introduction of stringent purification

**Table 6.6** Recombinant subunit vaccines that have gained approval for general medical use in the EU and/or the USA. Many are also approved for use in various other world regions.

Product	Company	Indication
Ambirix (combination vaccine containing recombinant HBsAg produced in <i>S. cerevisiae</i> as one component)	Glaxo SmithKline	Immunization against hepatitis A and B
Cervarix (recombinant C-terminally truncated major capsid L1 proteins from HPV types 16 and 18 produced in a baculovirus expression system)	GlaxoSmithKline	Prevention of cervical cancer
Comvax (combination vaccine, containing recombinant HBsAg produced in <i>S. cerevisiae</i> as one component)	Merck	Vaccination of infants against <i>Haemophilus influenzae</i> type B and hepatitis B
Dukoral (combination vaccine containing recombinant cholera toxin B subunit produced in a <i>Vibrio cholerae</i> strain as one component)	Crucell Sweden AB	Active immunization against diseases caused by <i>V. cholerae</i>
Engerix B (recombinant HBsAg produced in <i>S. cerevisiae</i> )	Smithkline Beecham	Vaccination against hepatitis B
Gardasil/Silgard (recombinant HPV vaccine, containing recombinant surface antigens from types 6, 11, 16, 18 produced in <i>S. cerevisiae</i> )	Merck Sharp & Dohme	Vaccine against cervical cancer and related conditions caused by HPV
HBVAXPRO (recombinant HBsAg produced in <i>S. cerevisiae</i> )	Sanofi-Pasteur	Immunization of children and adolescents against hepatitis B
Infanrix-Hexa (combination vaccine containing recombinant HBsAg produced in <i>S. cerevisiae</i> as one component)	Smithkline Beecham	Immunization against diphtheria, tetanus, pertussis, polio, <i>Haemophilus influenzae</i> b and hepatitis B
Infanrix-Penta (combination vaccine containing recombinant HBsAg produced in <i>S. cerevisiae</i> as one component)	Smithkline Beecham	Immunization against diphtheria, tetanus, pertussis, polio and hepatitis B
Pediarix (combination vaccine containing recombinant HBsAg produced in <i>S. cerevisiae</i> as one component)	Glaxo Smithkline	Immunization of children against various conditions, including hepatitis B
Recombivax (recombinant HBsAg produced in <i>S. cerevisiae</i> )	Merck	Hepatitis B prevention
Tritanrix-HB (combination vaccine containing recombinant HBsAg produced in <i>S. cerevisiae</i> as one component)	Smithkline Beecham	Vaccination against hepatitis B, diphtheria, tetanus and pertussis
Twinrix, adult and paediatric forms in EU (combination vaccine containing recombinant HBsAg produced in <i>S. cerevisiae</i> as one component)	Smithkline Beecham (EU) Glaxo Smithkline (USA)	Immunization against hepatitis A and B

HPV, human papillomavirus.

procedures to ensure complete inactivation and/or removal of any intact viral particles from the product stream. A final product quality control test to confirm this traditionally involves a 6-month safety test on chimpanzees.

The HBsAg gene has been cloned and expressed in a variety of expression systems, including *E. coli*, *Saccharomyces cerevisiae* and a number of mammalian

cell lines. The product used commercially is produced in *Saccharomyces cerevisiae*. The yeast cells are not only capable of expressing the gene, but also assembling the resultant polypeptide product into particles quite similar to those found in the blood of infected individuals.

Dukoral is the trade name given to an additional recombinant protein-containing vaccine now on the market. Indicated for active immunization against



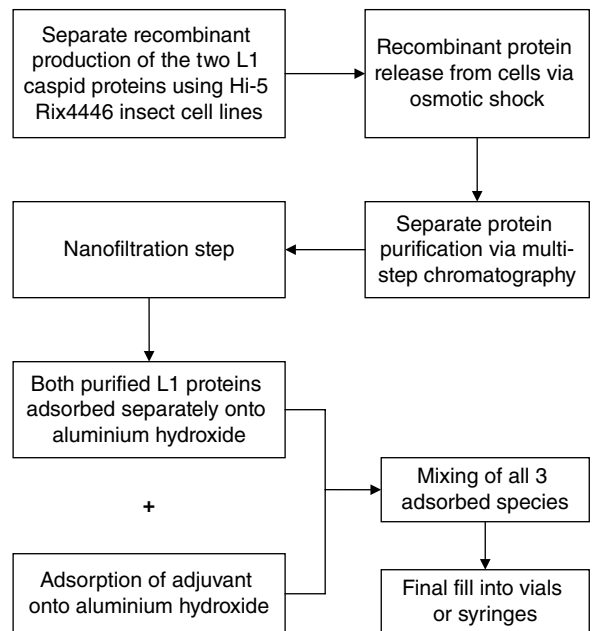
disease caused by *Vibrio cholerae* (serogroup 01), the product contains recombinant cholera toxin subunit B and four whole (heat or formalin-inactivated) *Vibrio cholerae* strains.

### 6.5.2 Cancer vaccines

To date two subunit cancer vaccines have been approved for medical use (Table 6.6). The anti-human papillomavirus (HPV) vaccines Gardasil (also sold as Silgard) and Cervarix target the prevention of cervical cancer, which is second only to breast cancer in global cancer incidences in women. HPV infections represent the most prevalent sexually transmitted disease worldwide, with 50% of young women being infected within 5 years of becoming sexually active. Two HPV strains (HPV 16 and HPV 18) are highly carcinogenic and are believed responsible for as many as 70% of invasive cervical cancers. Approximately half a million new cases of cervical cancer are diagnosed annually, culminating in an annual death rate approaching 300,000. Cervarix is a divalent vaccine, containing recombinant truncated major capsid L1 proteins from HPV types 16 and 18. The product, approved for general medical use in 2007, is also notable in that it was the first recombinant biopharmaceutical on the market produced using a baculovirus-based expression system. Its manufacture is overviewed in Figure 6.7.

Gardasil, on the other hand, is a quadrivalent vaccine containing recombinant forms of the major capsid protein from HPV types 6, 11, 16 and 18. In addition to vaccinating against cervical cancer, this latter product also affords protection against genital warts, 90% of which are caused by HPV 6 and 11.

The identification of antigens uniquely associated with the surface of cancer cells (i.e. tumour surface antigens) could also pave the way for the development of a range of cancer vaccines. Theoretically, administration of tumour-associated antigens may effectively immunize an individual against any cancer type characterized by expression of the tumour-associated antigen in question. Co-administration of a strong adjuvant would be advantageous as it would stimulate an enhanced

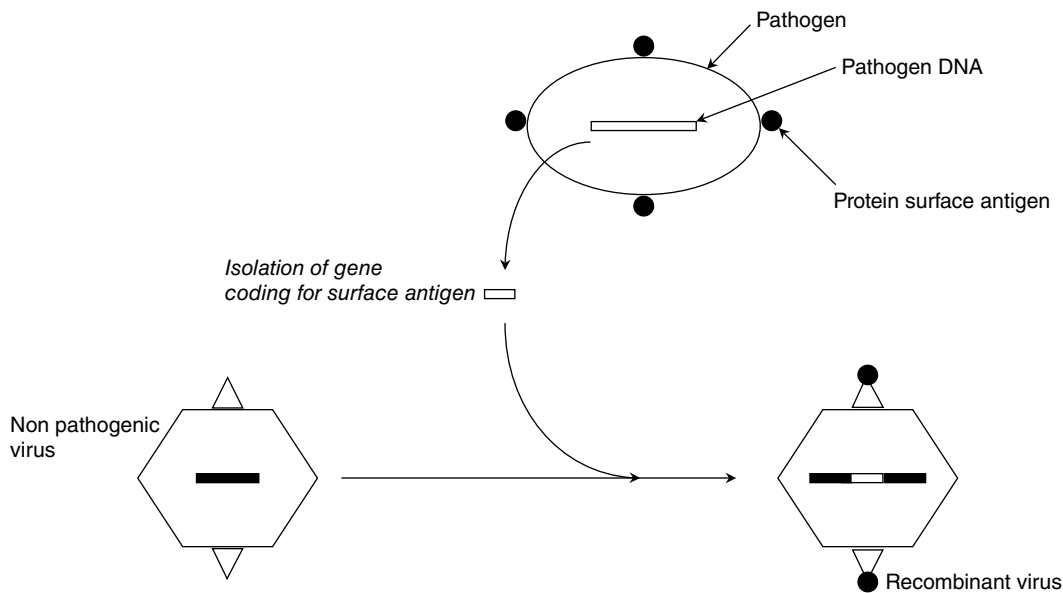


**Figure 6.7** Overview of the manufacture of Cervarix. The adjuvant used is MPL (monophosphoryl lipid A), a detoxified derivative of the lipopolysaccharide moiety of the Gram-negative bacterium *Salmonella minnesota*. Each dose of the final vaccine suspension contains 20 µg of both HPV type 16 and type 18 L1 capsid proteins.

immune response. (An adjuvant is defined as any material that enhances the cellular and/or humoral immune response to an antigen.) This is important as many tumour-associated antigens appear to be weak immunogens. Administration of subunit-based tumour-associated antigen vaccines would primarily stimulate a humoral immune response. The use of viral vectors (see section 6.5.3) may ultimately prove more effective, as a T-cell response appears central to the immunological destruction of such transformed cells.

### 6.5.3 Vaccine vectors

An alternative approach to the development of novel vaccine products entails the use of live vaccine vectors. The strategy followed involves incorporation of a gene/cDNA coding for a pathogen-derived antigen into a non-pathogenic species. If the resultant recombinant vector expresses the gene product



**Figure 6.8** Basic approach to developing recombinant vaccine vectors. The nucleotide sequence coding for most or all of a selected surface antigen on the target pathogen is identified and isolated. This is then introduced into the genome of a clinically safe virus (e.g. vaccinia). It is modified (e.g. by fusion with a viral gene coding for a viral surface protein) to ensure that the transferred gene product will be expressed on the viral cell surface.

on its surface, it may be used to immunize against the pathogen of interest (Figure 6.8).

Most vaccine vectors that have been developed are viral-based, with poxviruses, picornaviruses and adenoviruses being used most. In general, such recombinant viral vectors elicit both strong humoral and, in particular, cell-mediated immunity. The immunological response (especially the cell-mediated response) to subunit vaccines is usually less pronounced.

However, one potential complication centres around the possibility that previous recipient exposure to the virus being used as a vector would negate the therapeutic efficacy of the product. Such prior exposure would likely indicate the presence of circulating immune memory cells that could initiate an immediate immunological response on re-entry of the virus into the host. Studies involving repeat administration of vaccinia virus have, to some extent, confirmed this possibility. However, the degree to which such an effect limits the applicability of this approach in a clinical setting remains to be elucidated.

## 6.6 Therapeutic enzymes

A variety of enzymes are used clinically in the treatment of various medical conditions. Several, such as tPA and factors VIIa and IXa, have already been considered. Various additional enzyme preparations obtained by direct extraction from a naturally producing source have been in medical use for several decades (Table 6.7). Such traditional enzyme preparations are considered in this section. More recently, several recombinant preparations have gained marketing approval, as discussed in section 6.6.4.

### 6.6.1 Asparaginase

Asparaginase catalyses the hydrolysis of the amino acid asparagine, yielding aspartic acid and ammonia (Figure 6.9). The enzyme is a tetramer with a molecular mass in the region of 120 kDa. It may be purified from a wide variety of microorganisms including yeast, fungi and bacteria such as *E. coli*.

All cells require asparagine to sustain normal metabolic activity. Although most human cells are themselves capable of synthesizing this amino acid, certain malignant cells lack this ability. Intravenous administration of asparaginase results in the rapid depletion of serum asparagine levels, which normally range between 0.5 and 1.5 mg/dL. Protein synthesis in malignant cells incapable of synthesizing asparagine is thus severely compromised. In contrast, untransformed cells begin to synthesize their own asparagine (Figure 6.9).

Asparaginase preparations used clinically are normally purified from *E. coli* or from *Erwinia chrysanthemi*. *E. coli* produces two asparaginase isoenzymes of which only one is clinically effective. Recombinant DNA technology now facilitates the recombinant synthesis of asparaginase from a range of other sources, some of which may display clinical potential. This enzyme is mostly used in the treatment of certain forms of childhood leukaemia.

Side effects can include severe allergic reaction, as well as nausea, vomiting, fever, and compromised kidney as well as liver function. The latter effects may be attributable to the fact that most untransformed body cells do not themselves synthesize asparagine when it is available from the blood. The rapid depletion of serum asparagine on administration of asparaginase temporarily deprives such healthy cells of asparagine, thus disrupting protein synthesis. Ironically, this slows synthesis of the asparagine synthetase required by these cells to manufacture their own asparagine (Figure 6.9).

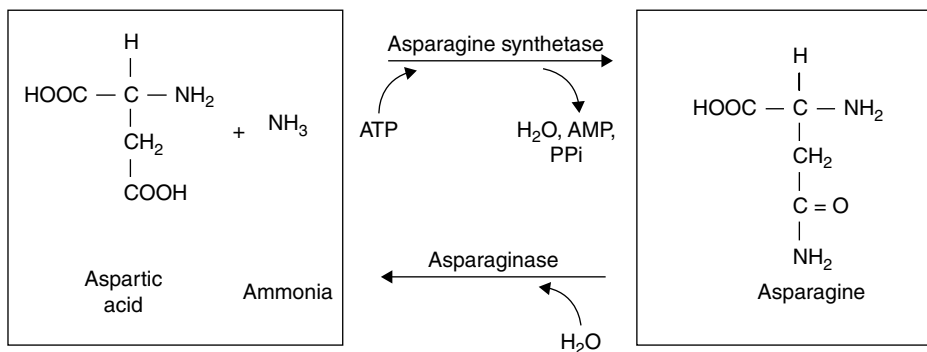
Given its microbial source it is not surprising that L-asparaginase elicits an immune response when administered to humans. This imposes obvious limitations on the enzyme's long-term clinical efficacy. Coupling the L-asparaginase to polyethylene glycol (PEG) has been shown to greatly reduce or eliminate the enzyme's immunogenicity, and PEGylated asparaginase is now used clinically.

**Table 6.7** Traditional (non-recombinant) enzymes used medically. Refer to text for details.

Enzyme	Therapeutic application
Asparaginase	Treatment of some types of cancer
Trypsin, papain, collagenase	Debriding/anti-inflammatory agents
Lactase, pepsin, pancrelipase, papain	Digestive aids

### 6.6.2 Debriding and anti-inflammatory agents

Certain enzymes are employed as debriding agents. These effectively clean open wounds by removal of foreign matter and any surrounding dead tissue. This allows for rapid healing of the wound. Enzymes such as trypsin, papain and collagenase have often been used as debriding agents. Such preparations are normally applied topically to the affected areas.



**Figure 6.9** The biosynthesis and degradation of L-asparagine.

Trypsin is a proteolytic enzyme synthesized by the mammalian pancreas. It has a molecular mass of 24 kDa and hydrolyses peptide bonds in which the carboxyl group has been contributed by either an arginine or lysine residue.

Papain is a proteolytic enzyme isolated from the leaves and the unripe fruit of the papaya tree. It catalyses the hydrolysis of peptide bonds involving basic amino acids such as lysine, arginine or histidine. In addition to its use as a debriding agent, papain has also been utilized as a meat tenderizer and for clearing beverages (see Chapter 12).

Chymopapain, a second proteolytic enzyme produced by the papaya tree, has also found medical application. Chemonucleolysis is the term used for the treatment of sciatica by injecting chymopapain into damaged intervertebral discs. Sciatica, a medical condition characterized by back and leg pain, is caused by the degradation of an intervertebral disc. The disc tends to protrude laterally, compressing spinal root neurones. The injection of chymopapain appears to speed up the underlying process of disc degradation, thereby more quickly reaching an end stage where it is stable and asymptomatic.

Collagenase is an enzyme that catalyses the proteolytic destruction of collagen. Although it may be isolated from culture extracts of various animal cells, it is normally obtained from the culture supernatant of various species of *Clostridium*. Some clostridial species are pathogenic, causing diseases such as gas gangrene. The ability of such microorganisms to produce tissue-degrading enzymes such as collagenase facilitates their rapid spread throughout the body.

Administration of certain enzymes has proven helpful in the reduction of various inflammatory responses. Such enzymes include chymotrypsin and bromelains. Chymotrypsin is a proteolytic enzyme produced in zymogen form (chymotrypsinogen) by the mammalian pancreas. Chymotrypsinogen is converted to chymotrypsin in the small intestine, where it functions to catalyse the proteolytic degradation of dietary proteins. Bromelains are plant proteases purified from the fruit or stem of the pineapple plant. The molecular mechanisms by which chymotrypsin and bromelains achieve an anti-inflammatory effect remains to be properly elucidated. However, it

is likely that the mode of action of these proteases centres around their ability to degrade protein-based inflammatory mediators, or proteins involved in promoting the synthesis of such mediators.

### 6.6.3 Enzymes as digestive aids

Various enzymatic preparations may be used as digestive aids. Most such enzymes are depolymerases, catalysing the enzymatic breakdown of a number of dietary components including polysaccharides, proteins and lipids. Some such enzyme preparations consist of a single enzyme that catalyses the degradation of a specific dietary substance. Others contain multiple enzymatic activities that exhibit broad digestive ability.

$\alpha$ -Amylase catalyses the hydrolysis of the  $\alpha 1 \rightarrow 4$  glycosidic bonds which form the main covalent linkage between glucose monomers in carbohydrates such as starch or glycogen.  $\alpha$ -Amylase produced by microorganisms such as *Bacillus subtilis* or members of the *Aspergillus* family have found widespread industrial application (discussed in Chapter 12). Amylase activity also plays an important digestive role in higher animals. The enzyme may be isolated from saliva or pancreatic tissue and various amylase preparations have been administered orally to aid the digestion of dietary carbohydrate.

Lactase catalyses the hydrolysis of the disaccharide lactose, the principal sugar of milk, yielding glucose and galactose. Preparations of lactase may be employed as a digestive aid, in particular to alleviate symptoms associated with lactose intolerance. While most infants exhibit high levels of intestinal lactase activity, the adult populations of many geographical regions have greatly reduced lactase activity. Such persons are unable to digest lactose, and often suffer from intestinal upset on consumption of milk.

Various proteolytic enzymes may also be employed as digestive aids. Such enzymes include papain, bromelains and pepsin. Pepsin is secreted naturally in the stomach of most animals, where it catalyses the proteolytic degradation of dietary protein. Pepsin preparations employed as digestive

aids are obtained by extraction from the mucous membranes of the stomach of various slaughterhouse animals.

Some enzymatic preparations employed clinically contain multiple enzymatic activities. Pancreatin, for example, is a proteinaceous preparation extracted from the pancreas. It contains amylase, protease, lipase and nuclease activities, and may be administered orally to patients suffering from conditions caused by deficient secretion of pancreatic enzymes. These include chronic pancreatitis (pancreatic inflammation and failure), pancreatic carcinomas, and cystic fibrosis (characterized partly by the blockage of pancreatic ducts by thick mucus). Pancreatin, and many other enzymatic digestive aids, display a pH versus activity profile which renders them maximally active in the upper portion of the small intestine (duodenum). Such enzymes, when administered orally, must pass through the stomach in order to reach their site of action. Inactivation of a large proportion of these enzymes can occur in the stomach, due to the low pH values encountered therein, and also possibly due to their proteolytic degradation by stomach pepsin. (The stomach secretes approximately 2 L of HCl daily, and pH values can be below 2.) A number of strategies can be adopted in order to minimize such gastric inactivation. The simplest approach entails coadministration of inhibitors of gastric acid secretion (e.g. cimetidine). However, by diminishing free acid levels in the stomach these agents can reduce the activity of pepsin and, more importantly, can compromise the natural bactericidal effects of low stomach pH (low pH normally kills dietary-derived microorganisms in the stomach). An alternative involves enzyme administration in enteric-coated tablet/capsule format (enteric coatings are impervious to acid but will dissolve at more neutral pH values, such as those characteristic of the duodenum). More recently, it has been shown that PEGylating some enzymes can potentially protect them from acid inactivation. Yet another approach under investigation is the use of microbial (mainly fungal) proteases, amylases or lipases derived from acidophilic microbes, who produce acid-stable, acid-active extracellular enzymes.

### 6.6.4 Enzymes produced by recombinant means

A number of therapeutic enzymes now used medically are produced by recombinant means (Table 6.8). Early examples include Pulmozyme and Cerezyme, although most such recombinant preparations have come on the market since 2000. Most of these more recently approved products (like Cerezyme) are used as enzyme replacement therapy for specific genetic diseases, and one such product is featured in Box 6.2.

#### 6.6.4.1 Nuclease treatment of cystic fibrosis

Cystic fibrosis (CF) represents one of the most common genetic diseases. The frequency of occurrence varies among populations, with persons of northern European extraction being most at risk. Within such populations approximately 1 in 2500 newborns are affected. It has been recognized for many years that excessive salt loss occurs in the sweat of persons suffering from CF. More recently, the gene that is defective in persons suffering from this disease has been identified. This gene codes for a 1480 amino acid, transmembrane, glycosylated polypeptide which functions as a chloride channel. The underlying cause of CF is therefore a malfunction in ion transport. In the region of 5% of white people carry this defective gene. However, the trait is recessive so the condition only occurs in cases where one inherits defective gene copies from both parents. The most common mutation associated with CF is the deletion of a single amino acid (Phe<sup>508</sup>) from the protein. In such individuals, expression of the aberrant gene results in compromised function of a number of tissue types, including the pancreas and sweat glands. However, the major clinical symptom of CF is undoubtedly the production of extremely viscous mucus in the respiratory tract. This compromises lung function.

The defective protein leads to a decrease in chloride transport from the lung epithelial cells into the surrounding respiratory mucus, and in an associated increased movement of sodium ions in the reverse direction. Overall this leads to an increased

**Table 6.8** Recombinant therapeutic enzymes now in medical use.

Product	Company	Indication	Approved
Aldurazyme (laronidase; recombinant human $\alpha$ -L-iduronidase produced in a CHO cell line)	Genzyme	Long-term enzyme replacement therapy in patients suffering from mucopolysaccharidosis I	2003 (EU)
Cerezyme (imiglucerase; recombinant $\beta$ -glucocerebrosidase, differs from native human enzyme by one amino acid – Arg 495 is substituted with His – also has modified oligosaccharide component, produced in a CHO cell line)	Genzyme	Treatment of Gaucher disease	1994 (USA) 1997 (EU)
Elelyso (taliglucerase alfa; recombinant human glucocerebrosidase, produced in engineered carrot root cell culture)	Pfizer/ Protalix Ltd	Gaucher disease	2012 (USA)
Elaprase (idursulfase; recombinant human iduronate-2-sulfatase, produced in a human cell line)	Shire Human Genetic Therapies	Mucopolysaccharidosis II (Hunter syndrome)	2007 (EU) 2006 (USA)
Fabrazyme (agalsidase beta; recombinant human $\alpha$ -galactosidase, produced in a CHO cell line)	Genzyme	Fabry disease ( $\alpha$ -galactosidase A deficiency)	2001 (EU)
Fasturtec (EU) or Elitek (USA) (rasburicase; recombinant urate oxidase produced in <i>S. cerevisiae</i> )	Sanofi-Aventis	Hyperuricaemia	2001 (EU) 2002 (USA)
Krystexxa (pegloticase; recombinant urate oxidase, produced in <i>E. coli</i> and PEGylated post synthesis)	Savient Pharmaceuticals	Gout	2010 (USA)
Lumizyme (alglucosidase alfa; recombinant human acid $\alpha$ -glucosidase, produced in a CHO cell line)	Genzyme	Pompe disease (glycogen storage disease type II)	2010 (USA)
Naglazyme (galsulfase; recombinant human <i>N</i> -acetylgalactosamine 4-sulfatase, produced in a CHO cell line)	BioMarin	Long-term enzyme replacement therapy in patients suffering from mucopolysaccharidosis VI	2006 (EU) 2005 (USA)
Pulmozyme (dornase alfa; recombinant human DNase produced in a CHO cell line)	Genentech	Cystic fibrosis	1993 (USA)
Replagal (agalsidase alfa; recombinant human $\alpha$ -galactosidase, produced in a human cell line)	Shire Human Genetic Therapies	Fabry disease	2001 (EU)
Voraxaze (glucarpidase; recombinant carboxypeptidase produced in <i>E. coli</i> )	BTG International	Treatment of methotrexate in blood due to kidney failure	2012 (US)
VPRIV (velaglucerase alfa, glucocerebrosidase; human fibroblast cell line)	Shire Human Genetic Therapies	Gaucher disease	2010 (US, EU)

**Box 6.2 Product case study: Elaprase**

Elaprase (idursulfase) is a recombinant form of the human lysosomal enzyme iduronate 2-sulfatase, produced in an engineered transformed human cell line (HT-1080). The 525 amino acid enzyme displays a molecular mass of 76 kDa and harbours two disulfide bonds, eight N-linked glycosylation sites and a formylglycine residue at position 59 that is necessary for biological activity. Elaprase is indicated for the long-term treatment of patients with Hunter syndrome (mucopolysaccharidosis II, MPS II). MPS II is a rare genetic lysosomal storage disease caused by a lack of functional lysosomal iduronate 2-sulfatase. This enzyme functions naturally in the body to cleave sulfate groups from two glycosaminoglycans (GAGs), dermatan sulfate and heparan sulfate. GAGs are unbranched polysaccharides consisting of various uronic acids and hexosamine residues and which largely play structure-related roles in the extracellular space, particularly those associated with connective tissue. Lack of the enzyme results in GAG accumulation in most organs and tissues, resulting in organ/tissue dysfunction. Clinical manifestations usually become apparent within the first 12–36 months of life and are progressive, severe and life-limiting.

Manufacture entails initial culture of the producer cell line, chosen for production due to its ability to attach complex, high mannose-type oligosaccharide side chains to the enzyme's polypeptide backbone. Specifically, the presence of mannose 6-phosphate (M6P) facilitates product binding to cell-surface M6P receptors, with subsequent cellular internalization and delivery to lysosomes, the target organelle.

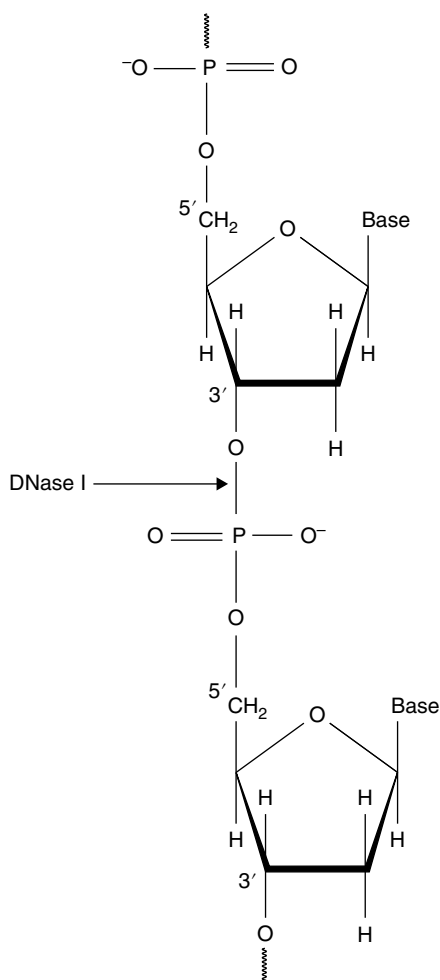
Product purification incorporates six chromatographic steps as well as two ultrafiltration and a viral filtration step. Formulation entails the addition of sodium chloride (to provide an isotonic solution for subsequent administration), sodium phosphate (buffering

agent) and polysorbate 20 (stabilizing agent and protection against agitation-induced aggregation). The final product is filter sterilized and aseptically filled into pre-sterile vials. Each vial contains 3 mL of a 2 mg/mL active ingredient solution.

level of water absorption from the mucus into the epithelial cells, thereby increasing viscosity and elasticity of the mucus. This in turn impedes clearance of the mucus, allowing any inhaled pathogens to remain in the lung, which is conducive to the establishment of recurrent bacterial infections. Such infections trigger an immune response in which large numbers of neutrophils are attracted to the site of infection. Ingestion and destruction of bacterial populations by such white blood cells results in the liberation of large amounts of DNA. This released DNA interacts with a variety of additional extracellular substances present in the infected lung, thus further increasing mucus viscosity.

In order to prevent build-up of such mucus, CF patients suffering from bacterial infections were traditionally subjected to percussion therapy. This basically involves physically pounding on the patient's chest for extended periods of time in order to dislodge the mucus and hence allow the sufferer to expel it. It was postulated a number of years ago that treatment of the infected lung with a DNase preparation might alleviate such respiratory symptoms, by catalysing degradation of the extracellular DNA and hence promoting a reduction in mucus viscosity.

DNase I is one of the most prominent and well-characterized members of the DNase family. This endonuclease catalyses the hydrolysis of internal phosphodiester linkages at the bond between P and the 3' O (Figure 6.10). Its preferred substrate is double-stranded DNA, although it will also degrade single-stranded DNA, albeit more slowly. Bovine DNase I was first (partially) purified from pancreatic extracts in the late 1940s. Experiments over the subsequent decades clearly illustrated the enzyme's ability to significantly reduce the *in-vitro* viscosity of lung mucus from CF patients, and



**Figure 6.10** Cleavage of DNA by DNase I. Hydrolysis generates two fragments, one with a free 3' hydroxy terminus, the other with a free 5' phosphate terminus.

bovine pancreatic DNase was approved for the treatment of CF in the USA in the 1950s. While initially well tolerated, adverse reactions were reported on prolonged administration. In part this was due to the enzyme's bovine origin (and hence its immunogenicity in humans). However, the major cause of such adverse reactions was most likely the presence of proteolytic contaminants in the product (these preparations were subsequently shown to contain up to 2% trypsin and chymotrypsin).

The nucleotide sequence coding for human DNase I was first isolated from a pancreatic cDNA

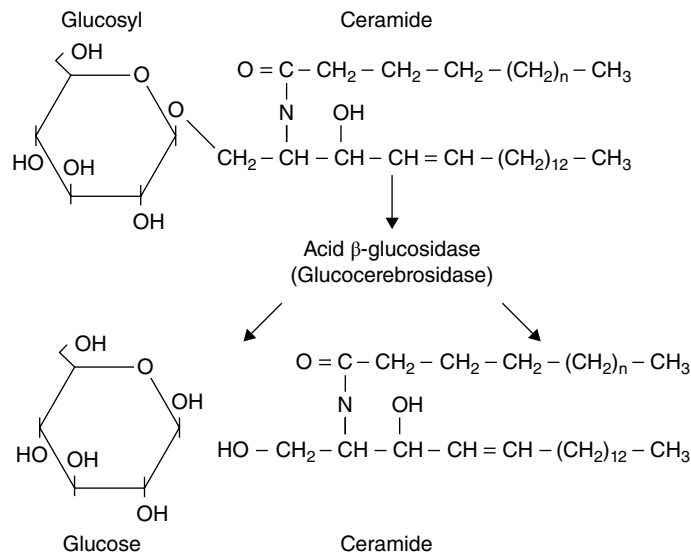
library in the 1980s. It codes for a 260 amino acid glycoprotein that shows high (77%) homology to bovine DNase I. The cDNA has been expressed in recombinant CHO cell lines, and DNase produced from this source (Pulmozyme) has been approved for medical use.

#### 6.6.4.2 Glucocerebrosidase

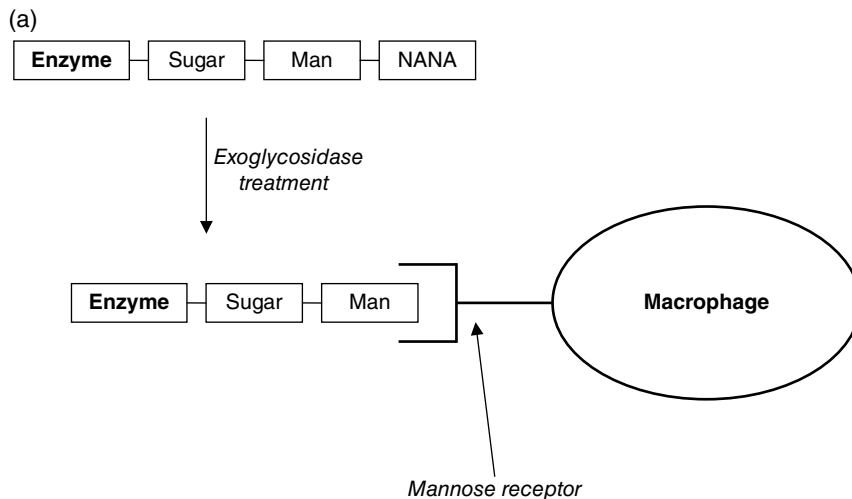
Gaucher disease is a hereditary metabolic disorder caused by a lack of the lysosomal enzyme  $\beta$ -glucocerebrosidase. This 60-kDa glycosylated enzyme normally catalyses the hydrolysis of a glycolipid molecule known as glucosylceramide (Figure 6.11) and deficiency of this enzyme activity results in the accumulation of glucosylceramide in tissue-based macrophages. This gives rise to the lysosomal storage disease known as Gaucher disease, characterized by swollen organs (due to accumulation of affected macrophages therein), anaemia, bone pain and sometimes neuronal damage. The most common form of the disease is caused by a point mutation, resulting in an asparagine to serine substitution at amino acid residue 370 (N370S) of the 497 amino acid mature protein.

The disease may be treated by enzyme replacement therapy, and glucocerebrosidase purified directly from human placenta was initially used for this purpose. Concerns relating to cost, source availability and accidental transmission of disease hastened development of a recombinant glucocerebrosidase product. Cerezyme is the trade name of such a recombinant product, produced in an engineered CHO cell line (Table 6.8). Product manufacture includes an enzyme-based downstream processing step using an exoglycosidase. The exoglycosidase removes outer sugar residues that cap the oligosaccharide side chains of the  $\beta$ -glucocerebrosidase. This exposes mannose residues underneath, facilitating specific uptake by macrophages via macrophage cell-surface mannose receptors (Figure 6.12). In this way the product is specifically targeted to the cell type most affected by the disease. Unmodified glucocerebrosidase, if administered, is quickly removed from the bloodstream by the liver.

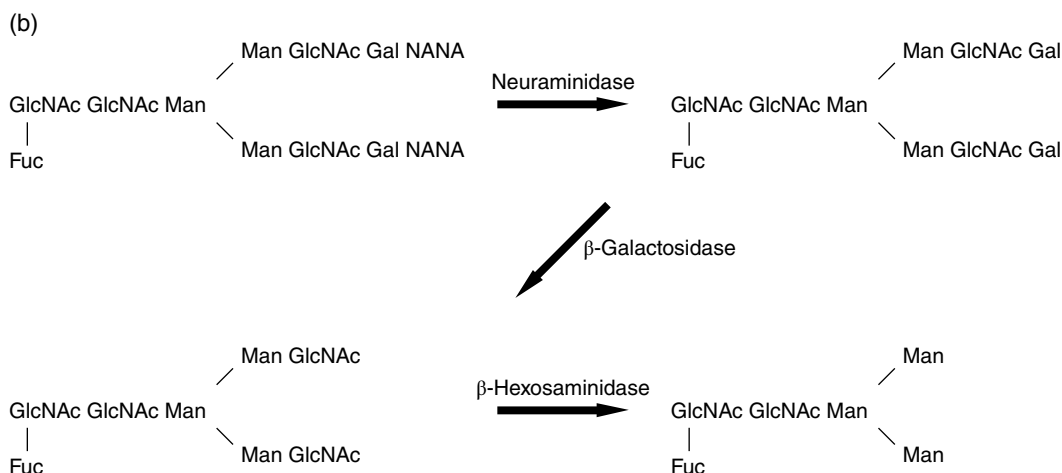




**Figure 6.11** The reaction catalysed by  $\beta$ -glucocerebrosidase.



**Figure 6.12** Simplified schematic overview of the glycoengineering of  $\beta$ -glucocerebrosidase (a). Enzymatic remodelling of the sugar chain removes terminal sialic acids, i.e. derivatives of the monosaccharide neuraminic acid such as *N*-acetylneuraminic acid (Neu5Ac or NANA), as well as galactose (Gal) exposing mannose (Man) residues underneath. Macrophages are phagocytes responsible for engulfing bacteria (and other pathogens/cell debris, etc.), followed by lysosomal destruction of the phagocytosed material. Bacteria often contain mannose residues on their surface, hence it is unsurprising that macrophages have evolved to express a mannose receptor on their surface. The carbohydrate remodelling undertaken therefore exploits this pathway, allowing for uptake of the enzyme with subsequent lysosomal delivery (a). The actual carbohydrate remodelling is likely more extensive than described above, with a necessity in practice to remove not only terminal sialic acids but also Gal residues. A likely remodelling process is presented in (b). Fuc, fucose; GlcNAc, *N*-acetylglucose.



**Figure 6.12** (Continued)

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# Chapter 7

## Therapeutic antibodies

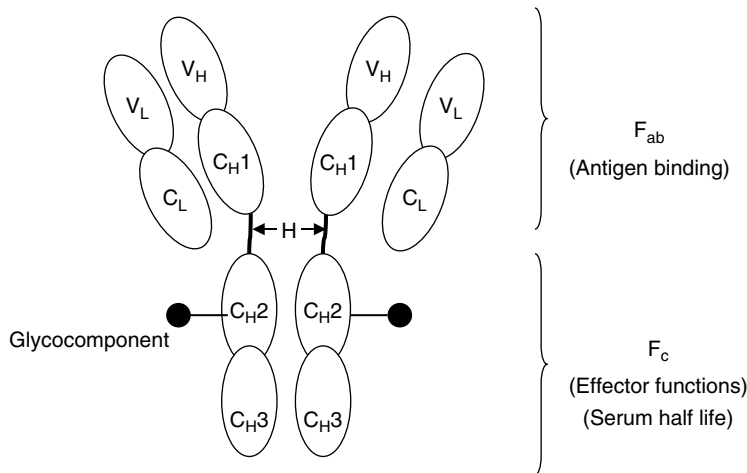
### 7.1 Antibodies

Five major classes of antibodies (immunoglobulins or Igs) have been characterized from mammalian serum (IgM, IgG, IgA, IgD and IgE). IgAs can be grouped into two subtypes or isotypes (IgA1 and IgA2), and IgG into four subtypes (IgG1–IgG4). Immunoglobulins of all classes and subtypes display a similar basic four-chain structure. IgGs are the predominant immunoglobulin class found in blood as well as in cerebrospinal fluid and peritoneal fluid. IgGs (with the exception of IgG3) are also noticeable in that they exhibit an unusually long serum half-life (approximately 23 days). Thus far all of the monoclonal antibody-based products approved for therapeutic use are of the IgG class.

### 7.2 IgG structure and activity

IgG consists of four polypeptide chains: two identical heavy (H) and two identical light (L) chains. The four polypeptide units assemble to form a Y-shaped

molecule with the antigen-binding regions residing at the tips of the Y (Figure 7.1). The overall structure is stabilized by both interchain and intrachain disulfide bonds, and by non-covalent interactions. Treatment with certain proteolytic enzymes results in cleavage of the antibody at the flexible hinge region, yielding two antigen-binding fragments (Fab) and a constant fragment (Fc). The constant region (Fc) of an intact antibody mediates various biological effector functions, such as antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). Both H chains and L chains contain variable (V) regions and constant (C) regions. Variable regions contain the antigen-binding site. Variable regions of antibodies of different specificity differ in amino acid sequence. Constant regions, on the other hand, do not. Structurally, each H and L chain consists of a number of domains. One such domain forms the variable region of the H chain ( $V_H$ ). The constant region of the H chain consists of three such domains,  $C_{H1}$ ,  $C_{H2}$  and  $C_{H3}$ . L chains, on the other hand, consist only of two domains: a variable one ( $V_L$ ) and a constant one ( $C_L$ ). Each domain consists of about 110 amino acids and all domains exhibit strikingly similar underlying



**Figure 7.1** IgG structure. Refer to text for details.

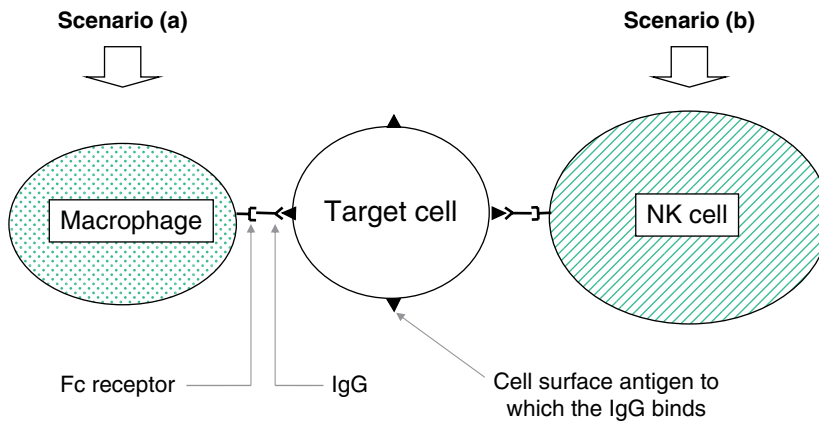
architectural features, consisting of  $\beta$ -pleated sheets joined by loop regions. These loops in the variable domains ( $V_H$  and  $V_L$ ) exhibit hypervariable sequences and form the antigen-binding sites of the antibody. These hypervariable sequences are referred to as complementarity determining regions (CDRs). The remaining areas of the variable domains are often termed framework regions. Framework regions exhibit reduced variability compared with CDR sequences. Immunoglobulins are also glycoproteins. The carbohydrate moiety is associated with the  $C_{H2}$  domains of the heavy chains. Removal of the carbohydrate moiety has no effect on antigen binding but it does affect various effector functions as well as the molecule's serum half-life.

The production of IgGs by specific white blood cells (B lymphocytes) in response to a foreign substance (antigen) represents a central element of our immune response. The antibodies display binding specificity towards the particular antigen which prompted their production. Binding of IgG to an antigen can promote a number of often overlapping responses.

- Neutralization of the biological activity of some antigens (e.g. toxins and viruses) or induction of apoptosis of some cells.
- Agglutination (physical clumping, made possible by the divalent antigen-binding nature of the antibody) of particulate antigens such as microbes, facilitating their subsequent uptake and destruction by phagocytic cells.

- Opsonization (meaning 'to prepare to eat'): binding marks antigens such as microbes for destruction by phagocytic cells such as macrophages and polymorphonuclear phagocytes.
- Induction of ADCC: binding of antibody to the surface of virally infected cells or cancer cells can facilitate the docking of certain immune cells, particularly natural killer (NK) cells, close to the target cell surface, thereby facilitating the efficient destruction of such target cells.
- Activation of CDC: the complement system consists of a number of blood proteins that when activated by various immunological signals generate a membrane attack complex, which triggers target cell lysis. Additionally, activation of complement can enhance opsonization and trigger the generation of chemotactic signals that attract immune cells such as macrophages and neutrophils.

While antibody binding to antigen underpins neutralization and agglutination, the additional 'effector function' mechanisms leading to antigen destruction are mediated by the antibody Fc region. Phagocytes, NK cells and other immune system cells all contain various antibody Fc receptors, allowing these cells to dock on the surface of antigen-bound antibody, hence facilitating efficient and targeted antigen destruction. Figure 7.2 illustrates how antibody binding to a target cell can trigger destruction of that cell.



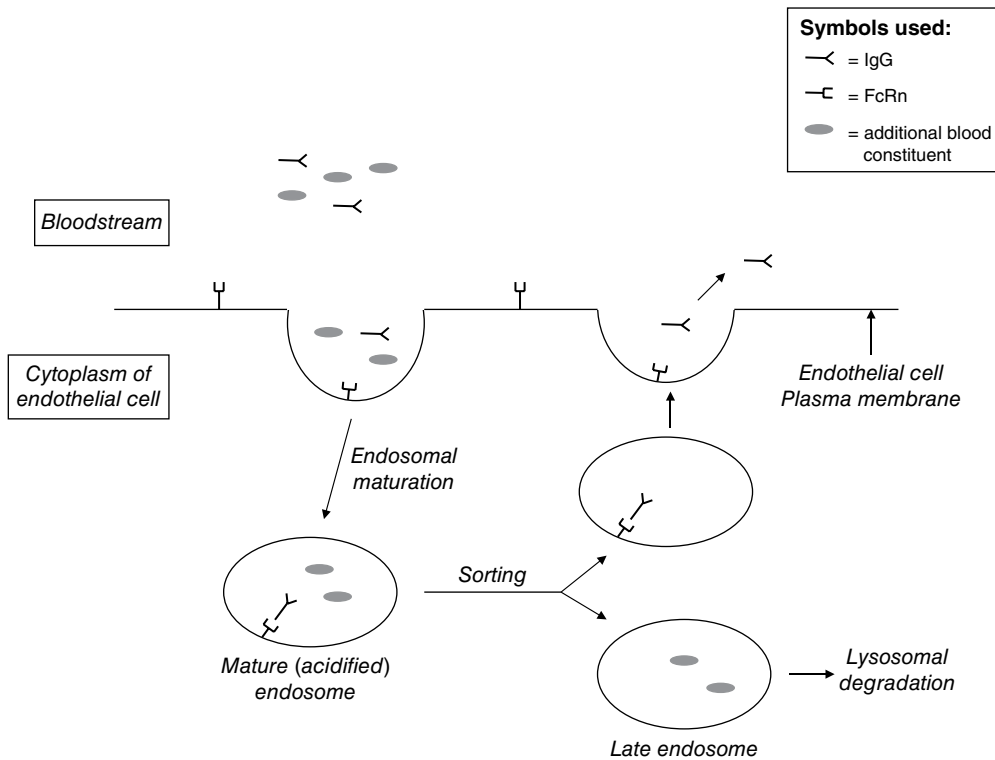
**Figure 7.2** Prominent effector mechanisms by which antibody binding to an antigen on the surface of a target cell (e.g. a microbial cell, a cancer cell or a virally infected cell) can trigger destruction of that cell. In scenario (a) phagocytic cells such as macrophages and neutrophils, because they display antibody Fc receptors on their surface, can dock on the surface of an opsonized target cell (often a microorganism), and subsequently engulf (phagocytose) the target cell. Opsonization refers to the binding of antibodies or certain other immunological mediators to an antigen, marking the antigen for recognition by immune cells. In scenario (b) certain immune system cells, in particular natural killer (NK) cells, dock at the target cell surface, also via binding to the antibody Fc region, inducing the latter's destruction via a process called antibody-dependent cell-mediated cytotoxicity (ADCC). This process often targets cancer cells and virally infected cells in the body. NK cells are cytotoxic lymphocytes that secrete proteins which insert themselves into the membrane of the target cell, forming a pore. This in turn lyses the cell. NK cells also secrete various serine proteases that are also capable of inducing cell death.

The antibody Fc region also underpins the long serum half-life of IgGs, by facilitating antibody recycling (Figure 7.3). Antibodies and other serum components are removed from the blood by mechanisms which generally rely on endocytosis. Many cells, including vascular endothelial cells which line blood vessels, express a neonatal Fc receptor (FcRn) on their surface. The FcRn binds IgG via its Fc region. Interestingly however, the FcRn does not bind antibody at physiological pH values characteristic of blood (~7.4), only binding antibody at lower pH (<6.5). During initial endocytotic uptake, therefore, the antibody is not actually bound to the FcRn (Figure 7.3). However, during subsequent endosomal maturation acidification occurs, triggering antibody binding. A sorting process then returns the membrane-bound FcRn with attached antibody to the cell surface, where the higher blood pH triggers release of free IgG back into the blood. Meanwhile, the remaining blood components in the 'late' endosome are usually destined for lysosomal degradation.

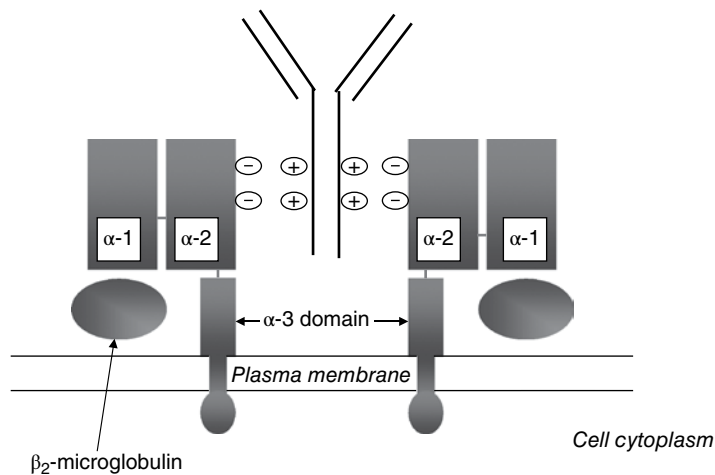
The human FcRn is a heterodimeric protein (Figure 7.4). The larger 44-kDa glycosylated

polypeptide is encoded by a gene located on chromosome 19. It spans the plasma membrane and consists of a three-domain extracellular portion, a single pass transmembrane domain and a short (44 amino acid) cytoplasmic tail. The smaller subunit is  $\beta_2$ -microglobulin, a polypeptide that also forms part of a whole range of additional protein complexes known as major histocompatibility complex (MHC) proteins. At pH values below 6.5, two FcRn molecules bind a single IgG. The rat FcRn has been crystallized and its three-dimensional structure resolved by X ray diffraction. Structural studies coupled with site-directed mutagenesis indicate that receptor-ligand binding is largely stabilized by the formation of salt bridges (i.e. a combination of hydrogen bonds and electrostatic interactions) between positively charged (at the lower pH) histidine side chains in the antibody's Fc region (His<sup>310</sup> and His<sup>435</sup> in particular, as well as Ile<sup>253</sup> and His<sup>433</sup>) and negatively charged residues found on the FcRn  $\alpha_2$  domain (Glu<sup>117</sup> and Glu<sup>132</sup> and, potentially, Glu<sup>135</sup> and Asp<sup>137</sup>).

FcRn-mediated endocytotic recycling is also responsible for the extended half-life (approximately



**Figure 7.3** The proposed process of antibody recycling via endocytosis. Several aspects of the exact molecular mechanisms underpinning this overall process remain to be fully elucidated. Refer to text for further details.



**Figure 7.4** Schematic overview of IgG-FcRn interaction. Refer to text for details.

20 days) exhibited by albumin in human serum. Although the pH-dependent endocytotic albumin recycling process is similar to that of IgG recycling in overall terms, the FcRn albumin-binding site is

distinct from that of the IgG-binding site and albumin-FcRn binding is a 1 : 1 stoichiometric ratio.

In addition to protecting IgG and albumin from degradation, the FcRn mediates several additional



physiological processes, including transport of maternal IgG to the fetus, and transport of IgG across mucosal epithelia (e.g. intestinal and pulmonary mucosa).

## 7.3 Antibody therapeutics: polyclonal antibody preparations

A wide variety of polyclonal antibody preparations have been used therapeutically for many decades to induce passive immunity. They usually function to afford immediate immunological protection against specific pathogens or other harmful antigenic substances. Administration of such specific antibody preparations is termed passive immunization. The purified antibody preparations administered are normally termed antisera. A distinction is often made between antisera and immunoglobulin preparations. The former refers to antibodies isolated from animals, while the latter refers to antibody preparations specifically obtained from human sources. In both cases the purified preparations consist predominantly of IgG molecules. Some of the

more commonly used antiserum/immunoglobulin preparations are listed in Table 7.1.

Passive immunization is generally used as a therapeutic measure if the patient is already suffering from a harmful condition caused or exacerbated by the presence of a known antigenic substance. Such antigenic substances include viruses, microorganisms or toxins/venom produced by certain spiders and snakes. The antibody preparations specifically recognize and bind the offending antigenic substances, thereby neutralizing or inactivating them. The exogenous antibody also helps to initiate a full immunological response against the foreign substance.

In contrast to this, the process of active immunization (the administration of a specific antigen, a vaccine, which stimulates the immune system to generate its own immunological response; see Chapter 6) is normally used as a prophylactic measure. The term 'prophylactic' or 'prophylaxis' refers to measures taken to prevent the future occurrence of specific diseases. Under certain circumstances, passive immunization may also be employed as a prophylactic measure. For example, if a person is likely to come in contact with pathogens because of work or travel, prior administration of antibodies

**Table 7.1** Polyclonal antibody preparations most commonly used to induce passive immunity.

Antibody preparation	Source	Antibody specificity
Normal immunoglobulin	Human	Exhibits a wide range of specificities against pathogens which are prevalent in the general population
Hepatitis B immunoglobulin	Human	Antibodies exhibiting a specificity for hepatitis B surface antigen
Measles immunoglobulin	Human	Antibodies exhibiting a specificity for measles virus
Rabies immunoglobulin	Human	Antibodies exhibiting a specificity for rabies virus
Cytomegalovirus immunoglobulin	Human	Antibodies exhibiting a specificity for cytomegalovirus
Varicella zoster immunoglobulin	Human	Antibodies exhibiting a specificity for the causative agent of chickenpox
Tetanus immunoglobulin	Human	Antibodies exhibiting a specificity for the toxin of <i>Clostridium tetani</i>
Tetanus antitoxin	Horse	Antibodies raised against the toxin of <i>Clostridium tetani</i>
Botulism antitoxin	Horse	Antibodies raised against toxins formed by type A, B or E <i>Clostridium botulinum</i>
Diphtheria antitoxin	Horse	Antibodies raised against diphtheria toxin or toxoid
Gas gangrene antitoxins	Horse	Antibodies raised against the $\alpha$ -toxin of <i>Clostridium novyi</i> , <i>C. perfringens</i> or <i>C. septicum</i>
Scorpion venom antisera	Horse	Antibodies raised against venom of one or more species of scorpion
Snake venom antisera	Horse	Antibodies raised against venom of various poisonous snakes
Spider antivenins	Horse	Antibodies raised against venom of various spiders, in particular the black widow spider

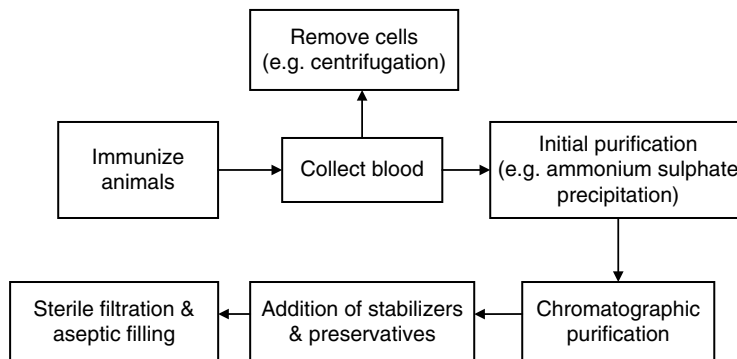
capable of recognizing the pathogenic agent will afford transient immunological protection.

Specific antiserum preparations are raised in large animals, of which horses are the most popular. This is achieved by injecting the antigen of interest into the animal, thus initiating an immunological response. Booster shots of the antigen in question may be administered subsequently in order to further heighten the antibody response. Samples of blood are withdrawn from the immunized animal at regular intervals and the antibody titre measured by an appropriate assay. When large quantities of antibodies which specifically recognize the antigen of interest are detected, the animal is normally bled.

When large animals such as horses are used, several litres of blood may be withdrawn during each bleed. The blood is then allowed to clot and the resulting antiserum subsequently recovered. Alternatively, the blood may be withdrawn directly into containers containing suitable anticoagulants and centrifuged to remove blood cells, thus producing plasma. The serum/plasma is then subjected to fractionation in order to recover a purified antibody preparation. Traditional purification protocols employ several sequential precipitation steps, usually using ethanol and/or ammonium sulfate as precipitants. More recent purification protocols employ various chromatographic steps (Figure 7.5) including ion-exchange, hydrophobic interaction, or protein A chromatography. All procedures used in the collection, manipulation and purification of antiserum preparations are carried out in accordance with the principles of good manufacturing practice.

Following antibody purification, the potency of the product is determined and adjusted to a suitable strength by dilution or concentration, as appropriate. Stabilizing agents such as NaCl or glycine are often added, as are antimicrobial agents such as phenol or thiomersal. The antiserum preparation is sterilized by filtration and filled by aseptic technique into sterile containers which are subsequently sealed to preserve sterility. The antiserum preparation may also be lyophilized. The antibody solution is often filled under an oxygen-free nitrogen atmosphere in order to prevent oxidative degradation of the product during long-term storage. The product is normally stored at 2–8°C. Under such conditions it should have a shelf-life of anything up to 3–5 years.

Immunoglobulin preparations are purified from donated blood obtained from human volunteers. The methods of purification used are broadly similar to those used in the purification of antibodies from animal sources. Immunoglobulins purified from blood donations obtained from normal healthy individuals usually contain a wide variety of antibody specificities. Such specificities have been produced over the years as the donor came into contact, either naturally or artificially (by vaccination), with a variety of antigens. Alternatively, high titres of antibodies that recognize specific antigens may be purified from donated blood obtained from individuals who have been immunized with that antigen or who have recently recovered from an illness caused by the antigenic substance. For example, persons recently vaccinated against hepatitis B or who have



**Figure 7.5** Overview of a generalized production protocol used to enrich/purify polyclonal antibodies for therapeutic use.

suffered from hepatitis B infection would generally exhibit high titres of anti-hepatitis B antibodies.

Administration of antibody preparations may sometimes result in adverse clinical reactions. This is especially true if the antiserum preparation used is of animal origin. Adverse reactions potentially associated with administration of animal serum include serum sickness and, in severe cases, anaphylactic shock. For this reason it is preferable to use immunoglobulin preparations of human origin whenever possible. Serum from which antibody preparations are purified should only be obtained from healthy animals or humans, and should be screened for the presence of potential pathogens prior to processing. The range of pathogens for which donated blood is screened before use usually includes those listed here, although exact screening profiles differ from world region to world region:

- HIV;
- hepatitis B and C viruses;
- *Treponema pallidum* (syphilis);
- cytomegalovirus;
- Epstein–Barr virus (EBV);
- Creutzfeldt–Jakob disease.

## 7.4 Antibody therapeutics: monoclonal antibodies

Polyclonal antibodies and their mode of manufacture suffer from a number of potential disadvantages in the context of their therapeutic use, including:

- variability of initial and ongoing serum antibody levels achieved in the immunized animals;
- disease/death of the producer animals signals the end of the antibody source;
- the antibody preparations are a heterologous mixture of dozens or perhaps hundreds or more of different antibodies, each produced by a specific B lymphocyte;
- each individual antibody recognizes a different antigenic epitope (region on the antigen surface) and binds to the epitope with its own characteristic specificity and affinity.

If a single antibody-producing B lymphocyte could be isolated and cultured, it would represent a source of homogeneous monospecific (monoclonal) antibody. From a therapeutic perspective such a monoclonal antibody would be a more defined, specific product compared with a polyclonal preparation. Moreover, ongoing cell culture of the B-lymphocyte clone could also surmount several of the disadvantages listed above in the context of polyclonal antibody production. However, such antibody-producing cells display a finite lifespan and therefore cannot act as an indefinite antibody source.

Despite this, a number of approaches have been successfully developed that allow the production of monoclonal antibodies for therapeutic use, and the majority of antibody preparations that have been approved over the last two decades are monoclonal in nature. First-generation monoclonal antibodies were generated via hybridoma technology, while the vast majority of more recently approved products, as well as those currently in clinical trials, are produced by recombinant means.

### 7.4.1 Hybridoma technology

In the mid-1970s, a technique was developed that facilitates the production of monospecific antibodies derived from a single antibody-producing cell. This process is termed hybridoma technology (Box 7.1.) and first-generation therapeutic monoclonal antibodies were produced by this means (Table 7.2).

However, murine hybridoma-derived antibodies generally proved to be a therapeutic disappointment, for several reasons:

- murine monoclonal antibodies are themselves recognized as foreign by the human immune system;
- murine antibodies do not trigger effector functions in humans;
- because their Fc region does not recognize the human FcRn, murine monoclonal antibodies are not recycled into the blood after cellular uptake and as a result they display a short half-life (typically 30–40 hours) in humans.

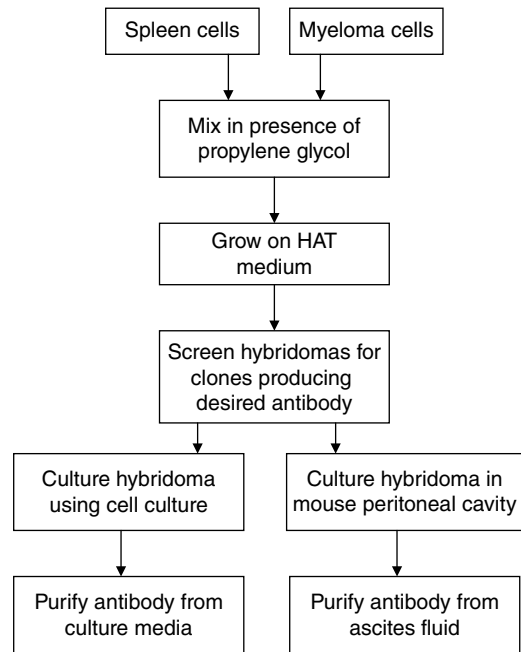
**Box 7.1 Hybridoma technology**

Hybridoma technology derives from the observation that if a population of antibody-producing cells are fused with immortal myeloma cells, a certain fraction of the resultant hybrid (hybridoma) cells will retain the immortal characteristics of the myeloma cell while secreting large quantities of monospecific antibody. The process begins with the immunization of a mouse with the antigen against which antibodies are required. The mouse is subsequently sacrificed (usually 1–4 weeks after initial immunization) and its spleen is removed. The spleen is the major anatomical site in the body where activation of B lymphocytes by blood-borne antigens occurs. Lymphoid cells are washed out of the spleen. Typically,  $10^8$  spleen-derived lymphocytes are fused with myeloma cells by co-incubation in the presence of polyethylene glycol. The myeloma cells used in the fusion protocol do not produce the enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT). Lack of this enzyme means they cannot synthesize DNA if grown in a medium containing hypoxanthine, aminopterin and thymidine (HAT medium).

A proportion of the daughter cells formed on incubation will be derived from the fusion of a single lymphocyte and a single myeloma cell. These daughter cells (hybridoma cells) often retain the antibody-producing characteristics of the parent lymphocyte and the immortal characteristics of the parent myeloma cell. Screening of such hybridoma cells after fusion is possible by culture in HAT medium. Unfused myeloma cells cannot grow on this medium, but fused cells (by inheriting the HGPRT enzyme from the parent lymphocyte) can. Unfused lymphocytes do not grow well on this medium and either die or are rapidly overgrown by hybrid cells, which double every 18–48 hours. Hybridoma cells growing on HAT medium can be separated from each other by serial dilution, with subsequent culture of individual cells to form clones. Screening of

these clones pinpoints those producing the antibody of interest. Clones can be stored deep frozen under liquid nitrogen if required.

Growth of these cells to produce monoclonal antibodies can be undertaken by two means. Direct cell culture *in vitro* results in monoclonal antibody production to microgram per millilitre levels. Alternatively, the hybridoma cells can be injected into the peritoneal cavity of live mice, where the hybridomas grow as an ascitic tumour. The ascitic fluid produced contains the monoclonal antibodies, usually at levels 50–100 times those achievable by *in vitro* cell culture. However, large-scale production of monoclonal antibodies by this route has raised ethical issues and is compromised by the fact that the ascitic fluid is also contaminated by mouse immunoglobulins.



Antibody immunogenicity remains one of the inherent therapeutic limitations associated with administration of murine monoclonal antibodies to humans. A single injection of a murine monoclonal antibody will elicit an immune response in 50–80% of patients. Human anti-mouse antibodies (HAMA)

**Table 7.2** First-generation murine therapeutic monoclonal antibodies (Mabs) produced via hybridoma technology.

Product	Company	Indication	Approved
NeuroSpec (fanolesomab). Mab raised against CD15 surface antigen of selected leukocytes	Palatin Technologies, Inc./ Covidien Pharmaceuticals	Imaging of equivocal appendicitis	2004 (USA) Withdrawn 2005
OncoScint CR/OV (satumomab pentetide). Mab directed against TAG-72, a high-molecular-weight tumour-associated glycoprotein	Cytogen Corp	Detection/staging/follow-up of colorectal and ovarian cancers	1992 (USA) Withdrawn 2002
Orthoclone OKT3 (muromomab CD3). Mab directed against the T-lymphocyte surface antigen CD3	Janssen	Reversal of acute kidney transplant rejection	1986 (USA) Withdrawn 2011
ProstaScint (capromab pentetate). Mab directed against the tumour surface antigen PSMA	EUSA Pharma Inc.	Detection/staging/follow-up of prostate adenocarcinoma	1996 (USA)
Scintimun (besilesomab). Mab specific for NCA-95 found on surface of granulocytes	Cisbio International	<i>In vivo</i> diagnosis/investigation of sites of inflammation/infection via scintigraphic imaging	2010 (EU)
Zevalin* (ibritumomab tiuxetan). Mab targeted against the CD20 antigen	Bayer Pharma AG/Spectra Pharmaceuticals Inc.	Non-Hodgkin lymphoma	2002 (USA) 2004 (EU)

\* Zevalin is a murine monoclonal antibody originally produced via hybridoma technology. However, at an early stage of product development the antibody genes were isolated from the hybridoma and expressed in a CHO cell line, in which the commercialized product is produced.

will generally be detected within 14 days of antibody administration. The HAMA response will effectively and immediately destroy subsequent doses of monoclonal antibody administered. In practice, therefore, therapeutic efficacy of murine monoclonal antibodies is usually limited to the first and, at most, the second dose administered.

### 7.4.2 Overcoming the limitations of murine monoclonal antibodies

An obvious strategy for overcoming the immunogenicity problem would be the generation and use of monoclonal antibodies of human origin. This is possible but difficult, using traditional technologies at least. Human antibody-producing lymphocytes can potentially be rendered immortal by:

- transformation by EBV infection;
- fusion with murine myelomas;
- fusion with human lymphoblastoid cell lines.

However, a number of technical hurdles remain that prevent routine production of human monoclonal preparations by such means, including:

- source of antibody-producing cell;
- reliable methods for lymphocyte immortalization;
- stability and antibody-producing capacity of resulting immortalized cells.

Initial stages in the production of murine monoclonal antibodies entail administration of the antigen of interest to a mouse. This is followed by sacrifice and recovery of activated B lymphocytes from the spleen. A similar approach to the production of human monoclonal antibodies would be unethical. Administration of some antigens to humans could endanger their health. Although B lymphocytes could be obtained from the peripheral circulation, the majority of these are unstimulated, and recovery of (stimulated) B lymphocytes from the spleen is impractical.

Although EBV is capable of inducing cellular transformation, few antibody-producing B lymphocytes display the viral cell-surface receptor. Most, therefore, are immune to EBV infection. Even on successful transformation, most produce low-affinity IgM antibodies, and the cells are often unstable. Having said that, one monoclonal antibody approved for medical use (Humaspect) is produced by a human lymphoblastoid cell line originally transformed by EBV.

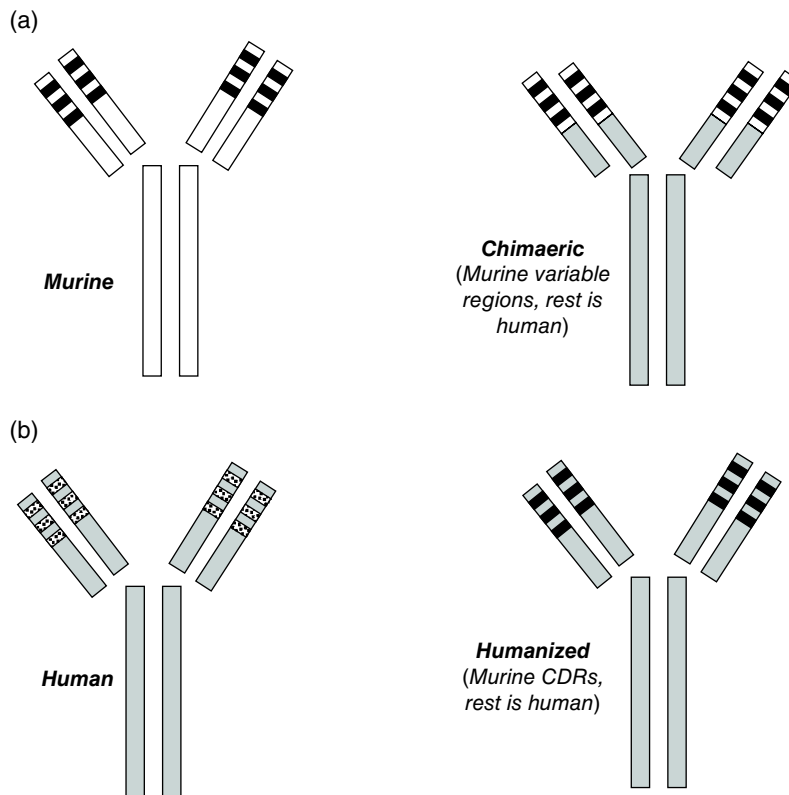
Fusion of human lymphocytes with human lymphoblastoid cell lines is a very inefficient process. Similarly, fusion of human lymphocytes with murine myeloma cells leads to very unstable hybrids. On fusion, preferential loss of human genetic elements is often observed. Unfortunately, particularly common is the loss of chromosomes 2, 14 and 22, which encode antibody light and heavy chain loci. The production yields of human monoclonal antibodies on immortalization of the human B lymphocyte (by whatever means) are also low.

### 7.4.3 Chimeric and humanized antibodies

Recombinant DNA technology has provided an effective means of reducing the innate immunogenicity of murine monoclonal antibodies. The approach entails

initial production of a murine monoclonal antibody against the target antigen via standard hybridoma technology. The nucleotide sequences coding for the murine antibody can thus be identified. The sequences coding for the antigen-binding regions of this murine antibody can be isolated and fused to nucleotide sequences coding for the remaining regions of a human antibody. In this way, the engineered antibody demonstrates considerable human (i.e. non-immunogenic) sequences while retaining the original murine antibody-binding specificity. Moreover, this approach allows the genetic manipulation of the murine nucleotide sequence, potentially facilitating the development of variant antibodies with improved antigen-binding properties.

Initial genetic manipulations centred around production of hybrid or chimeric antibody molecules consisting of mouse variable regions and human constant regions (Figure 7.6). Such



**Figure 7.6** Production of (a) chimeric and (b) humanized antibodies (via recombinant DNA technology). Chimeric antibodies consist of murine monoclonal  $V_H$  and  $V_L$  domains grafted onto the Fc region of a human antibody. Humanized antibody consists of murine CDR regions grafted into a human antibody.

antibodies are significantly less immunogenic compared with unaltered murine monoclonal antibodies. However, chimeric antibodies still retain significant portions of mouse sequence (the entire variable region) and these sequences generally still induce substantial immunological responses in humans.

The immunogenicity of murine monoclonal antibodies may be further reduced by 'grafting' only the DNA sequences coding for the antigen-binding CDR regions of the parent murine monoclonal antibody into the DNA sequences coding for a human antibody (Figure 7.6). The resultant humanized monoclonal antibody, while retaining the antigen-binding specificity of the murine parental antibody, is almost entirely human in sequence. It therefore does not elicit a significant antigenic response when administered to humans.

The successful production of chimeric and humanized monoclonal antibodies also overcomes the inability of first-generation fully murine monoclonal antibodies to mediate a variety of antibody effector functions in recipient human patients. Chimeric and humanized antibodies approved for general medical use are presented in Table 7.3.

#### **7.4.4 Fully human monoclonal antibodies**

Advances in molecular techniques now facilitate the production of entirely human antibodies, and a significant and growing proportion of more recently approved monoclonal antibodies fall into this category (Table 7.4). The overall approach to fully human monoclonal antibody production entails the initial isolation of a human antibody gene repertoire, often from human peripheral blood lymphocytes, the cloning and expression of a library of these genes, with subsequent screening in order to identify the antibody of interest. The coding sequence for the desired human antibody can then be expressed in a cell line suitable for ongoing routine product manufacture (Figure 7.7).

In practice, two molecular approaches have come to dominate human antibody library expression, that of phage display and the use of transgenic mice. Phage display allows the efficient expression of a library of genes coupled with convenient screening of the gene products for the target protein of choice (Box 7.2.). Thus, in the context of human monoclonal antibody development, the coding sequences of the antibody gene library (usually only the sequences coding for variable regions, which dictate antigen-binding specificity) are expressed in the phage-based system, facilitating screening using immobilized antigen. After the appropriate binding specificities are identified, their coding sequences are ligated into a whole human IgG expression vector. Subsequent transfection into appropriate producer cells facilitate routine monoclonal antibody manufacture (Figure 7.7).

The generation of a human monoclonal antibody via transgenic mouse technology involves the use of mice in which human IgG genes replace the endogenous mouse antibody genes. These knockout/knockin mice can then be vaccinated with any antigen of interest, their spleens isolated, with subsequent generation of human IgGs via traditional hybridoma techniques.

#### **7.4.5 Purification of therapeutic monoclonal antibodies**

The preceding sections outline the various approaches that may be taken to generate monoclonal antibodies for therapeutic use. Whatever the developmental approach, the full-length antibodies are generally produced in appropriate mammalian cell lines. CHO, NS0 or Sp2/0 cells are most typically used (see Tables 7.2, 7.3 and 7.4) as these not only grow robustly to high cell densities but achieve the most appropriate glycosylation patterns on the antibody Fc component (see discussion of the glycocomponent in section 7.9). The exact details of downstream processing vary from antibody product to antibody product, but most incorporate a protein A affinity chromatography step (see Chapter 3). The steps employed in the purification of one monoclonal antibody (Avastin) is overviewed in Box 7.3.

**Table 7.3** Chimeric and humanized monoclonal antibodies (Mabs) that have gained approval for general medical use in the EU and/or the USA. Many are also marketed in other world regions.

Product	Company	Indication	Approved
Actemra/RoActemra (tocilizumab). Humanized Mab specific for IL-6. Produced in a CHO cell line	Genentech/Roche	Rheumatoid arthritis	2010 (USA) 2009 (EU)
Adcetris (brentuximab vedotin). Chimeric Mab conjugate specific for human CD30 (expressed on the surface of lymphoma cells). Produced in a CHO cell line	Seattle Genetics Inc.	Lymphoma	2011 (USA)
Avastin (bevacizumab). Humanized Mab raised against vascular endothelial growth factor. Produced in a CHO cell line	Genentech/Roche	Carcinoma of the colon or rectum, metastatic breast cancer	2004 (USA) 2005 (EU)
Erbix (cetuximab). Chimeric Mab raised against human EGF receptor. Produced in a Sp2/0 cell line	ImClone Systems/ Bristol-Myers Squibb	Treatment of EGF receptor-expressing metastatic colorectal cancer	2004 (USA, EU)
Herceptin (trastuzumab). Humanized Mab directed against human epidermal growth factor receptor (HER)2. Produced in a murine hybridoma cell line	Genentech/Roche	Treatment of metastatic breast cancer if tumour overexpresses HER2 protein	1998 (USA) 2000 (EU)
MabCampath (EU) or Campath (USA) (alemtuzumab). Humanized Mab directed against CD52 surface antigen of B lymphocytes. Produced in a CHO cell line	Genzyme	Chronic lymphocytic leukaemia	2001 (EU, USA)
Mabthera (EU) or Rituxan (USA) (rituximab). Chimeric Mab directed against CD20 surface antigen of B lymphocytes. Produced in a CHO cell line	Roche/Genentech	Non-Hodgkin lymphoma	1997 (USA) 1998 (EU)
Mylotarg (gemtuzumab zogamicin). Humanized Mab-toxic antibiotic conjugate targeted against CD33 antigen found on leukaemic blast cells. Produced in an NS0 cell line	Pfizer/Wyeth	Acute myeloid leukaemia	2000 (USA) Withdrawn 2010
Perjeta (pertuzumab). Recombinant humanized Mab specific for HER2. Produced in a CHO cell line	Genentech	Breast cancer	2012 (USA)
Raptiva (efalizumab). Humanized Mab that binds to the LFA-1 antigen, which is expressed on all leukocytes. Produced in a CHO cell line	Genentech, Serono	Treatment of adult patients with chronic moderate to severe plaque psoriasis	2003 (USA) 2004 (EU) Withdrawn 2009
Remicade (infliximab). Chimeric Mab directed against TNF- $\alpha$ . Produced in a Sp2/0 cell line	Janssen	Treatment of Crohn's disease	1998 (USA) 1999 (EU)
Simulect (basiliximab). Chimeric Mab directed against the $\alpha$ -chain of the IL-2 receptor. Produced in a murine myeloma cell line	Novartis	Prophylaxis of acute organ rejection in allogeneic renal transplantation	1998 (EU, USA)
Soliris (eculizumab). Humanized IgG that binds human C5 complement protein. Produced in a murine myeloma cell line	Alexion	Paroxysmal nocturnal haemoglobinuria	2007 (EU, USA)
Synagis (palivizumab). Humanized Mab directed against an epitope on the surface of respiratory syncytial virus. Produced in a murine myeloma cell line	MedImmune, Abbott	Prophylaxis of lower respiratory tract disease caused by respiratory syncytial virus in paediatric patients	1998 (USA) 1999 (EU)
Tysabri (natalizumab). Humanized Mab raised against selected leukocyte integrins. Produced in a murine myeloma cell line	Biogen Idec Inc. Elan	Treatment of patients with relapsing forms of multiple sclerosis	2006 (EU) (USA: approved 2004, suspended 2005, resumed 2006)
Xolair (omalizumab). Humanized Mab that binds IgE at the site of high-affinity IgE receptor binding. Produced in a CHO cell line	Genentech/Roche Novartis	Treatment of adults/adolescents with moderate to severe persistent asthma	2003 (USA)
Zenapax (daclizumab). Humanized Mab directed against the $\alpha$ -chain of the IL-2 receptor. Produced in a NS0 murine cell line	Roche	Prevention of acute kidney transplant rejection	1997 (USA) 1999 (EU) Withdrawn 2009

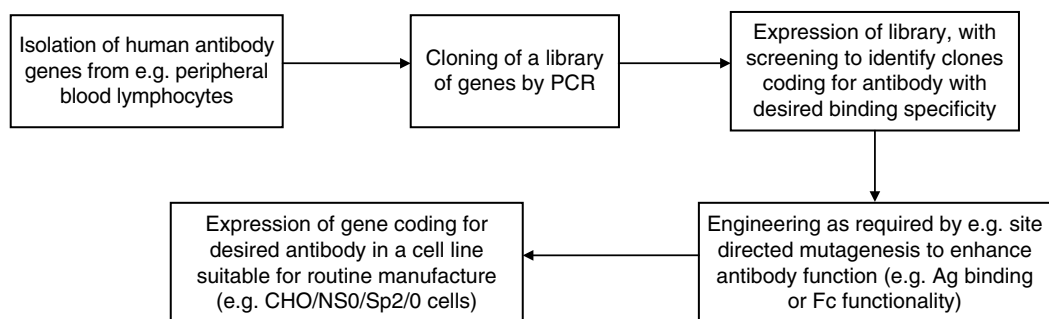
TNF, tumour necrosis factor.



**Table 7.4** Fully human monoclonal antibodies (Mabs) that have gained approval for general medical use in the EU and/or the USA. Many are also marketed in other world regions.

Product	Company	Indication	Approved
Azerra (ofatumumab). Mab specific for CD20 antigen. Produced in an NS0 cell line	Glaxo	Chronic lymphocytic leukaemia	2010 (EU) 2009 (USA)
Benlysta (belimumab). Mab specific for human B-lymphocyte stimulator (BLyS), a B-cell survival factor. Produced in an NS0 cell line	Glaxo, Human Genome Sciences	Lupus	2011 (USA, EU)
Humaspect (votumumab). Mab specific for cytokeratin tumour-associated antigen. Produced in a human lymphoblastoid cell line	KS Biomedix	Detection of carcinoma of the colon or rectum	1998 (EU) Withdrawn, 2004
Humira (adalimumab). Mab specific for TNF- $\alpha$ . Produced in a CHO cell line	Abbott	Rheumatoid arthritis	2002 (USA) 2003 (EU)
Ilaris (canakinumab). Mab specific for IL-1 $\beta$ . Produced in an Sp2/0 cell line	Novartis	Cryopyrin-associated periodic syndromes (CAPS)	2009 (USA, EU)
Prolia/Xgeva (denosumab). Mab specific for 'RANK' ligand. Produced in a CHO cell line	Amgen	Osteoporosis in postmenopausal women	2010 (EU, USA)
Raxibacumab. Mab specific for the <i>Bacillus anthracis</i> toxin. Produced in a murine cell line	GlaxoSmithKline	Inhalational anthrax	2012 (USA)
Simponi (golimumab). Mab specific for TNF- $\alpha$ . Produced in a Sp2/0 cell line	Janssen/Centocor	Rheumatoid arthritis, psoriatic arthritis and ankylosing spondylitis	2009 (USA, EU)
Stelara (ustekinumab). Mab specific for the p40 subunit of IL-12 and IL-23. Produced in a Sp2/0 cell line	Janssen	Moderate to severe plaque psoriasis	2009 (USA, EU)
Vectibix (panitumumab). Mab specific for EGFR. Produced in a CHO cell line	Amgen	EGFR-expressing colorectal carcinoma	2007 (EU) 2006 (USA)
Yervoy (ipilimumab). Mab specific for CTLA-4 (a negative regulator of T-cell activation), thereby enhancing T-cell activation and proliferation. Produced in a CHO cell line	Bristol-Myers Squibb	Melanoma	2011 (USA, EU)

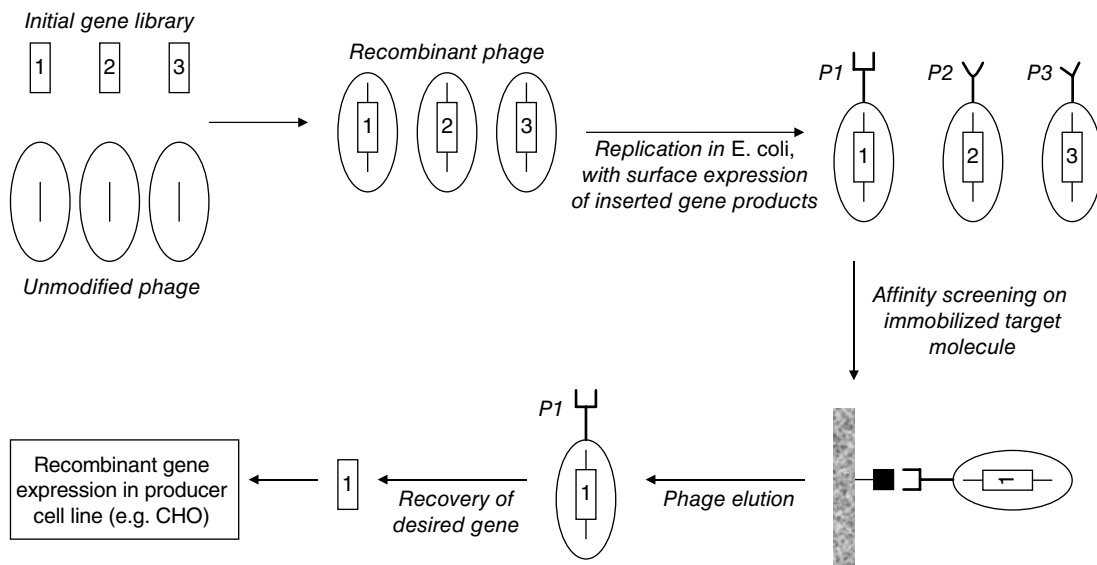
EGFR, epidermal growth factor receptor; TNF, tumour necrosis factor.

**Figure 7.7** Overview of the general approach undertaken to produce fully human monoclonal antibodies. Refer to text for further details.

**Box 7.2 Phage display technology**

Phage display technology provides an efficient way to express a library of protein ligands and subsequently to conveniently screen these ligands for their ability to bind a selected target molecule. The technique uses filamentous phage (bacteriophage) which replicate in *Escherichia coli*. A library of genes (one of which codes for the protein of interest) is first generated/obtained. These genes are inserted (batch cloned) into a phage library fused to a gene encoding one of the phage coat proteins (pIII, pIV or pVIII). The phage are then incubated with *E. coli*, which facilitates phage replication. Expression of the fusion gene product during replication and the subsequent incorporation of the fusion product into the mature phage coat results in the gene product being 'presented' on the phage surface. The entire phage library can then be screened in order to identify the one(s) coding for the protein of interest. This is usually achieved by affinity selection (biopanning). Biopanning entails passing the library over immobilized target molecules, usually in immobilized column format. Only the phage expressing the protein of desired

specificity should be retained in the immobilized column. The bound phage can subsequently be eluted by reducing the pH of the elution buffer or by inclusion of a competitive ligand, usually free target molecules, in the buffer. Eluted phage can then be re-passed over the affinity column in order to isolate those binding the immobilized ligand with the highest specificity/affinity. Once this is achieved, the gene coding for the protein of interest can be excised from the phage genome by standard techniques. It can then be incorporated into an appropriate microbial/animal cell/transgenic expression system, facilitating large-scale production of the gene product. In the simplified diagrammatic representation below, the initial library consists of three genes, with gene number 1 coding for the protein of interest. In reality, libraries can consist of millions to tens of millions of different genes. Variations of the phage display approach have been developed, some of which utilize engineered phage (phagemids) while others achieve library expression not on the surface of phage but on the surface of bacteria.



Modified from Walsh, G. (2007) *Pharmaceutical Biotechnology: Concepts and Applications*. John Wiley & Sons Ltd, Chichester.

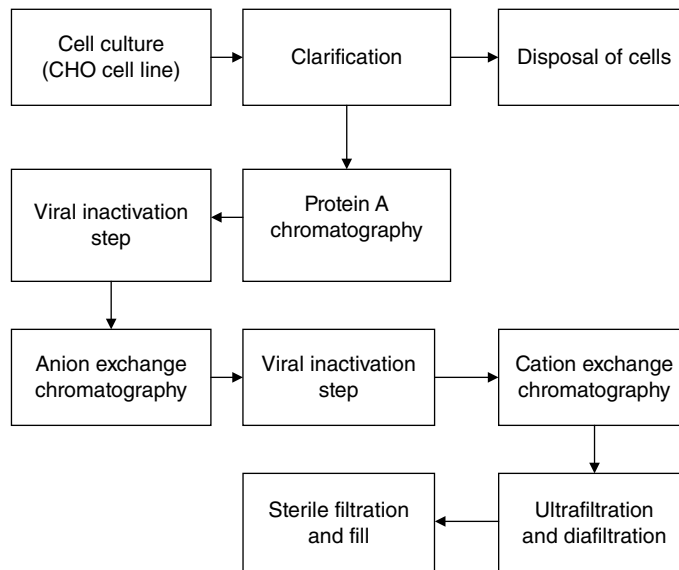
**Box 7.3 Product case study: Avastin**

Avastin (bevacizumab) is a humanized monoclonal antibody produced in an engineered CHO cell line. Approximately 93% of its sequence is human in origin, with the remaining 7% being murine-derived. It is manufactured and marketed by Genentech/Roche and is one of the best-selling biopharmaceuticals globally, generating revenues of just over \$6 billion in 2012. Avastin is indicated for first-line treatment of patients with metastatic carcinoma of the colon or rectum. The incidence of colorectal cancer in Western society is particularly high, with in the region of 190,000 deaths and 350,000 new cases diagnosed each year.

Avastin brings about its therapeutic effect by inhibiting angiogenesis (the formation of new blood vessels), a process required to support tumour growth. Angiogenesis is driven by binding of human vascular endothelial growth factor (VEGF) to VEGF receptors found on the surface of vascular endothelial cells. Binding results in receptor activation via triggering of a tyrosine phosphorylation event, which in turn leads to a series of mitogenic and pro-survival activity signals within the vascular endothelial cells. Binding of Avastin to VEGF prevents VEGF interaction with its cell-surface receptor, hence

preventing new blood vessel formation. VEGF receptor expression on most normal cells is low or undetectable but is significantly upregulated in the vasculature of colorectal and several other tumour types, contributing to the specificity of the antibody effect.

Production-scale manufacture involves culture of the CHO producer cell line in 12,000-L suspension cell culture vessels, using a serum-free, low-protein medium supplemented with recombinant human insulin. Following upstream processing the cells are removed (by centrifugation) and the product is then purified from the spent medium. An initial protein A affinity chromatography step is particularly effective in the removal of other CHO-derived proteins and DNA. The subsequent anion exchange step (using Q sepharose FF chromatographic media) is followed by a cation exchange step (using CM sepharose FF) and finally the product stream is subject to ultrafiltration/diafiltration. Downstream processing also incorporates two viral inactivation steps. The final product, formulated in a phosphate buffer, pH 6.2, also contains trehalose and polysorbate 20 as excipients and is filled into final-product containers after a sterilizing filtration step using a 0.22- $\mu\text{m}$  filter.



## 7.5 Therapeutic applications of monoclonal antibodies

Monoclonal antibodies continue to be used for various therapeutic purposes (see Tables 7.2, 7.3 and 7.4). In broad terms some applications are predicated on the ability of the antibody to neutralize or block the biological activity of a target antigen, while others rely on the ability of bound antibody to trigger target antigen destruction via the recruitment of effector functions such as ADCC (see Figure 7.2). In the latter case the target cell is most often a cancer cell.

### 7.5.1 Monoclonal antibody-mediated antigen neutralization

Many diseases are caused or exacerbated by the inappropriate expression or overexpression of specific signalling molecules, and the disease or its symptoms can be ameliorated by neutralization or blocking of the signalling molecule. A prominent example is that of certain inflammatory conditions such as rheumatoid arthritis and Crohn's disease, both conditions being initiated by the overproduction of proinflammatory cytokines, particularly tumour necrosis factor (TNF)- $\alpha$ . Administration of antibodies that neutralize or block the signalling activity of such molecules can therefore potentially treat or counteract the disease. Several such products are on the market (Table 7.5) and many more are in clinical trials.

### 7.5.2 Monoclonal antibody-mediated cell destruction

Several anticancer antibodies on the market bring about their therapeutic effect via binding to an antigen found on the target cancer cell surface (Table 7.6). Although the mode of action may be complex, antibody binding likely triggers cell death via the induction of effector functions, most notably ADCC and/or CDC. In an ideal scenario, the target

cell-surface antigen chosen would be uniquely associated with the target cell, though this level of specificity is rarely attained in practice.

When a healthy (untransformed) cell turns cancerous it begins to express a number of genes which are either unexpressed or expressed at lower levels in the pre-cancer state. Some of these newly synthesized proteins reside on the surface of the transformed cell and such tumour surface antigens (TSAs) can therefore represent an appropriate target for antibody binding.

An example is that of the human epidermal growth factor receptor (HER)2 protein, which is overexpressed in approximately 30% of human breast tumours. Moreover, HER2 overexpression is a prognostic factor of poor survival. HER2 is a 185-kDa transmembrane receptor that is structurally related to the epidermal growth factor receptor. As a result, cells overexpressing HER2 are especially sensitive to mitogenic stimulation by normal amounts of this growth factor. Its overexpression can be triggered by genomic amplification or by a mutation in the protein-enhancer control region of the encoding gene (the cellular *c-erbB2* proto-oncogene), leading to increased transcription. Herceptin (Table 7.6) is a humanized antibody that targets HER2 and hence is used to treat HER2-overexpressing breast tumours. Its likely mode of manufacture is presented in Figure 7.8.

The development of TSA-based anticancer antibody-based therapies is complicated by a number of factors.

- For many cancers appropriate TSAs have not been thus far identified.
- There is a lack of targeting specificity if the TSA is expressed by additional cell types.
- In some cases tumours secrete a portion of the TSAs they produce in soluble form into the bloodstream. These soluble TSAs will mop up some of the antibody administered.
- In some instances TSA expression is neither static nor uniform. At any given time a proportion of tumour cells may not be expressing the TSA at all.
- In other cases antibody cross-reacts with host antigens bearing a structural resemblance to the TSA.

**Table 7.5** Some approved therapeutic monoclonal antibodies that bring about their effect by neutralizing/blocking the biological activity of a target signalling ligand.

Antibody product	Target ligand	Comment
Actemra/RoActemra (see Table 7.3)	IL-6 receptor	This humanized antibody is used to treat rheumatoid arthritis. It binds specifically to the IL-6 cell-surface receptor. This prevents binding of IL-6, and hence effectively inhibits the biological activity of this proinflammatory cytokine
Avastin (see Table 7.3)	VEGF	This humanized antibody is used to treat patients with colorectal cancer. It binds specifically to human vascular endothelial growth factor (VEGF). Binding to VEGF prevents VEGF interaction with its cell-surface receptor, a process central to the triggering of new blood vessel growth, which is required to support tumour growth
Erbix (see Table 7.3)	EGFR	This chimeric antibody is used to treat (EGFR-expressing) metastatic colorectal cancer. The antibody is directed against the human epidermal growth factor receptor (EGFR). The product acts as a competitive antagonist, preventing binding of native EGF to its receptor, thereby blocking EGFR activation and signal transduction. This in turn inhibits cell growth and triggers apoptosis
Humira (see Table 7.4)	TNF- $\alpha$	This human antibody is used to treat rheumatoid arthritis. It binds specifically to tumour necrosis factor (TNF)- $\alpha$ , thus preventing the latter from binding to its cell-surface receptor, an event which would trigger various proinflammatory processes
Ilaris (see Table 7.4)	IL-1 $\beta$	This human antibody is used to treat cryopyrin-associated periodic syndromes (CAPS), an autoinflammatory condition characterized by the overproduction of IL-1 $\beta$ , driving inflammation. The antibody binds human IL-1 $\beta$ , neutralizing it
Remicade (see Table 7.3)	TNF- $\alpha$	This chimeric antibody is used to treat rheumatoid arthritis. It binds specifically to TNF- $\alpha$ , and thus has a mode of action similar to Humira
Simponi (see Table 7.4)	TNF- $\alpha$	This human antibody is used to treat rheumatoid arthritis. It binds specifically to TNF- $\alpha$ , and thus has a mode of action similar to Humira and Remicade
Stelara (see Table 7.4)	p40 subunit of IL-12 and IL-23	This human antibody is used to treat adults with moderate to severe plaque psoriasis under certain conditions. Psoriasis is a chronic inflammatory skin condition caused by migration and over-activation of T lymphocytes in the epidermis, a process that is fuelled in part by IL-12 and IL-23. Stelara, by binding to the p40 subunit of IL-12 and IL-23, prevents them from triggering a biological response
Xolair (see Table 7.3)	IgE	This humanized antibody is used to treat people with moderate to severe persistent asthma. It brings about its effect by binding and neutralizing IgE, a major mediator of many allergic reactions
Yervoy (see Table 7.4)	CTLA-4	This human antibody is used to treat melanoma. It binds cytotoxic T lymphocyte-associated antigen (CTLA)-4, a negative regulator of T-cell activation. Binding blocks CTLA-4 interaction with its receptor (CD80/CD86), thereby enhancing T-cell activation and proliferation
Zenapax (see Table 7.3)	$\alpha$ -chain of the IL-2 receptor	This humanized antibody is used as an immunosuppressant to prevent acute organ rejection in kidney transplant recipients. It binds the $\alpha$ -chain of the IL-2 receptor found on the surface of activated lymphocytes. This in turn blocks binding of IL-2 to the receptor, thus preventing IL-2 mediated stimulation of the lymphocytes involved in organ rejection

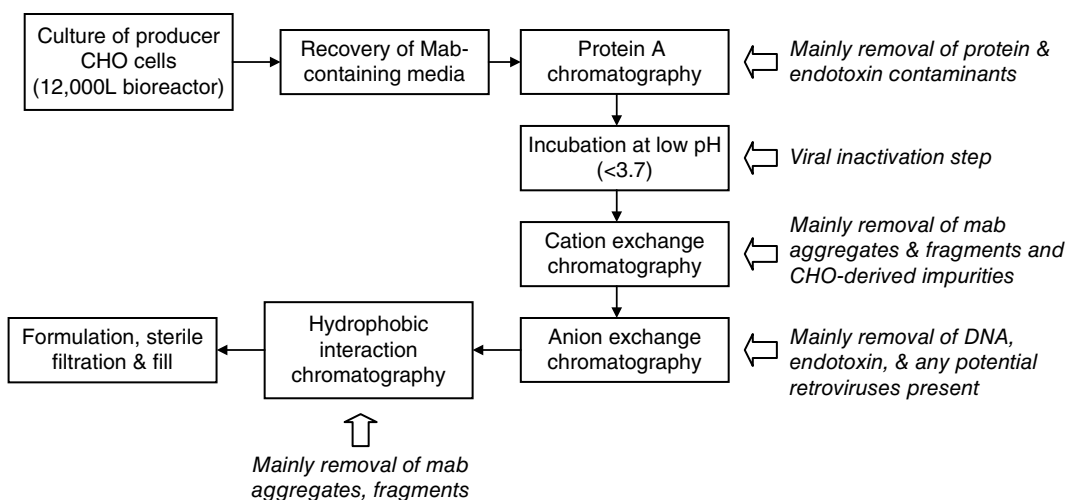
Carcinoembryonic antigen (CEA), a well-known TSA associated with colorectal cancer cells, illustrates some of these difficulties. CEA is also expressed by some breast and lung cancer cell types, and at low levels by some untransformed cells in the lactating breast, as well as colonic mucosal cells. In addition, cells that synthesize CEA also secrete a proportion of this antigen into the blood in soluble

form. Elevated levels of soluble serum CEA can also be associated with several medical conditions including inflammatory bowel disease, cirrhosis and hepatitis.

Another example of lack of specificity in the context of the TSA targeted is provided by the antibody product Mabcapth (Table 7.6), which targets a cell-surface antigen (CD52) present on the surface of

**Table 7.6** Some monoclonal antibody-based products approved for the treatment of cancer that bring about their effect via triggering of various antibody-mediated immune effector functions on binding to the cancer cell surface.

Antibody product	Target ligand	Comment
Herceptin (see Table 7.3)	HER2	This humanized antibody is used to treat patients with metastatic breast cancer whose tumours overexpress the human epidermal growth factor receptor (HER)2. Antibody binding to the cancer cell surface mediates ADCC, believed to be the principal mode by which the tumour cells are destroyed
MabCampath (see Table 7.3)	CD52	This humanized antibody is used to treat patients with B-cell chronic lymphocytic leukaemia. It binds to CD52, an antigen present on the surface of the transformed lymphocytes (as well as several additional cell types). The proposed mechanism of action is ADCC-mediated lysis following antibody cell surface binding
Mabthera/Rituxan (see Table 7.3)	CD20	This chimeric antibody is used to treat lymphoma and leukaemias. It binds specifically to the CD20 antigen (human B-lymphocyte-restricted differentiation antigen, Bp35), found on the surface of normal and malignant B cells, inducing cell lysis. Possible mechanisms of cell lysis include CDC and ADCC. The bound antibody may also induce cell apoptosis
Arzerra (see Table 7.4)	CD20	This human antibody is used to treat chronic lymphocytic leukaemia. It binds to the CD20 antigen, and thus likely has a mode of action similar to that of Mabthera/Rituxan



**Figure 7.8** Overview of the likely manufacturing scheme of the antibody product Herceptin.

transformed B lymphocytes. However, this antigen is also found on the surface of a whole range of non-transformed (mainly) immune cell types, including B and T lymphocytes, a majority of monocytes, macrophages, NK cells, and some granulocytes. A proportion of bone marrow cells also express variable levels of CD52. As a result, product administration typically also triggers depletion of untransformed lymphocytes and other immune

system cells, substantially increasing the risk of infections during treatment.

The use of antibodies to trigger cellular destruction is not only limited to cancer cells. In principle the approach can be applied to virtually any cell type. In 1986, OKT3 became the first monoclonal antibody to be approved for an *in vivo* therapeutic purpose. This antibody is used to promote reversal of acute kidney transplant rejection. OKT3

specifically recognizes a cell-surface antigen known as CD3 (CD denotes 'cluster of differentiation') that is associated with virtually all T cells. Binding of the antibody to CD3 can induce destruction of T cells (these cells usually mediate rejection of transplanted tissue).

## 7.6 Antibody conjugates

The binding of antibody alone to a cell surface can trigger cellular destruction via activation of effector functions. However, several approaches have been pursued with a view to potentiating the killing activity of such products (Figure 7.9):

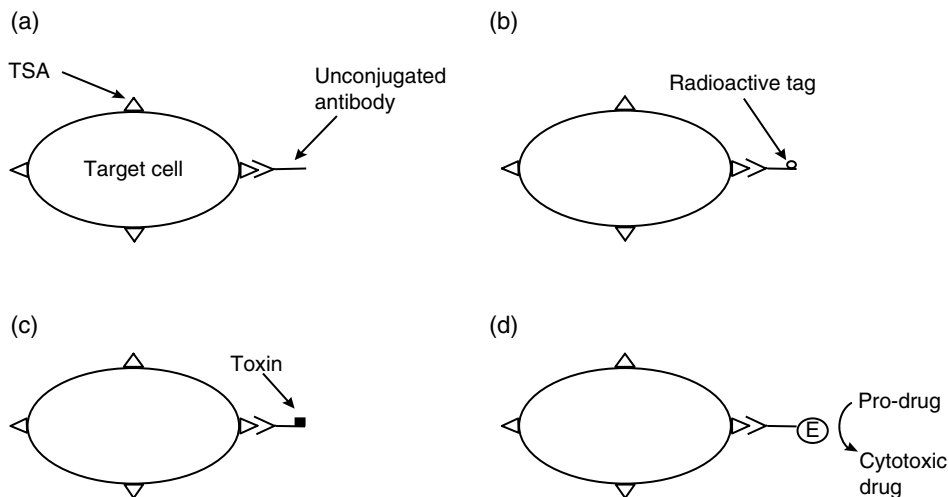
- administration of antibody to which a radioactive tag has been conjugated;
- administration of antibody to which a cytotoxic agent has been conjugated;
- administration of antibody to which a prodrug-activating enzyme has been conjugated.

In all cases the antibody, in addition to itself potentially triggering cell death, will target the conjugated substance to the cell surface, effectively providing targeted radiotherapy or chemotherapy.

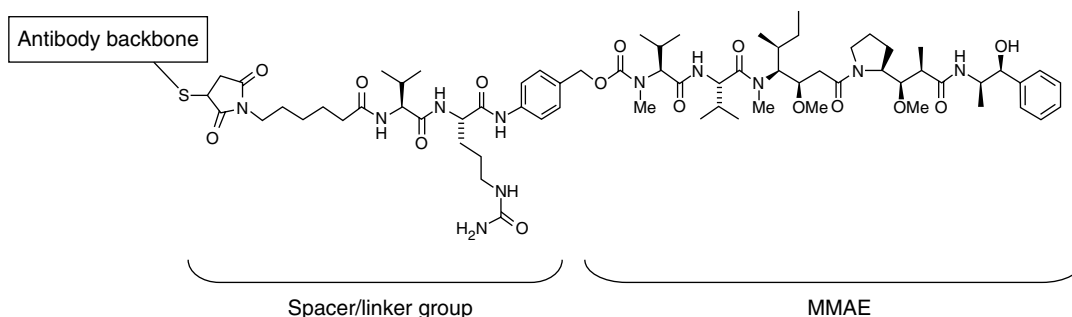
### 7.6.1 Radiolabelled antibody conjugates

Radioactively tagged antibodies are most often labelled with  $\beta$ -emitting (e.g. yttrium 90) or  $\alpha$ -emitting (e.g. bismuth 212 or astatine 211) isotopes.  $\beta$ -Particles can penetrate several layers of cells, and will kill these cells via the ionizing effects of radiation.  $\alpha$ -Particles have a shorter effective path length, but each  $\alpha$ -emission has a greater likelihood of killing all cells in its path. Antibody-mediated targeted radiotherapy has proven effective in promoting regression of various tumours, although obviously a proportion of healthy cells in the immediate vicinity of the tumour will also be irradiated.

Zevalin represents a radiolabelled antibody targeted against the CD20 surface antigen and used to treat non-Hodgkin's lymphoma (lymphomas are solid tumours derived from transformed B or T lymphocytes). The antibody is chemically conjugated to a chelator (tiurexan) during downstream processing and formulated as a solution for injection. Immediately prior to its administration, it is chelated to the radionuclide  $^{90}\text{Y}$ , which is purchased by the end user separately to the antibody product. The resultant irradiation of cells with  $^{90}\text{Y}$ -derived



**Figure 7.9** The various antibody-mediated approaches that may be used to induce target cell destruction. Refer to text for details. TSA, tumour surface antigen.



**Figure 7.10** Chemical structure of monomethyl auristatin E (MMAE), the antineoplastic agent conjugated to the CD30-directed monoclonal antibody Adcetris. Refer to text for further details.

high-energy  $\beta$  radiation contributes to its mode of action. Radiolabelled antibodies (or more commonly antigen-binding antibody fragments) can also be used to detect target cells (i.e. for an *in vivo* diagnostic purpose), as considered in section 7.8.

### 7.6.2 Antibody–toxin conjugates

Conjugation of toxins to anti-TSA antibodies will result in targeted toxin delivery to the tumour cell surface. In many instances binding of an antibody–toxin conjugate results in the subsequent internalization of the conjugate, thus delivering the toxin inside the cell. Adcetris is a CD30-directed antibody–drug conjugate used to treat patients with Hodgkin’s lymphoma. The conjugated toxin is monomethyl auristatin E (MMAE), a synthetic anticancer agent (Figure 7.10). On average four molecules of MMAE are attached to each antibody molecule. Binding of the antibody conjugate to CD30-expressing cells is followed by endocytotic internalization and subsequent release of MMAE via proteolytic cleavage. MMAE is a microtubule disrupting agent. Binding to tubulin disrupts the microtubule network within the cell, inducing cell cycle arrest and death via apoptosis.

### 7.6.3 Antibody–enzyme conjugates

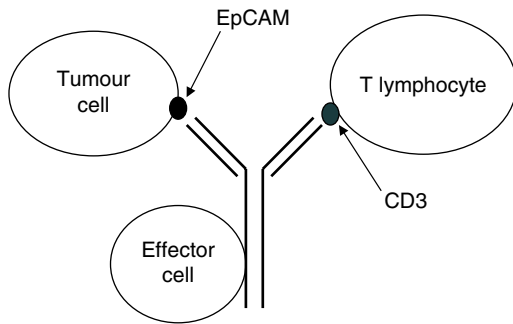
Antibodies to which prodrug-activating enzymes are conjugated are also being clinically assessed.

This approach to targeted cancer chemotherapy is termed antibody-directed catalysis or antibody-directed enzyme prodrug therapy (ADEPT). The enzyme used is chosen on the basis of its ability to catalytically convert an inactive prodrug into a cytotoxic agent. Alkaline phosphatase is one such example. It activates etoposide phosphate by dephosphorylating it. Clinically, the conjugate is first administered and allowed time to congregate at the tumour surface. The prodrug is then administered with its ensuing activation at the tumour surface.

## 7.7 Bispecific antibodies

Protein engineering makes it possible to combine the variable domains from two different parental antibody specificities by recombinant means. This generates so-called bispecific antibodies, in which each of the two monoclonal antibody arms bind different antigens. The first such bispecific antibody (trade name Removab) was approved initially within the EU in 2009 for the treatment of malignant ascites (fluid accumulation in the peritoneal cavity) in patients displaying EpCAM-positive carcinomas (cancers displaying the EpCAM surface antigen). The antibody comprises a mouse  $\kappa$  light chain, a rat  $\lambda$  light chain, a mouse IgG2a heavy chain and a rat IgG2b heavy chain. The antibody displays two different antigen-binding specificities: a mouse-derived epithelial cell adhesion (EpCAM)-binding Fab region and a rat-derived CD3-binding Fab region.





**Figure 7.11** Schematic of the bispecific antibody Removab, and the potential methods by which it may trigger the destruction of its tumour cell. One arm of the antibody binds the EpCAM antigen on the target cell surface, while the other binds the CD3 antigen found on the surface of T lymphocytes (cells which can mediate tumour destruction). Additionally, the Fc region of the antibody facilitates docking of various immune effector cells (e.g. phagocytes and NK cells) which can induce tumour cell destruction. Overall therefore the product may induce a twin tumoricidal effect.

EpCAM is overexpressed on the majority of epithelial tumours and the bispecific nature of the antibody effectively brings CD3-expressing T lymphocytes into close proximity with tumour cells. Additionally, the Fc region of the antibody facilitates docking of various immune effector cells (e.g. phagocytes and NK cells). This facilitates cancer cell destruction via not one but two distinct immune effector mechanisms (Figure 7.11). Several additional bispecific antibodies remain in clinical trials.

## 7.8 Antibody fragments

In some instances the use of an intact (whole) antibody is not necessary to achieve its pharmaceutical purpose. For example, antigen-binding antibody fragments find actual/potential application as *in vivo* diagnostic reagents and as reagents mediating directed radiotherapy for solid tumours.

Antibody application for *in vivo* diagnostic purposes is most often applied in the context of detecting and localizing or visualizing specific cell populations within the body. The antibody (either intact or an antigen-binding antibody fragment) is

raised against a cell-surface antigen that is, ideally, uniquely associated with the target cell surface. The antibody/antibody fragment is then conjugated to a  $\gamma$ -emitting radionuclide (to allow outward penetration from the body) immediately before administration to the patient. Metastable technetium ( $^{99m}\text{Tc}$ ) or indium ( $^{111}\text{In}$ ) are most often used for this purpose. The  $\gamma$  radiation can be detected using a planar gamma camera, and hence the presence and location of the target cells can be established. Such an application simply requires the antibody to congregate at the target cell surface. Binding is via the antibody's Fab region (see Figure 7.1) and the antibody constant regions are redundant in such a context.

The use of (intact) antibodies as anticancer agents has already been considered. In nearly all instances, antibodies approved for this application (see Tables 7.4 and 7.5) are applied to treat diffuse cancers such as lymphomas. One reason why antibody-based therapy of solid tumours is more challenging relates to the physical size of intact antibodies, which renders more difficult effective tumour penetration. Because of their smaller size, antigen-binding antibody fragments can more effectively penetrate the tumour mass than can intact antibodies.

Antibody fragments can be generated by one of two approaches: (i) incubation of the intact antibody with certain proteolytic enzymes; or (ii) by recombinant DNA technology. Treatment of intact IgG with certain proteolytic enzymes (e.g. papain) results in cleavage of the immunoglobulin at the hinge region (see Figure 7.1), yielding two separate antigen-binding fragments ( $2 \times \text{Fab}$ ) and a constant fragment (Fc). Each Fab fragment, while retaining its antigen-binding properties, is no longer capable of precipitating antigen *in vitro*. However, immunoglobulin incubation with other proteases (e.g. pepsin) results in antibody fragmentation immediately below the hinge region. This leaves intact two interchain disulfide linkages towards the C-terminus of the hinge region. This holds the two antigen-binding fragments together. The antigen-binding product of this fragmentation is termed  $\text{F}(\text{ab}')_2$ . Because of its bivalent nature,  $\text{F}(\text{ab}')_2$  retains the ability to precipitate antigen *in vitro*.

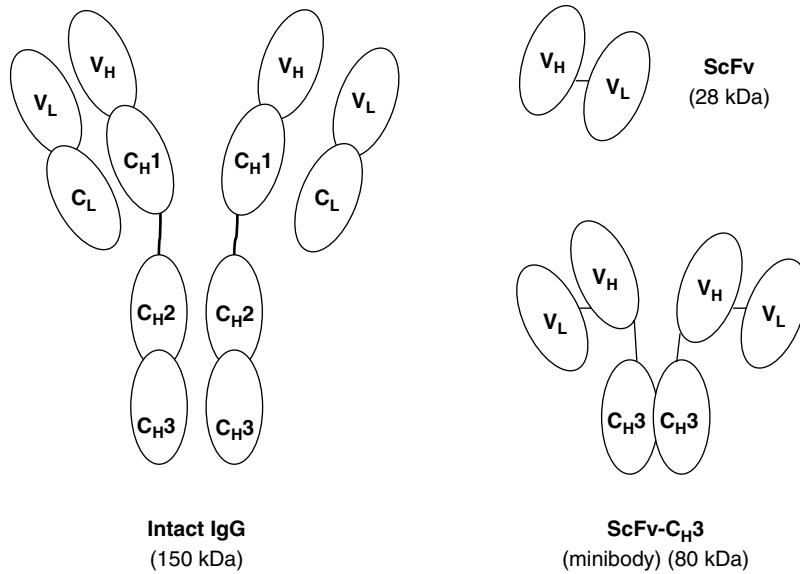
**Table 7.7** Antibody fragments approved for general medical use. For various commercial and technical reasons many of these products no longer remain on the market. Refer to text for details.

Product	Company	Indication	Approved
CEA-scan (arcitumomab). Murine Mab fragment (Fab) directed against human carcinoembryonic antigen (CEA). Produced in a hybridoma cell line	Immunomedics Inc.	Detection of recurrent/metastatic colorectal cancer	1996 (USA and EU) Withdrawn 2005
Cimzia (certolizumab pegol). Anti-TNF- $\alpha$ humanized antibody Fab' fragment, PEGylated. Produced in <i>E. coli</i>	UCB	Crohn's disease, rheumatoid arthritis	2009 (EU) 2008 (USA)
Indimacis 125 (igovomab). Murine Mab fragment (Fab <sub>2</sub> ) directed against the tumour-associated antigen CA125. Produced in a hybridoma cell line	Cisbio International	Diagnosis of ovarian adenocarcinoma	1996 (EU) Withdrawn 2009
LeukoScan (sulesomab). Murine Mab fragment (Fab) directed against NCA-90, a surface granulocyte non-specific cross-reacting antigen. Produced in a Sp2/0 cell line	Immunomedics GmbH	Diagnostic imaging for infection/inflammation in bone of patients with osteomyelitis	1997 (EU)
Lucentis (ranibizumab). Humanized Mab fragment. Binds and inactivates VEGF-A. Produced in <i>E. coli</i>	Novartis, Genentech	Neovascular (wet) age-related macular degeneration	2007 (EU) 2006 (USA)
MyoScint (imicromab pentetate). Murine Mab fragment directed against human cardiac myosin. Produced in a hybridoma cell line	Janssen	Myocardial infarction imaging agent	1996 (USA) Withdrawn 1999
ReoPro (abciximab). Fab fragments derived from a chimeric Mab directed against the platelet surface receptor GPIIb/IIIa. Produced in a mammalian cell line	Eli Lilly	Prevention of blood clots	1994 (USA)
Tecnemab K1 (anti-melanoma Mab fragments). Murine Mab fragments (Fab/Fab <sub>2</sub> mix) directed against high-molecular-weight melanoma-associated antigen. Produced in murine ascites culture	Sorin	Diagnosis of cutaneous melanoma lesions	1996 (EU) Withdrawn 2000
Verluma (nofetumomab). Murine Mab fragments (Fab) directed against carcinoma-associated antigen. Produced in murine hybridoma cell line	NeoRx	Detection of small cell lung cancer	1996 (USA) Withdrawn 1999

Mab, monoclonal antibody; TNF, tumour necrosis factor; VEGF, vascular endothelial growth factor.

The Fc fragment can subsequently be chromatographically separated from the antigen-binding fragment(s). Many of the earlier antibody fragments approved for medical use (Table 7.7) were produced via proteolysis of intact antibody but this approach has now been very largely superseded by the use of recombinant technology to directly produce antibody fragments. Several of these earlier products

have subsequently been withdrawn from the market. Reasons for their discontinuation are invariably a mix of commercial and technical, for example lack of sufficient binding specificity for the target cell and the availability of alternative technologies for the intended application. Some of the more common small antibody formats now produced by recombinant means are shown in Figure 7.12. ScFv



**Figure 7.12** Structure of two of the more common antibody fragments (ScFv and the ‘minibody’) which are produced by recombinant means. Refer to text for details.

fragments, for example, consist of  $V_H$  and  $V_L$  domains and are monovalent in nature, while an ScFv- $C_{H3}$  dimeric fragment is termed a ‘minibody’.

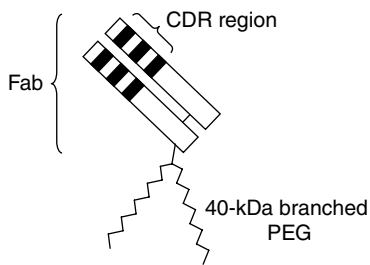
Relative to full-length antibodies, antibody fragments tend to have very short serum half-lives (usually less than a day). Lack of the antibody Fc region prevents antibody fragment recycling via the FcRn receptor-mediated route, as described earlier. Moreover, most fragments have molecular masses less than the kidney threshold value of 50–60 kDa, and hence are excreted via the renal route. A short serum half-life can be an advantage for some applications, for example in the context of radiolabelled conjugates used for diagnostic purposes. Alternative approaches may be pursued if a longer serum half-life is desirable, such as ensuring that the fragment molecular mass exceeds the kidney threshold value. This can be done at a protein engineering level (e.g. compare the masses of the minibody and ScFv fragments in Figure 7.12). Alternatively smaller fragments can be PEGylated (see Chapter 2). Cimzia is the trade name of one such recently approved PEGylated antibody fragment (Box 7.4.). The routine manufacture of antibody fragments is also generally more straightforward than is the case for full-length antibodies.

#### Box 7.4 Product case study: Cimzia

Cimzia is the trade name given to a recombinant humanized anti-TNF- $\alpha$  binding (Fab) fragment covalently linked to a 40-kDa polyethylene glycol. The product first came on the market in 2008 and is used in the treatment of chronic inflammatory conditions such as rheumatoid arthritis and Crohn’s disease. These conditions are generally triggered by overactive T-cell activity, including the overproduction of proinflammatory cytokines, most notably tumour necrosis factor (TNF)- $\alpha$ .

The 48-kDa antibody fragment is expressed in *E. coli* and consists of a 214 amino acid light chain and a 229 amino acid heavy chain fragment, held together by a single intrachain disulfide bond between Cys<sup>214</sup> of the light chain and Cys<sup>221</sup> of the heavy chain. Conjugated to the Fab via a thioester linkage is a single branched PEG molecule, consisting of two 20-kDa PEG chains linked via a reactive maleimide group, giving the overall molecule an apparent molecular mass of 91 kDa. Cimzia

brings about its therapeutic benefit by binding TNF- $\alpha$ , preventing its binding to its receptor and hence reducing the proinflammatory effect. Unlike additional full-length antibody-based therapies for inflammatory disease (Remicade and Humira), Cimzia is devoid of the antibody Fc component and therefore cannot trigger Fc-mediated functions such as complement fixation or ADCC, nor is it recycled via the FcRn mechanism. The PEG moiety, however, extends the product's plasma half-life, allowing an initial dosage regimen of once-fortnightly subcutaneous injection, with subsequent once-monthly maintenance dosages. Manufacture entails chromatographic purification subsequent to product recovery from *E. coli*-based fermentation and the final conjugated product is formulated in lyophilized form, containing sucrose, lactic acid and polysorbate as excipients.

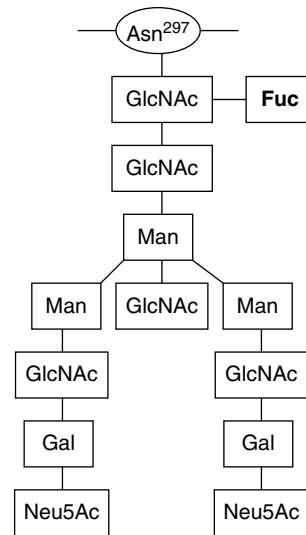


Apart from being significantly smaller, antibody fragments have no glycosylation requirement, and hence can be expressed in microbial as opposed to mammalian cells.

## 7.9 Engineering the antibody glycocomponent

Antibodies continue to be engineered by various means in order to better tailor their therapeutic attributes. The main engineering rationales include:

- reducing immunogenicity (e.g. production of chimeric and humanized antibodies);



**Figure 7.13** Typical diantennary structure of the IgG glycocomponent, as attached to the antibody's backbone at Asn<sup>297</sup> of both heavy chains. The fucose (Fuc) residue discussed in the text is highlighted in bold.

- enhancing binding specificity (e.g. by modifying the amino acid sequences in the CDR or surrounding framework regions);
- generating novel binding specificities (e.g. bispecific antibodies);
- reducing size (e.g. antibody fragments);
- enhancing plasma half-life (e.g. by altering the amino acid sequence of the Fc region involved in binding the FcRn or by conjugating PEG to the protein backbone);
- enhancing effector functions (e.g. by altering the amino acid sequence of the Fc region involved in triggering ADCC and/or CDC).

Traditionally, antibody engineering has focused on modifications at the protein level. More recently, significant research effort has been expended on engineering the antibody glycocomponent. Intact IgGs are N-glycosylated at Asn<sup>297</sup> of the antibody's heavy chain (see Figure 7.1). The glycocomponent plays an indirect but central role in triggering ADCC which, as we have seen, is a principal mechanism by which antibodies can trigger the destruction of cancer cells. The glycocomponent has a diantennary structure which contains, among other sugars, the monosaccharide fucose (Figure 7.13). Removal of the

**Table 7.8** Fc-based fusion products approved for general medical use in the EU and/or the USA. Many are also available in several other world regions.

Product	Company	Indication	Approved
Amevive (alefacept). A fusion protein consisting of the extracellular human leukocyte functional antigen 3 domain linked to an IgG fragment. Produced in a CHO cell line	Astellas Pharma	Chronic plaque psoriasis	2003 (USA) Withdrawn 2011
Arcalyst/Rilonacept (rilonacept). A dimeric fusion protein with each monomer consisting of the ligand-binding domains of the IL-1 receptor and the IL-1 receptor accessory protein, and the Fc region of IgG-1. Produced in a CHO cell line	Regeneron	Cryopyrin-associated periodic syndromes (CAPS)	2009 (EU) 2008 (USA)
Enbrel (etanercept). A TNF receptor-IgG fragment fusion protein. Produced in a CHO cell line	Amgen, Pfizer	Rheumatoid arthritis	1998 (USA) 2000 (EU)
Eylea (aflibercept). A fusion protein consisting of the extracellular ligand-binding domains of VEGF receptor fused to IgG Fc. Produced in a CHO cell line	Regeneron	Neovascular (wet) age-related macular degeneration	2011(USA)
Nplate (romiplostim). A dimeric fusion protein with each monomer consisting of two thrombopoietin receptor-binding domains and the Fc region of IgG. Produced in <i>E. coli</i>	Amgen Inc.	Thrombocytopenia	2009 (EU) 2008 (USA)
Nulojix (belatacept). A fusion protein consisting of the extracellular domain of human CTLA-4 fused to IgG Fc. Produced in a CHO cell line	Bristol-Myers Squibb	Prophylaxis of organ rejection following kidney transplant	2011 (USA, EU)
Zaltrap (ziv-aflibercept). A combination drug containing a fusion protein. The fusion construct consists of binding domains of VEGF receptors 1 and 2 fused to an IgG Fc. Produced in a CHO cell line	Regeneron/Sanofi-Aventis	Metastatic colorectal cancer	2012 (USA)

CTLA, cytotoxic T lymphocyte-associated antigen; TNF, tumour necrosis factor; VEGF, vascular endothelial growth factor.

fucose enhances ADCC activity by up to 100-fold. Therefore, generating non-fucosylated anticancer antibodies could be of obvious therapeutic benefit. One approach entails the application of so-called glycomab technology, which is based on producing the antibody in an engineered producer mammalian cell line which itself overexpresses a specific glycosyltransferase III enzyme. This alters the cell's glycosylation capacity, resulting in the generation of bisected oligosaccharide side chain devoid of fucose.

An alternative engineering approach achieving the same effect involves the development of a knockout CHO cell line which is devoid of the *FUT8* gene. This gene codes for the fucosyltransferase enzyme that normally attaches the fucose residue to the growing antibody sugar backbone. These so-called 'Potelligent' cells are therefore capable of generating completely defucosylated antibody with

consequent improved cancer killing ability. Defucosylated antibodies continue to be investigated in clinical trials.

## 7.10 Fc fusion proteins

As described earlier, the FcRn, associated with the antibody's Fc region, greatly extends antibody serum half-life. This FcRn-mediated antibody recycling process has been imaginatively used to extend the serum half-life of other proteins via protein engineering. The approach entails the generation of a fusion product in which a target protein is fused to an antibody Fc region. Fc-based fusion products approved for medical use to date are outlined in Table 7.8, while once such product, trade name Enbrel, is described more fully in Box 7.5.

**Box 7.5 Product case study: Enbrel**

Enbrel (etanercept) is a fusion protein generated by recombinant DNA technology. It consists of the extracellular domain of the tumour necrosis factor (TNF) receptor (p75) and the Fc region of a human IgG1. During product development, the TNF receptor gene was cloned from human fibroblasts while the Fc region of an IgG1, which includes the C<sub>H</sub>2 and C<sub>H</sub>3 but not C<sub>H</sub>1 domains, was cloned using PCR amplification and published nucleotide sequence information. The fusion product consists of 934 amino acids and displays a molecular mass of 150 kDa. Enbrel is expressed in a CHO cell line and downstream processing involves several chromatographic steps, ultrafiltration and procedures for removing viral particles. The final product is presented in both liquid and lyophilized form and contains sucrose, sodium chloride, arginine and sodium phosphate buffer components as excipients.

Enbrel is indicated for the treatment of certain forms of arthritis and psoriasis, both of which are inflammatory conditions. It brings about its therapeutic effect by binding TNF, thereby blocking the activation of TNF receptors and therefore the TNF-mediated inflammatory process. Enbrel is administered as a subcutaneous injection once or twice a week. The presence of the immunoglobulin domain as part of the fusion protein increases the half-life of the product in the blood to 70 hours. The product is one of the best selling of all biopharmaceuticals, commanding a global sales value of \$8.4 billion in 2012.

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# Chapter 8

## Hormones and growth factors used therapeutically

A number of hormone preparations have been used clinically for many decades. While some (e.g. adrenaline, peptide hormones and many steroid hormones) may be prepared synthetically, protein hormones used medically were (initially at least) extracted directly from biological source material. Problems associated with source availability and accidental transmission of disease hastened the development of recombinant forms of many such hormones. In addition, protein engineering has facilitated the development of modified forms of polypeptide hormones and several engineered products have recently gained approval for general medical use. The major polypeptide hormones and growth factors used clinically are summarized in Table 8.1, and these form the basis of this chapter. Some interleukins and interferons also stimulate the growth/differentiation of some cell types, but these are considered separately in Chapter 9.

### 8.1 Insulin

Insulin is a polypeptide hormone produced by the  $\beta$  cells of the islets of Langerhans in the pancreas. The hormone was first isolated in 1921, and its

amino acid sequence was determined in the late 1950s. Insulin exerts a wide variety of metabolic effects: it plays a central regulatory role in the metabolism of carbohydrates, protein and lipid. The level of secretion of insulin from the  $\beta$  cells is primarily determined by blood glucose levels. Increases in the concentration of blood glucose induce insulin secretion, which promotes the uptake of glucose by a number of tissues, particularly liver, and by muscle. This reduces blood glucose levels to normal values which in turn decreases the rate of insulin release.

#### 8.1.1 Type 1 diabetes

Failure to produce insulin results in the development of type 1 diabetes, formerly known as insulin-dependent diabetes mellitus (IDDM) or childhood-onset diabetes. This is distinguished from type 2 diabetes, formerly known as non-insulin-dependent diabetes mellitus (NIDDM) or adult-onset diabetes, which results from the body's ineffective use of insulin.

Type 1 diabetes, which affects approximately 35 million people worldwide, is a chronic disease characterized by an elevated level of blood glucose

**Table 8.1** Major polypeptide hormones and growth factors used therapeutically.

Hormone or growth factor	Main or sole therapeutic use
Insulin	Type 1 diabetes
Glucagon	Hypoglycaemia
Human growth hormone	Growth hormone deficiency (e.g. dwarfism)
Follicle-stimulating hormone	Some forms of subfertility/infertility
Erythropoietin	Anaemia
Colony-stimulating factors	Neutropenia (low blood neutrophil count)
Platelet-derived growth factor	Slow-healing skin ulcers sometimes associated with diabetes
Insulin-like growth factor-1	Growth failure in children
Keratinocyte growth factor	Oral mucositis (ulcers of the mouth)

and by the presence of glucose in the urine. Increased rates of glycogenolysis, gluconeogenesis, fatty acid oxidation, ketone body production and urea formation are also observed. Type 1 diabetes also results in a decrease in fatty acid and protein biosynthesis as well as decreased uptake of glucose by peripheral tissues. The condition is usually caused by irreversible damage to the insulin-producing  $\beta$  cells in the pancreas. The underlying molecular events inducing such damage remain to be fully characterized, although autoimmunity, viral infection and genetic predisposition are all believed to be contributory factors. Diabetes mellitus may be controlled by the parenteral administration of insulin. Insulin was first administered to diabetics in 1922, just one year subsequent to its initial isolation.

### 8.1.2 *Insulin synthesis in vivo*

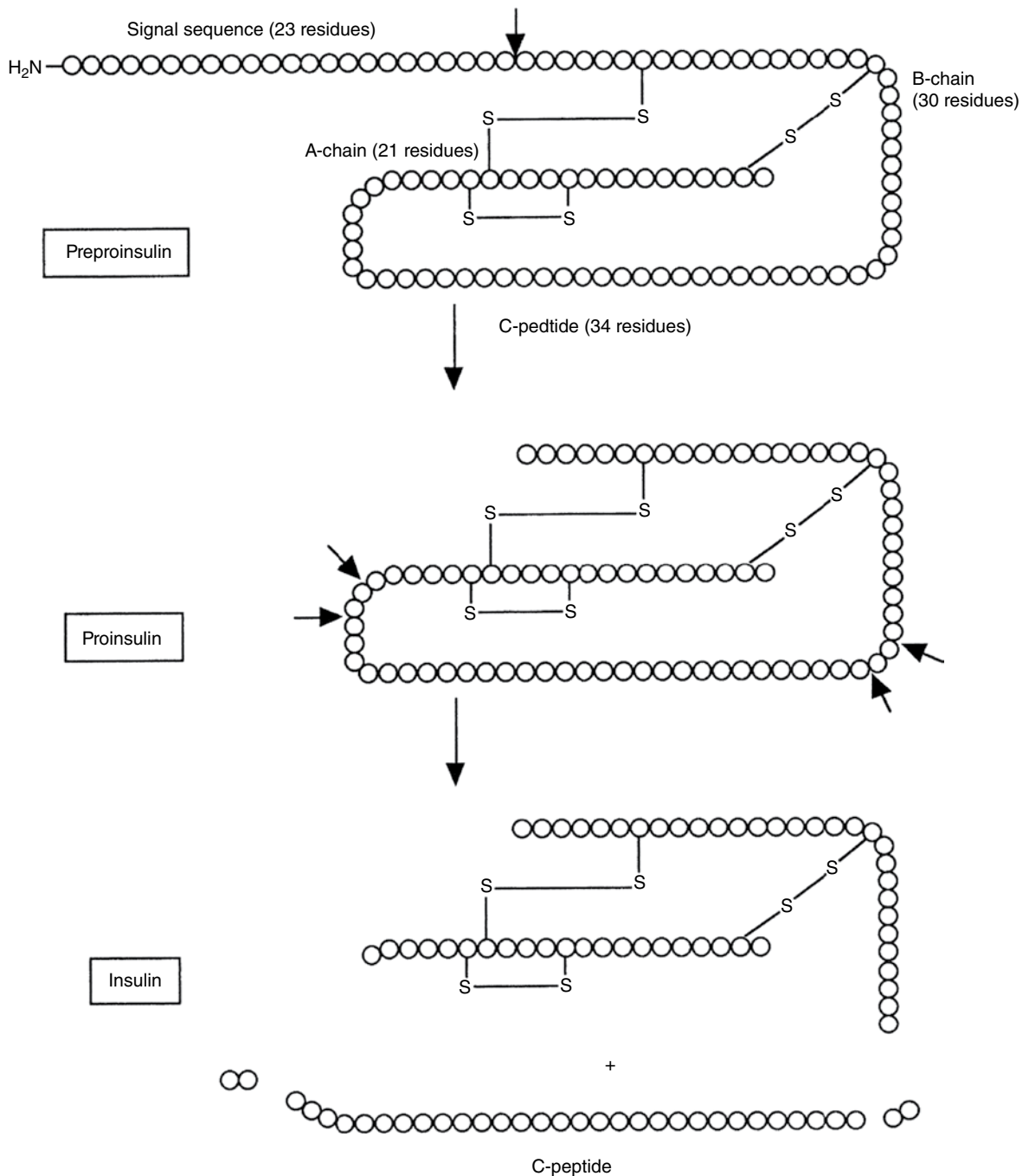
Insulin is initially synthesized in the pancreatic  $\beta$  cells as proinsulin (Figure 8.1). This molecule contains a 23 amino acid amino-terminal signal sequence that directs the protein through the rough endoplasmic reticulum (ER) membrane to the

lumen of the ER. Here, the leader sequence is removed from the proinsulin molecule by a specific signal peptidase, yielding proinsulin.

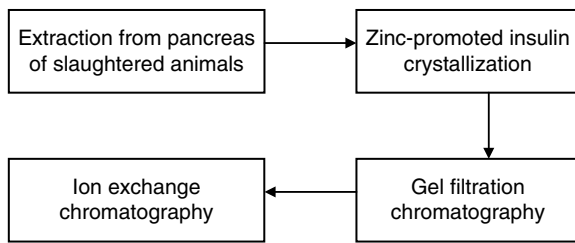
The proinsulin molecule remains within the lumen of the ER. Small vesicles containing proinsulin subsequently bud off from the ER and fuse with membranous structures termed Golgi apparatus. Proinsulin-containing vesicles in turn pinch off from the Golgi apparatus. These vesicles are often termed coated secretory granules, as they exhibit a coat composed of a protein, clathrin, on their outer surface. As they move further away from the Golgi body, these vesicles lose their clathrin coat, thus forming non-coated secretory granules.

The conversion of proinsulin into insulin takes place in the coated secretory vesicles. This process involves the proteolytic cleavage of the proinsulin molecule, yielding mature insulin and the C or connecting peptide. The mature insulin consists of two polypeptide chains, the A and B chains, joined by two disulfide cross-links and has a molecular mass of 5.8 kDa. The human insulin A chain consists of 21 amino acid residues whereas the B chain consists of 30 residues. The A chain also contains one intrachain disulfide linkage. Conversion of proinsulin to insulin generates a polypeptide sequence that originally bridged the insulin A and B chains of the proinsulin molecule. On the formation of mature insulin, two dipeptides are removed from either end of this bridge peptide yielding a slightly shorter peptide, termed C or connecting peptide.

In addition to mature insulin, secretory granules also contain low levels of proinsulin as well as the C peptide and some proinsulin-derived amino acids. Non-coated secretory granules serve as the storage depot of insulin in the  $\beta$  cells. The insulin is normally stored as a hexamer, consisting of six molecules of insulin stabilized by two atoms of zinc. Indeed the addition of a small quantity of zinc to purified insulin results in the formation of characteristic rhombohedral crystals, with the basic crystal unit consisting of the zinc-containing insulin hexamer. The secretory granules release their contents into the blood by the process of exocytosis. Insulin is released in this manner only on stimulation by specific secretory signals, the most significant of which is an increase in the blood glucose concentration.



**Figure 8.1** Synthesis of human insulin from preproinsulin. The initial proteolytic event involves removal of a 23 amino acid signal sequence from the amino-terminal end of preproinsulin, thus yielding proinsulin. Proinsulin is converted into insulin by additional proteolytic events, resulting in the generation of not only insulin but also a 30 amino acid sequence (termed C or connecting peptide) and two dipeptide moieties. Mature human insulin thus consists of two polypeptides, the A and B chains. The B chain contains 30 amino acid residues whereas the A chain contains 21 amino acids. The chains are covalently linked via two interchain disulfide linkages. One intrachain disulfide linkage is also present in the A chain.



**Figure 8.2** A typical purification scheme for the production of traditional insulin preparations derived from the pancreatic tissue of slaughtered animals.

Insulins obtained from various different species are similar, though not identical, in their overall amino acid sequence. Rats and mice synthesize two insulin types that differ slightly from each other in amino acid sequence. Porcine insulin differs in sequence from human insulin by only one amino acid, the C-terminal residue of the B chain. Bovine insulin differs from the human hormone by three amino acids while sheep insulin differs by four amino acids. However, the amino acid sequence of the insulin C-peptide differs greatly from species to species.

Insulin initiates its characteristic molecular effects by binding to a receptor found on the surface of insulin-sensitive cells. The binding of insulin to the extracellular portion of this receptor results in the activation of tyrosine kinase activity associated with its intracellular domains. This in turn results in the phosphorylation of a number of target intracellular proteins, ultimately resulting in transcriptional regulation of various insulin-responsive genes.

### 8.1.3 The industrial production of insulin

Insulin preparations administered to diabetic patients were traditionally obtained by direct extraction from the pancreas of healthy slaughterhouse animals, in particular pigs (Figure 8.2). Indeed, some products still on the market are produced in this way.

Human insulin can also be produced by the enzymatic modification of porcine insulin. The human molecule differs from porcine insulin by a single amino acid. Threonine forms the carboxyl terminus (residue number 30) of the human insulin B chain,

whereas an alanine residue is found in this position in the porcine molecule. Treatment of intact porcine insulin with trypsin results in the proteolytic cleavage of the B chain between residues 22 and 23 (and also between residues 29 and 30), effectively removing the carboxy-terminal octapeptide from the B chain. A synthetic octapeptide whose sequence is identical to the analogous human octapeptide may then be coupled to the trypsin-treated porcine insulin. Human insulin produced by this means has been used clinically for a number of years, but the majority of insulin preparations used today are produced via recombinant DNA technology.

### 8.1.4 Insulin production by recombinant means

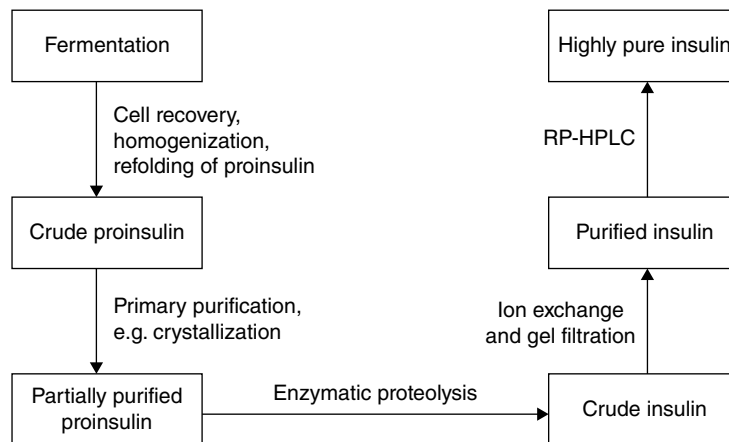
The majority of modern insulin preparations currently in therapeutic use are produced by recombinant DNA technology (Table 8.2). Indeed, insulin represented the first commercial healthcare product produced by such means to be approved for clinical use. Recombinant production has a number of potential advantages over traditional purification approaches.

- *Source availability*: the quantity of purified insulin obtained from the pancreas of one pig would satisfy the insulin requirement of one diabetic patient for only 3 days. The growing incidence of diabetes – likely to double by 2030 – could eventually lead to a shortage of suitable pancreatic material.
- *Safer product*: production in recombinant organisms all but eliminates the risk of accidental transmission of disease to diabetics from infected pancreatic material.
- *Engineered insulins*: as described below, genetic engineering has allowed the development of a number of insulin variants whose onset and/or duration of activity in the body differs from native human insulin.

The initial approach to recombinant insulin production entailed inserting the nucleotide sequence coding for the insulin A and B chains into two different *Escherichia coli* cells (both strain K12). These

**Table 8.2** Some human insulin preparations produced via recombinant DNA technology that have been approved for general medical use.

Product name	Description	Company	Year and region initially approved
Actrapid, Insulatard, Protaphane, Mixtard, Actraphane	All contain recombinant human insulin produced in <i>S. cerevisiae</i>	Novo Nordisk	2002 (EU)
Humulin R	Recombinant human insulin produced in <i>E. coli</i>	Eli Lilly	1982 (USA)
Insulin Human Winthrop	Recombinant human insulin produced in <i>E. coli</i>	Sanofi-Aventis	2007 (EU)
Insuman	Recombinant human insulin produced in <i>E. coli</i>	Sanofi-Aventis	1997 (EU)
Novolin	Recombinant human insulin produced in <i>S. cerevisiae</i>	Novo Nordisk	1991 (USA) (now withdrawn from the market)

**Figure 8.3** A likely purification scheme for human insulin produced via the 'proinsulin' route. Refer to text for further details.

cells were then cultured separately in large-scale fermentation vessels, with subsequent chromatographic purification of the insulin chains produced. The A and B chains are then incubated together under appropriate oxidizing conditions in order to promote interchain disulfide bond formation.

An alternative method involves inserting a nucleotide sequence coding for human proinsulin into recombinant *E. coli*. This is followed by purification of the expressed proinsulin and subsequent proteolytic excision of the C-peptide *in vitro*. This approach has become more popular, largely due to the requirement for a single fermentation and subsequent purification scheme.

While recombinant product produced by either method is identical in sequence to native insulin, any impurities present will be derived from the host microbial cells and hence potentially highly immunogenic in humans. Stringent purification of the recombinant product must thus be undertaken, entailing multiple chromatographic steps (Figure 8.3). This can involve the use of process-scale reverse-phase high-pressure liquid chromatography (RP-HPLC). The C8 or C18 RP-HPLC column typically used has a volume of 80 L or more, and up to 1200 g of insulin may be loaded during a single purification run. This 'polishing' step yields a final product of approximately 99% purity. Over

95% of the insulin activity loaded onto the column can be recovered. A single column run takes in the order of 1 hour.

### 8.1.5 Insulin formulations

Healthy humans typically secrete insulin continuously at a low basal level, with rapid but transient increases triggered by elevated blood glucose levels. Endogenous insulin levels generally peak 1 hour after consumption of a meal, returning to basal levels within a further 2 hours. Conventional insulin therapy cannot accurately reproduce this endogenous secretion pattern. When present in blood at physiologically normal levels (about 0.1 nmol/L), biologically active insulin exists in monomeric form. Insulin is present in commercial formulations at significantly higher concentrations (about 1 mmol/L). At such concentrations it exists primarily in oligomeric form, as zinc-containing insulin hexamers. The hexamers comprise three identical dimers coordinated to the zinc ions and the strongest subunit interactions lie between the dimerizing monomers.

When injected directly into the bloodstream, insulin has a half-life in the order of minutes. Clearly such a short half-life would render the clinical management of diabetes extremely difficult. Administration by subcutaneous injection facilitates a more prolonged release of hormone from the site of injection into the bloodstream. However, the hexameric complexes must first dissociate in order to be absorbed from the site of injection into the blood. As a result peak plasma insulin concentrations are not witnessed for up to 2 hours, with levels remaining elevated for up to 5 hours. In order to best mimic physiological patterns, conventional insulins were traditionally administered 30–40 minutes or more before mealtimes. This can be inconvenient, and can lead to hypoglycaemia if the person subsequently skips the meal for any reason.

In addition to such 'fast' (short)-acting products, commercial products can also be formulated in order to retard the rate of insulin entry into the bloodstream from the site of injection. Such 'slow'

**Table 8.3** Some therapeutic characteristics of short- and longer-acting insulin preparations.

Category	Onset (hours after administration)	Peak activity (hours after administration)	Duration (hours)
Short-acting	0.5–1	2–5	6–8
Intermediate-acting	2	4–12	up to 24
Long-acting	4	10–20	up to 36

(long)-acting insulins may also be administered to diabetics in order to mimic low (baseline) endogenous insulin secretion patterns. Long-acting insulins are usually prepared by formulating soluble insulins so as to generate insulin suspensions. This further prolongs the rate of dissociation into individual insulin monomers after injection (Table 8.3).

Long-acting insulins were traditionally prepared by, for example, complexing with a protein such as protamine which further retards its release into the general circulation. Protamines are a group of basic proteins found in association with nucleic acids in the sperm of certain species of fish. Alternatively, zinc may be added to the final preparation in order to promote the growth of insulin crystals in the resultant zinc–insulin suspension.

### 8.1.6 Engineered insulins

Recombinant DNA technology facilitates not only production of native human insulin but also the generation of insulins displaying modified amino acid sequences. Such a protein engineering approach had led to the identification of insulin analogues with altered activity-over-time (pharmacokinetic) profiles, i.e. both faster-acting and slower-acting insulins.

Attempts to generate faster-acting insulins have centred on developing analogues that do not dimerize or form higher polymers at therapeutic dose concentrations. The contact points between individual insulin molecules in insulin dimers/oligomers include amino acids at positions B8, B9, B12–13, B16 and B23–28. Thus, analogues with various

**Table 8.4** Engineered rapid and long-acting insulin products now used therapeutically.

Product name	Description	Company	Year and region first approved
Apidra (insulin glulisine)	Rapid-acting insulin analogue produced in <i>E. coli</i>	Sanofi-Aventis	2004 (USA, EU)
Humalog (insulin lispro)	Rapid-acting insulin analogue produced in <i>E. coli</i>	Eli Lilly	1996 (USA, EU)
Lantus/Optisulin (insulin glargine)	Long-acting insulin analogue produced in <i>E. coli</i>	Sanofi-Aventis	2000 (USA, EU)
Levemir (insulin detemir)	Long-acting insulin analogue produced in <i>S. cerevisiae</i>	Novo Nordisk	2004 (EU)
Liprolog Bio-Lysprol (insulin lispro)	Rapid-acting insulin analogue produced in <i>E. coli</i>	Lilly	1997 (EU)
NovoRapid/Novolog (insulin aspart)	Rapid-acting insulin analogue produced in <i>S. cerevisiae</i>	Novo Nordisk	1999 (EU)

substitutions at these positions have been generated. The approach adopted generally entails insertion of charged or bulky amino acids, in order to promote charge repulsion or steric hindrance between individual insulin monomers. Several are absorbed from the site of injection into the bloodstream far more quickly than native soluble (fast-acting) insulin. Such modified insulins could thus be injected at mealtimes rather than 1 hour before and several such fast-acting engineered insulins have now been approved for medical use (Table 8.4).

For example, insulin aspart is a fast-acting engineered human insulin analogue now approved for general medical use. It differs from native human insulin in that the Pro<sup>B28</sup> residue has been replaced by aspartic acid. This single amino acid substitution also decreases the propensity of individual molecules to self-associate, ensuring that they enter the bloodstream from the site of injection immediately on administration.

A number of studies have also focused on the generation of longer-acting insulin analogues. Zinc-insulin suspensions, or protamine-zinc-insulin suspensions, generally display a plasma half-life of 20–25 hours. Selected amino acid substitutions have generated insulins which, even in soluble form, exhibit plasma half-lives of up to 35 hours.

Optisulin and Lantus are the trade names given to one such analogue (also known as insulin

glargine; see Table 8.4). This molecule differs from native human insulin in that the C-terminal asparagine residue of the A chain has been replaced by a glycine residue and the  $\beta$ -chain has been elongated (again from its C-terminus) by two arginine residues. The overall effect is to increase the molecule's isoelectric point (the pH at which the molecule displays a net overall zero charge and consequently is least soluble) from 5.4 to a value approaching 7.0. The engineered insulin is expressed in a recombinant *E. coli* K12 host strain and is produced via the 'pro-insulin route' as previously described. The purified product is formulated at pH 4.0, a value at which it is fully soluble. On subcutaneous injection, the insulin experiences an increase in pH towards more neutral values and consequently precipitates in the subcutaneous tissue. It resolubilizes very slowly and hence a greatly prolonged duration of release into the bloodstream is noted. Thus a single daily injection supports the maintenance of acceptable basal blood insulin levels, and insulin molecules are still detected at the site of injection in excess of 24 hours after administration. An alternative approach to engineering insulin in order to generate a long-acting analogue is exemplified by Levemir (Box 8.1).

The generation of engineered insulin analogues raises several important issues relating to product safety and efficacy. Alteration of a native protein's amino acid sequence could render the engineered product immunogenic. Such an effect would be

**Box 8.1 Product case study: Levemir**

Levemir (insulin detemir) is a long-acting insulin analogue produced in an engineered *S. cerevisiae*. The product is engineered in two ways. Its amino acid sequence differs from native human insulin in that the threonine residue at position 30 of the insulin B chain (B30) has been omitted. In addition, a C<sub>14</sub> saturated fatty acid (myristic acid) has been covalently attached (acylated) to the amino group of the lysine residue found at position B29. Albumin harbours three high-affinity fatty acid-binding sites and, as such, Levemir binds tightly but reversibly to albumin, both at the site of injection and in the blood. This in turn ensures a constant and prolonged release of free insulin, bestowing on it an extended duration of action of up to 24 hours. Product manufacture entails an initial yeast fermentation step, followed by product recovery, an ion-exchange-based concentration step and two crystallization steps. The insulin is then further purified via several chromatographic steps, followed by product acylation. Final polishing purification and precipitation are then undertaken and the product is formulated as a solution for subcutaneous administration.

particularly significant in the case of insulin as the product is generally administered daily for life. In addition, alteration in structure could have unintended influences on the therapeutic properties of the drug by, for example, altering its receptor-binding affinity. Such issues must be assessed during product development and in particular during clinical trials.

## 8.2 Glucagon

Glucagon is a polypeptide hormone synthesized in the  $\alpha$  cells of the islets of Langerhans in the pancreas. It is also synthesized by related cell types found in the gastrointestinal tract. Glucagon consists of 29 amino acids and has a molecular mass of

approximately 3.5 kDa. The amino acid sequence of the hormone isolated from different animal species is almost invariably the same. As in the case of insulin, glucagon is synthesized initially as proglucagon. Proglucagon is converted to glucagon by two separate proteolytic events.

Glucagon is a hyperglycaemic hormone, inducing an increase in the blood glucose concentration of recipients. In this way it opposes one of the major physiological actions of insulin. The increase in blood glucose is promoted by stimulating an increase in the rate of gluconeogenesis and glycogen breakdown in the liver. It also promotes an increase in the rate of lipolysis and a decrease in the rate of glucose utilization by adipose tissue and muscle. Glucagon is occasionally used clinically in order to reverse insulin-induced hypoglycaemia in diabetic patients.

Although glucagon obtained by direct extraction from pancreatic tissue has been available for many years, more recently recombinant glucagon products have come on the market. GlucaGen is the trade name given to one such product, produced by Novo Nordisk using an engineered *Saccharomyces cerevisiae* strain. Upstream processing (aerobic batch-fed fermentation) is followed by an upward adjustment of media pH in order to dissolve precipitated product (glucagon is insoluble in aqueous-based media between pH 3 and 9.5). This facilitates subsequent removal of the yeast by centrifugation. Glucagon is then recovered and purified from the medium by a series of further precipitation as well as high-resolution chromatographic steps.

In addition to their therapeutic use in counteracting hypoglycaemia, these products may also be used as a diagnostic aid in radiological examinations of the stomach. The hormone relaxes smooth muscle in the stomach wall, thus reducing stomach motility, so that improved images may be obtained during radiological examinations.

## 8.3 Gonadotrophins

Gonadotrophic hormones, as the name suggests, exert their primary effect on the male and female gonads. A number of gonadotrophins may be used clinically in the treatment of various human clinical



conditions and to induce a superovulatory response in female animals. The most commonly utilized gonadotrophins are follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which is also referred to as interstitial cell stimulating hormone. Both LH and FSH are synthesized by the pituitary. Another gonadotrophin, human chorionic gonadotrophin (hCG), is produced by the placenta of pregnant women.

### 8.3.1 FSH, LH and hCG

FSH, LH and hCG are glycoproteins of molecular mass 34, 28 and 36kDa, respectively. Both LH and FSH consist of approximately 16% carbohydrate while hCG contains over 30% carbohydrate. All are dimers composed of an  $\alpha$  and  $\beta$  subunit. In any one animal species, the  $\alpha$  subunit of all three is identical, whereas the  $\beta$  subunit differs. It is thus the  $\beta$  subunit which confers on the particular molecule its distinct biological characteristics. The  $\beta$  subunit of hCG exhibits significant amino acid sequence homology with the  $\beta$  subunit of LH. It is therefore not surprising that hCG displays similar biological activities to LH.

In the male, FSH stimulates the production of spermatocytes by the Sertoli cell of the seminiferous tubules. LH stimulates production of testosterone, the principal male sex hormone, by the Leydig cells of the testes. In the female, FSH and LH play critical roles in regulating the reproductive cycle.

The ovary represents the primary female reproductive organ. It houses egg cells (ova) and synthesizes a range of steroid hormones. The ovary contains numerous follicles, each follicle consisting of a single egg cell (ovum) surrounded by two layers of cells. The inner layer of cells are termed granulosa cells and these respond primarily to FSH. The granulosa cells produce estrogens, the major female sex hormones. The outer follicular layer is composed of theca cells. These cells, which fall primarily under the hormonal influence of LH, synthesize a variety of steroids that are subsequently utilized by the granulosa cells during synthesis of estrogen.

At the commencement of a normal menstrual cycle a group of follicles begin to mature, a process largely stimulated by FSH. Shortly thereafter a single

dominant follicle emerges, and the remaining follicles regress. This first half of the menstrual cycle is often referred to as the follicular phase. The growing follicle begins to secrete increasing levels of estrogens, which ultimately trigger a surge in the concentration of LH at mid-cycle, approximately day 14 of the human female cycle. This in turn triggers ovulation – the release of the egg from the mature follicle – with the resultant conversion of the ruptured follicle into a structure known as the corpus luteum. The menstrual cycle has now entered its second phase, also known as the luteal phase. The corpus luteum secretes estrogens and the steroid hormone progesterone. Progesterone serves to prepare the lining of the uterus, the endometrium, for implantation of the fertilized ovum, and is required to support the growing embryo.

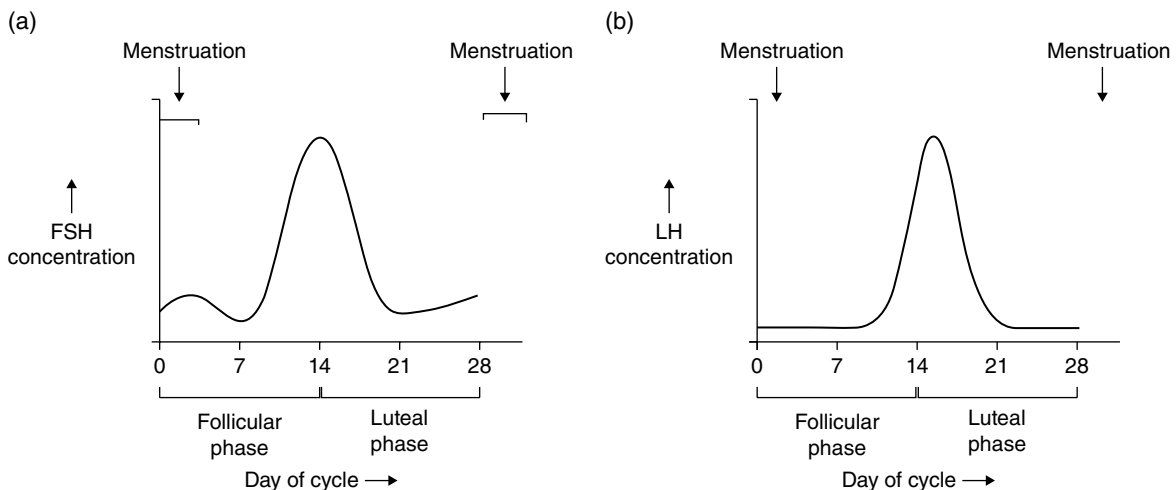
If fertilization does not occur, the maximum lifespan of the corpus luteum in humans is normally 14 days, during which time it steadily regresses. This in turn results in a decrease in the levels of the corpus luteal hormones, estrogen and progesterone. Withdrawal of such hormonal support results in shedding of the endometrial tissue, which is discharged from the body during menstruation.

If follicular rupture is followed by fertilization of the released ovum, the corpus luteum does not regress and continues to produce progesterone. In the pregnant female, the corpus luteum is maintained by hCG, which is secreted by the placenta. hCG is also found in urine and detection of this hormone forms the basis of pregnancy detection kits (see Chapter 10).

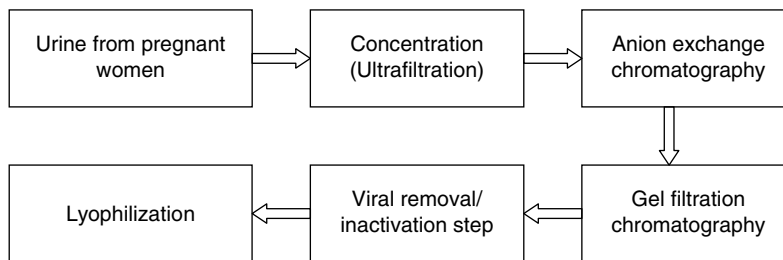
FSH and LH essentially regulate the reproductive process in females (Figure 8.4). In addition, FSH and LH also regulate the development and maintenance of male fertility. These hormones may be employed therapeutically to treat some forms of sterility, or other medical conditions caused by low circulatory levels of the hormones.

### 8.3.2 Sources of FSH, LH and hCG

FSH may be isolated from the pituitary gland. Supply of human FSH from such sources is obviously very limited and is no longer used due to the



**Figure 8.4** Changes in plasma (a) FSH and (b) LH levels during the menstrual cycle of a healthy human female.



**Figure 8.5** Overview of a typical scheme by which hCG may be purified from the urine of pregnant females.

potential for accidental transmission of pathogens. FSH is also found in the urine of postmenopausal women. Preparations obtained from this source also contain LH activity, and are termed menotropin. Menotropin is purified from urine by a variety of fractionation procedures, as well as by chromatographic techniques such as ion-exchange chromatography. Menotropin preparations may be subjected to further chromatographic steps in order to remove most or all of the contaminating LH activity. Menotropin is often used clinically to treat a variety of reproductive complications such as anovulatory infertility, which is caused or exacerbated by low levels of circulating FSH.

Human LH may also be isolated from the pituitary. As in the case of FSH, this does not represent a commercially viable source of this hormone. hCG, as mentioned earlier, exhibits many biological activities which are similar if not identical to those

of LH. hCG may be purified relatively easily from the urine of pregnant women (Figure 8.5). Such hCG preparations have traditionally found widespread medical application in the treatment of conditions caused by lack of LH activity and it has been employed clinically to treat infertility and delayed puberty in males. It is also administered to females together with FSH in the treatment of anovulatory infertility. In such cases, the administered FSH stimulates development of the follicle while subsequent administration of LH initiates the mid-cycle LH surge, stimulating final maturation and subsequent rupturing of the follicle.

Recombinant versions of all these gonadotrophins are now available (Table 8.5), thereby overcoming problems of source availability and worries about potential transmission of pathogens sometimes associated with earlier products directly extracted from a native source. With one exception

**Table 8.5** Recombinant gonadotrophins now used to treat various fertility-related conditions. All the products are expressed in CHO cell lines, which facilitate appropriate product glycosylation.

Name	Description and use	Company	Year and region approved
Elonva	Engineered recombinant human FSH used to achieve controlled ovarian stimulation (see Box 8.2)	N.V. Organon	2010 (EU)
Fertavid	Recombinant human FSH used to treat some forms of infertility	Merck, Sharp & Dohme	2009 (EU)
Follistim	Recombinant human FSH used to treat some forms of infertility	Schering Corporation	1997 (USA)
Gonal F	Recombinant human FSH used to treat anovulation	Merck Serono	1995 (EU) 1997 (USA)
Luveris	Recombinant human LH used to treat some forms of infertility	Merck Serono	2000 (EU)
Ovitrelle/Ovidrel	Recombinant hCG. Used in selected assisted reproductive techniques	Merck Serono	2001 (EU) 2000 (USA)
Puregon	Recombinant human FSH used to treat anovulation	N.V. Organon	1996 (EU)

(Elonva), the recombinant products exhibit amino acid sequences identical to those of the native human protein, although their glycosylation patterns vary somewhat compared to the native products. Elonva is noteworthy in that it is an engineered gonadotrophin (Box 8.2).

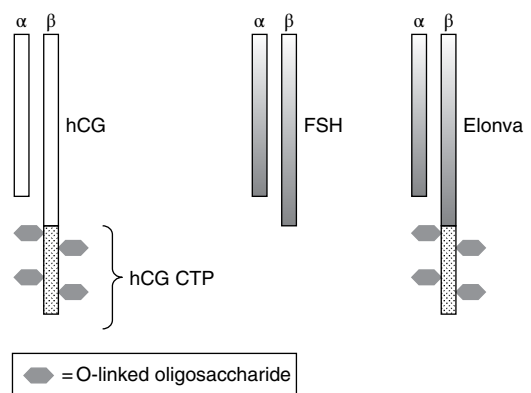
## 8.4 Growth hormone

Growth hormone (GH) is a species-specific hormone also known as somatotrophin or somatotropin. Somatotrophin is produced by the anterior pituitary and is required for normal body growth and lactation. The hormone promotes general protein synthesis and also plays a role in the regulation of carbohydrate and lipid metabolism, and promotes bodily retention of various minerals and other elements essential for normal growth. Secretion of GH from the pituitary is stimulated by a hypothalamic regulatory factor termed growth hormone releasing factor (GHRF). GH release is inhibited by a second hypothalamic factor termed growth hormone release-inhibiting hormone or somatostatin.

GH initiates its anabolic effect by binding to specific cell-surface receptors. A truncated form of the GH receptor, which approximates to the extracellular ligand-binding domain, is also found in

### Box 8.2 Product case study: Elonva

Elonva (corifollitropin alfa) is an engineered recombinant human FSH used to achieve ovarian stimulation in some fertility treatments. The modification entails the addition, to the C-terminal end of the FSH  $\beta$ -subunit, of a short stretch of 31 amino acids found at the C-terminal end of the hCG  $\beta$  subunit, known as the hCG C-terminal peptide (hCG CTP; see diagram below). Native hCG has a significantly longer serum half-life than that displayed by native FSH and the increased half-life appears largely due to the CTP.



The  $\beta$ -subunit of Elonva is 139 amino acids in length, compared with 111 amino acids in the native FSH  $\beta$ -subunit. Moreover, the C-terminal peptide contains four O-linked carbohydrate side chains, attached to specific serine residues in the CTP backbone. Each of these side chains has two terminal sialic acid sugars. Overall, therefore, the engineered molecule is larger and more negatively charged than the native FSH. This likely contributes to the longer serum half-life of the product, by decreasing its glomerular filtration rate in the kidney. The modification described increases the serum half-life of Elonva to about 65 hours, approximately twice that of native FSH. The resultant prolonged duration of activity means that a single subcutaneous injection of Elonva can replace the first seven daily injections of recombinant unengineered FSH characteristic of fertility programmes.

Elonva is expressed in an engineered CHO cell line. Product manufacture entails initial culture of the producer cells in a chemically defined culture medium. Downstream processing involves an 11-step process that incorporates several chromatographic steps, as well as ultrafiltration/diafiltration steps and steps to inactivate and remove any potential viral contaminants. The final product is presented as a solution for subcutaneous administration.

serum. This serum protein is capable of binding growth hormone and may play an important role in its clearance from the body. GH receptors have been detected in a number of tissues. The hormone exerts its primary influence on the liver, where it stimulates the synthesis of somatomedin or insulin-like growth factor (IGF)-I. IGF-I directly mediates most of the growth-promoting effects of GH.

Insufficient production of GH in humans leads to dwarfism. Excessive production of the hormone result in gigantism or acromegaly, a condition characterized by an increase in the size of the hands, feet and face. GH may therefore be employed

clinically to treat hypopituitary dwarfism. The species-specific nature of GH makes it essential to use human GH (hGH) in human medicine.

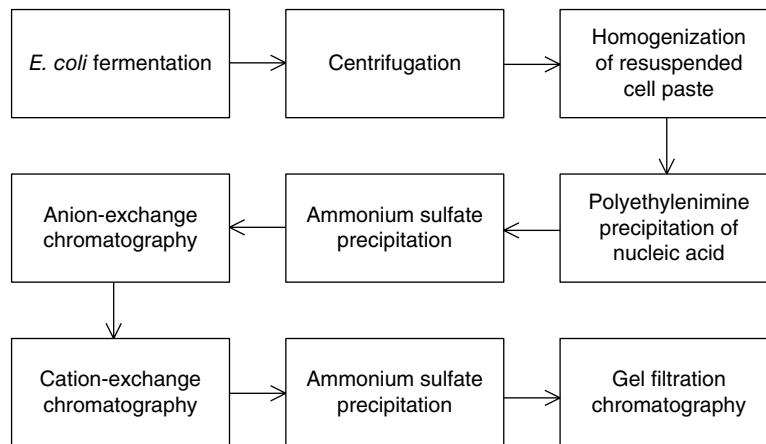
hGH is a polypeptide consisting of 191 amino acid residues. It contains two intrachain disulfide links and has a molecular mass in excess of 21 kDa. Until the mid 1980s the sole source of hGH was human pituitary glands and for this reason preparations of hGH were not widely available. Furthermore, such preparations were generally not homogeneous, containing contaminants including a number of modified forms of GH in addition to unrelated proteins. The clinical use of hGH extracted from human pituitaries was banned in 1985. In that year it was shown that several people suffering from Creutzfeldt–Jakob disease (CJD) had contracted the condition via hGH preparations contaminated with the CJD causative agent.

The expression of the cDNA for hGH in *E. coli* or other cell lines facilitated the large-scale production of ample quantities of GH free of such infectious agents and many such products have been approved for medical use (Table 8.6). The initial recombinant preparations produced in *E. coli* differed from the native human hormone only in that they contained an extra methionine residue (due to the AUG start codon inserted at the beginning of the gene). Subsequently, a different cloning strategy allowed production in *E. coli* of products devoid of this terminal methionine. *In vitro* analysis, including tryptic peptide mapping, amino acid analysis and comparative immunoassays, shows the native and recombinant form of the molecule to be identical. Clinical trials in humans have also confirmed that the recombinant version promotes identical biological responses to the native hormone. Recombinant hGH was first purified (on a laboratory scale) by Genentech scientists using the strategy outlined in Figure 8.6. A somewhat similar strategy is likely used in its process-scale purification.

Somavert represents an interesting hGH-based product in that it is an engineered antagonist used to treat acromegaly. Its engineering entailed the introduction of nine mutations into the hGH amino acid sequence. The resulting analogue binds to the

**Table 8.6** Representative recombinant human growth hormone (rhGH)-based products now approved for general medical use.

Product	Company	Therapeutic indication	Approved
Accretropin (somatotropin; rhGH produced in <i>E. coli</i> )	Cangene Corporation	Growth failure or short stature associated with Turner syndrome in paediatric patients	2008 (USA)
Genotropin (rhGH produced in <i>E. coli</i> )	Pharmacia and Upjohn (Pfizer Inc.)	hGH deficiency in children	1995 (USA)
Humatrope (rhGH produced in <i>E. coli</i> )	Eli Lilly	hGH deficiency in children	1987 (USA)
Norditropin (rhGH produced in <i>E. coli</i> )	Novo Nordisk	Treatment of growth failure in children due to inadequate GH secretion	1995 (USA)
Nutropin AQ (rhGH produced in <i>E. coli</i> )	Ipsen Pharma Genentech, Inc.	Growth failure, Turner syndrome	2001 (EU) 2008 (USA)
Omnitrope (rhGH produced in <i>E. coli</i> )	Sandoz	GH deficiency/growth failure	2006 (EU and USA)
Saizen (rhGH produced in a mouse C127 cell line)	EMD Serono	hGH deficiency in children	1996 (USA)
Somavert (pegvisomant; engineered rhGH analogue, antagonist, produced in <i>E. coli</i> )	Pfizer	Treatment of selected patients suffering from acromegaly	2002 (EU) 2003 (USA)
Valtropin (rhGH produced in <i>S. cerevisiae</i> )	BioPartners GmbH	Growth failure/GH deficiency	2007 (USA) 2006 (EU)

**Figure 8.6** Production of recombinant human growth hormone (rhGH) in *E. coli*. Subsequent to fermentation, the cells are collected by centrifugation or filtration. After homogenization (the product accumulates intracellularly), nucleic acids as well as some membrane constituents are precipitated by the addition of polyethylenimine. Ammonium sulfate precipitation of the supernatant concentrates the crude rhGH preparation. Chromatographic purification follows, as illustrated.

hGH cell-surface receptor, but does not trigger an intracellular response. As such it effectively acts in an antagonistic fashion, reducing the effects of endogenous hGH. The molecule is also PEGylated

*in vitro* in order to increase its serum half-life (see Chapter 2). Valtropin and Omnitrope are also noteworthy in that they were the first 'biosimilar' products to gain approval (Box 8.3).

**Box 8.3 Biosimilars**

When a new drug first comes on the market it is generally protected by one or more patents/regulatory exclusivity periods, which effectively gives its developer a market monopoly for a number of years. On expiry of protection, other pharmaceutical companies can then manufacture and market this product. Such 'generic' products are generally priced well below that of the original product. In the case of traditional pharmaceutical drugs (low-molecular-mass substances, made by direct chemical synthesis), it is possible to manufacture a generic copy identical to the original drug. However, in the case of biopharmaceuticals (because of their size and the fact that they are synthesized in biological systems), it is all but impossible to generate a product guaranteed to be identical to the original product. While the amino acid sequence of such a copy can be identical to the original product, the profile of biological impurities will likely differ. Also, if the products are glycosylated, the exact composition of the glyco-components are likely to differ somewhat. What is technically possible is to generate a copy product that is very substantially similar to the original product, hence the term 'biosimilar'. Many earlier biopharmaceutical products (e.g. hGH, colony-stimulating factors and erythropoietin) have lost patent protection, hence facilitating the development of biosimilar versions of these products.

The European Union has led the way in terms of developing a legislative framework supporting the approval of biosimilars and such products approved in Europe (and the manufacturers) are listed below.

**Human growth hormones**

Omnitrope (Sandoz)  
Valtropin (Biopartners)

**Erythropoietins**

Binocrit (Sandoz)

Epoetin alfa hexal (Hexal)  
Abseamed (Medice Arzneimittel)  
Retacrit (Hospira)  
Silapo (Stada)

**Filgrastims (granulocyte colony-stimulating factors)**

Ratiograstim (Ratiopharm)  
Biograstim (CT Arzneimittel)  
Tevagrastim (Teva)  
Zarzio (Sandoz)  
Filgrastim hexal (Hexal)  
Nivestim (Hospira)

## 8.5 Erythropoietin

Erythropoietin (EPO) is a glycoprotein hormone produced by the kidneys that stimulates the production of red blood cells, the erythrocytes, from their precursor stem cells. Human EPO exhibits a molecular mass in the region of 36 kDa, 60% of which is carbohydrate. The hormone displays four potential glycosylation sites and the glyco-component contains a high level of sialic acid and varying amounts of hexosamines and hexoses. Removal of the sugar residues dramatically decreases *in vivo* biological activity by promoting rapid removal of EPO from plasma. EPO is found in plasma and low levels are found in urine, from which it may be purified.

Production of EPO *in vivo* is regulated by a number of factors, most notably tissue oxygen tension. Its plasma concentration can increase by 100-fold in highly anaemic individuals due to insufficient oxygen supply to the tissues. EPO production can plummet as a result of certain conditions such as chronic renal failure. Such a decrease in EPO levels can in turn lead to the development of secondary anaemia. The condition may also be triggered by various additional chronic conditions, including rheumatoid arthritis and chronic infection. Moreover, anaemia is often associated with chemotherapy, due to the negative effect of many chemotherapeutic agents on fast-growing bone

**Table 8.7** Representative recombinant human erythropoietin (rhEPO)-based products now approved for general medical use.

Product	Company	Therapeutic indication	Approved
Abseamed (rhEPO produced in a CHO cell line)	Medice Arzneimittel Putter GmbH	Anaemia associated with chronic renal failure	2007 (EU)
Aranesp (long-acting rEPO analogue, produced in a CHO cell line)	Amgen	Anaemia associated with chronic renal failure and in cancer patients receiving chemotherapy	2001 (EU, USA)
Biopoin (rhEPO produced in a CHO cell line)	CT Arzneimittel GmbH	Anaemia associated with chronic renal failure and patients receiving chemotherapy	2009 (EU)
Epoetin alfa Hexal (rhEPO produced in a CHO cell line)	Hexal AG	Anaemia associated with chronic renal failure	2007 (EU)
Epogen (rhEPO produced in a CHO cell line)	Amgen	Treatment of anaemia	1989 (USA)
Mircera (recombinant PEGylated EPO produced in a CHO cell line)	Roche	Anaemia associated with chronic kidney disease	2007 (EU, USA)
Retacrit (rhEPO produced in a CHO cell line)	Hospira	Anaemia associated with chronic renal failure	2007 (EU)
Silapo (rhEPO produced in a CHO cell line)	STADA Arzneimittel AG	Anaemia associated with chronic renal failure	2007 (EU)

marrow stem cells, from which mature red blood cells are ultimately derived. EPOs may therefore be used to treat a wide variety of prevalent medical conditions. This is reflected in the global sales value of EPO-based therapeutic products, which collectively stood at \$7.2 billion in 2012. Those used clinically are produced by recombinant DNA technology. The cDNA coding for human EPO was first expressed as a heterologous protein product in 1984. Various companies have developed recombinant human EPO preparations that have gained regulatory approval (Table 8.7).

Of these products, Mircera and Aranesp are noteworthy in that they are both engineered in order to increase their serum half-life. Aranesp (also known as Nespo) is engineered in order to introduce two additional N-glycosylation sites along its backbone. Mircera, on the other hand, has an amino acid sequence identical to that of native human EPO but is PEGylated (Box 8.4). The increased molecular mass of the products and, in the case of Aranesp, the additional negative charges

due to sialic acid likely reduce the rate of elimination of the products from the blood via renal filtration.

## 8.6 Other hormones

Additional polypeptide hormones now produced by recombinant means include parathyroid hormone (Box 8.5), calcitonin and thyroid-stimulating hormone.

Fortical is the trade name given to a recombinant salmon calcitonin, a 32 amino acid, single-chain polypeptide hormone produced by recombinant means in *E. coli*. Calcitonin is secreted by the parafollicular cells of the thyroid gland in mammals. The hormone increases the amount of calcium and phosphate deposited in bones and also reduces serum calcium levels. Interestingly, salmon calcitonin is some 30 times more potent in humans when compared with the endogenous human hormone, hence its application in medicine. Fortical is

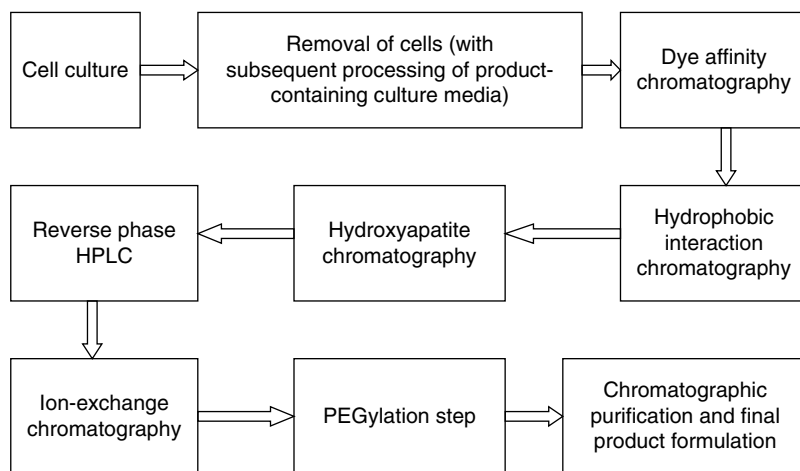
**Box 8.4 Product case study: Mircera**

Mircera is a PEGylated recombinant form of human EPO. The PEG used is methoxypolyethylene glycol-succinimidyl butanoic acid (PEG-SBA), a 30-kDa, linear, chemically activated PEG. When it is co-incubated with EPO, the PEG-SBA spontaneously forms amide chemical linkages with either EPO's N-terminal amino group or with the  $\epsilon$ -amino group of an accessible surface lysine residue (Lys<sup>45</sup> or Lys<sup>52</sup>). The final product generated is a 60-kDa monopegylated product.

The recombinant EPO is manufactured (see diagram below) by initial cell culture of the producer CHO cell line in serum-free medium,

followed by product purification and PEGylation. Phosphate buffer constituents, mannitol, poloxamer 188, sodium sulfate and methionine are added as excipients and the filter-sterilized product is filled into glass vials or pre-filled syringes.

*In vitro* studies indicated that the PEGylated EPO has a lower affinity for the EPO receptor compared with unmodified EPO. However, *in vivo* studies have shown the PEGylated product to be a more potent stimulator of erythropoiesis in terms of magnitude and particularly duration of response, presumably due to the product's extended serum half-life.



also interesting in that it is one of the only biopharmaceuticals administered intranasally, although mean product bioavailability is low (3%). Fortical is used for the treatment of postmenopausal osteoporosis, a condition characterized by low bone mass and increased bone fragility caused by a disproportionate rate of bone resorption over bone formation. Consequently there is an increased risk of fracture. Calcitonin is known to inhibit the bone resorption process. Chemically synthesized calcitonin of both human and salmon sequence has been used for over 20 years and a different

recombinant salmon calcitonin (trade name Forcaltonin) is also used for the treatment of the bone disorder Paget disease.

Thyroid-stimulating hormone (thyrotrophin or TSH) is a glycoprotein hormone produced by the anterior lobe of the pituitary. Structurally TSH is a member of the gonadotrophin family, although functionally it targets the thyroid gland as opposed to the gonads. Like other gonadotrophins, TSH is a dimer consisting of  $\alpha$  and  $\beta$  subunits. Its overall molecular mass is in the order of 28 kDa. TSH stimulates an increase in iodine uptake by the thyroid



**Box 8.5 Product case study: Preotact**

Preotact is the trade name given to a recombinant form of human parathyroid hormone (PTH). The 9.4-kDa, 84 amino acid hormone produced in an engineered *E. coli* cell is identical in sequence to the native human molecule and is devoid of disulfide bonds or a glycocomponent. It is used to treat osteoporosis in postmenopausal women at high risk of fractures. PTH functions as a primary regulator of calcium and phosphate metabolism in bones. It stimulates bone formation by osteoblasts, which display high-affinity cell-surface receptors for the hormone. It also increases intestinal absorption of calcium.

Manufacture involves initial (1100 L) fermentation of the producer *E. coli* (cell line PAL 1000). The cells contain an expression plasmid (pJT42) housing a synthetic gene coding for human PTH fused to a 21 amino acid *E. coli* outer membrane protein A signal peptide. As a result the fusion product is secreted into the culture medium, with automatic removal of the leader sequence from the fusion product by an *E. coli* signal peptidase. Following fermentation, the cells are removed from the product stream via a combination of centrifugation and filtration, followed by an ultrafiltration/diafiltration step. Purification entails five chromatographic steps, followed by excipient addition and lyophilization.

A truncated version of PTH (trade names Forsteo and Forteo), also produced by recombinant means in *E. coli*, has also been approved for the treatment of osteoporosis in postmenopausal women. This 4-kDa polypeptide is identical in sequence to the N-terminal residues 1–34 of endogenous hPTH and it binds to the native PTH receptor and triggers the same effects.

gland and an increase in the rate of synthesis and release of the thyroid hormones thyroxine ( $T_4$ ) and triiodothyronine ( $T_3$ ). It can be used as a diagnostic aid to detect thyroid cancer/function. A recombinant

version of human TSH produced in an engineered CHO cell line (trade name Thyrogen) has been on the market for a number of years.

## 8.7 Growth factors

The differentiation, growth and division of eukaryotic cells is modulated by various growth factors. Each growth factor has a mitogenic (promotes cell division) effect on a characteristic range of cells. While some such factors affect only a few cell types, most stimulate growth of a wide range of cells. A wide variety of polypeptide growth factors have been identified (Table 8.8) and more undoubtedly

**Table 8.8** Some polypeptide growth factors. Many can be grouped into families on the basis of amino acid sequence homology, or the cell types affected. Most growth factors are produced by more than one cell type and display endocrine, paracrine or autocrine effects on target cells by interacting with specific cell-surface receptors.

Growth factor	Major target cells
Colony-stimulating factors	Mainly haemopoietic cells
Epidermal growth factor	Various, including epithelial and endothelial cells and fibroblasts
Erythropoietin	Erythroid precursor cells
Fibroblast growth factors	Various, including fibroblasts, osteoblasts and vascular endothelial cells
Insulin	Various
Insulin-like growth factors	A very wide range of cells found in various tissue types
Interferon- $\gamma$	Mainly lymphocytes and additional cells mediating immunity (and inflammation)
Interleukins	Various, mainly cells mediating immunity and inflammation
Leukaemia inhibitory factor	Mainly various haemopoietic cells
Neurotrophic factors	Several, but mainly neuronal cell populations
Platelet-derived growth factor	Various, including fibroblasts, glial cells and smooth muscle cells
Thrombopoietin	Mainly megakaryocytes
Transforming growth factor- $\alpha$	Various

remain to be characterized. Factors that inhibit cell growth also exist, for example interferons and tumour necrosis factor (TNF) inhibit proliferation of some cell types. Several such growth factors are used therapeutically. We consider some of these in this chapter, while others (interferons and interleukins) are considered in Chapter 9.

### 8.7.1 Colony-stimulating factors

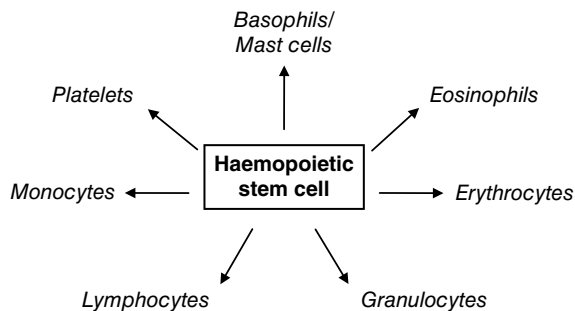
The serum of healthy individuals normally contains three distinct leukocyte (white blood cell) types, lymphocytes, granulocytes and monocytes. Lymphocytes may be subdivided into B and T cells, which function to promote antibody- and cell-mediated immunity, respectively. Granulocytes may be further subcategorized as basophils, neutrophils and eosinophils. These cells are capable of detecting and destroying bacteria and other foreign particles. Some

may also play a role in allergic responses. Monocytes are also capable of engulfing and destroying (phagocytosing) bacteria and other foreign particles, in addition to tissue debris. Derived from monocytes, macrophages are found in tissues and like monocytes phagocytose pathogens and other particles. As previously discussed, all circulating blood cells are ultimately derived from a single cell type, the bone marrow haemopoietic stem cells. A variety of haemopoietic growth factors stimulate the proliferation and differentiation of such stem cells, ultimately yielding this variety of blood cell (Figure 8.7).

Colony-stimulating factors (CSFs) play a prominent role in the proliferation and differentiation of certain precursor cells derived from stem cells, ultimately yielding mainly mature granulocytes and macrophages. These two cell types play important roles in defending the body against a wide range of pathogens. Any factor that decreases the circulating level of these cells renders an individual extremely susceptible to infectious disease.

The term 'colony-stimulating factor' reflects the ability of these substances to promote the *in vitro* growth of various leukocytes in clumps or colonies. A number of different CSFs have been identified and characterized (Table 8.9). These include granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF) and multipotential colony-stimulating factor (multi-CSF), now known to be interleukin (IL)-3.

The various CSFs exhibit little amino acid sequence homology and receptors for all four subtypes have been identified on susceptible cells. All the receptors are transmembrane glycoproteins having an extracellular ligand-binding domain,



**Figure 8.7** Major blood cell types/derivatives generated from haemopoietic stem cells. The differentiation of a stem cell into any single mature blood cell type is a complex multistage process, promoted by a cocktail of various growth and differentiation factors.

**Table 8.9** Human colony-stimulating factors (CSFs) and their sources.

Name	Composition	Molecular mass (kDa)	Sources
GM-CSF	127 amino acid, single-chain glycoprotein	22	T cells, macrophages, fibroblasts, endothelial cells
G-CSF	Single-chain glycoprotein, 177 and 174 amino acid variants exists	21	Macrophages, fibroblasts, endothelial cells
M-CSF	522 amino acid, dimeric glycoprotein	90	Multiple
Multi-CSF (IL-3)	133 amino acid, single-chain glycoprotein	14–30	Activated T cells, mast cells, eosinophils

**Table 8.10** Colony-stimulating factors (CSFs) approved for general medical use.

Product	Company	Therapeutic indication	Approved
Biograstim (filgrastim; recombinant human G-CSF produced in <i>E. coli</i> )	CT Arzneimittel GmbH	Neutropenia	2008 (EU)
Filgrastim hexal (filgrastim; recombinant human G-CSF produced in <i>E. coli</i> )	Hexal AG	Neutropenia	2009 (EU)
Leukine (recombinant GM-CSF; differs from the native human protein by one amino acid, produced in <i>S. cerevisiae</i> )	Genzyme Corporation	Autologous bone marrow transplantation	1991 (USA)
Neulasta/Neupogen (pegfilgrastim; recombinant PEGylated filgrastim, produced in <i>E. coli</i> )	Amgen	Neutropenia	2002 (USA EU)
Neupogen (filgrastim; recombinant G-CSF differs from human protein by containing an additional N-terminal methionine, produced in <i>E. coli</i> )	Amgen	Neutropenia	1991 (USA)
Nivestim (filgrastim; recombinant human G-CSF produced in <i>E. coli</i> )	Hospira UK Ltd	Neutropenia	2010 (EU)
Ratiograstim (filgrastim; recombinant human G-CSF, produced in <i>E. coli</i> )	Ratiopharm GmbH	Neutropenia	2008 (EU)
Tevagrastim (filgrastim; recombinant human G-CSF produced in <i>E. coli</i> )	Teva GmbH	Neutropenia	2008 (EU)
Zarzio (filgrastim; recombinant human G-CSF, produced in <i>E. coli</i> )	Sandoz GmbH	Neutropenia	2009 (EU)

a transmembrane segment and an intracellular effector domain. CSF signal transduction entails tyrosine phosphorylation, although additional mechanisms may also be involved.

GM-CSF displays haemopoietic activity, specifically promoting the differentiation, growth and activation of granulocytes and monocytes. It also serves as a growth factor for T cells, endothelial cells and megakaryocytes. G-CSF serves as a growth, differentiation and activation factor for neutrophils and their precursors. These cells display a short circulatory lifespan (6–8 hours) and are normally synthesized in the body at a rate of some 70,000 cells per second. G-CSF also promotes growth of endothelial cells. M-CSF stimulates the growth, differentiation and activation of macrophages.

### 8.7.2 Production and medical applications of CSFs

The advent of recombinant DNA technology has facilitated the large-scale production of CSFs and several such products are now approved for general

medical use (Table 8.10). G-CSF and GM-CSF have proven useful in the treatment of neutropenia. All three CSF types are (or are likely to be) useful also in the treatment of infectious diseases, some forms of cancer and the management of bone marrow transplants, as they stimulate the differentiation/activation of the white blood cell types most affected by such conditions.

Neutropenia is a condition characterized by a decrease in blood neutrophil count below  $1.5 \times 10^9/L$  (normal neutrophil count  $2.0\text{--}7.5 \times 10^9/L$ ). Its clinical symptoms include the occurrence of frequent and usually serious infections, often requiring hospitalization. Neutropenia may be caused by a number of factors. Particularly noteworthy is neutropenia triggered by administration of chemotherapeutic drugs to cancer patients. Chemotherapeutic agents, when administered at therapeutically effective doses, often induce the destruction of stem cells and/or compromise stem cell differentiation.

Various companies now market recombinant G-CSF (filgrastim) usually for the treatment of chemotherapy-induced neutropenia. Neulasta is the trade name given to a PEGylated form of filgrastim

approved for general medical use. Manufacture of this product entails covalent attachment of an activated monomethoxy polyethylene glycol molecule to the N-terminal methionyl residue of filgrastim. The product is formulated in the presence of acetate buffer, sorbitol and polysorbate and is presented in prefilled syringes for subcutaneous injection. The rationale for PEGylation is to increase the drug's plasma half-life, thereby reducing the frequency of injections required.

G-CSF and GM-CSF have also found application after allogeneic or autologous bone marrow transplantation in order to accelerate neutrophil recovery. ('Allogeneic' indicates that donor and recipient are different individuals, while 'autologous' indicates that donor and recipient are the same.)

### 8.7.3 Some additional growth factors

A number of additional recombinant growth factors are also used therapeutically (Table 8.11). These include IGF-1 (Box 8.6), glucagon-like peptide (GLP)-1 (Box 8.7), platelet-derived growth factor (PDGF) and various bone morphogenetic proteins (BMPs).

PDGF, as the name suggests, was first isolated from platelets. Subsequently, however, it has been established that this growth factor is synthesized and secreted by a number of other cell types. PDGF is a powerful mitogen for a variety of cell types, including fibroblasts, glial cells and smooth muscle cells. PDGF has a molecular mass of 30 kDa and consists of two polypeptide chains termed A and B, which are covalently linked by an interchain disulfide bond. Three isoforms of the growth factor have been isolated: the homodimers AA and BB in addition to the heterodimeric form AB. The A chain consists of 124 amino acids whereas the B chain contains 140 amino acids.

Two distinct, though related, PDGF receptors have also been identified, termed  $\alpha$  and  $\beta$  PDGF receptors respectively. Both receptor types have tyrosine kinase activity. The  $\alpha$  receptor binds all three PDGF isoforms with high affinity. The  $\beta$  receptor on the other hand binds the differing PDGF isoforms with varying degrees of affinity. Binding affinity increases in the order AA, AB, BB. PDGF plays an important role in the process of wound healing. Regranex is the trade name given to the recombinant PDGF used to help accelerate the wound healing process of diabetic skin ulcers (an ulcer is effectively a very slow healing wound).

**Table 8.11** Some additional recombinant growth factors approved for general medical use.

Product	Company	Therapeutic indication	Approved
Increlex (recombinant human IGF-1 produced in <i>E. coli</i> )	Tercica Inc./Baxter/Ipsen Pharma	Long-term treatment of growth failure in children with severe primary IGF-1 deficiency or with growth hormone gene deletion	2007 (EU) 2005 (USA)
InductOs (recombinant BMP-2 produced in a CHO cell line)	Medtronic BioPharma B.V.	Treatment of acute tibia fractures	2002 (EU)
Infuse (recombinant human BMP-2 produced in a CHO cell line)	Medtronic Sofamor Danek	Promotes fusion of vertebrae in lower spine	2002 (USA)
Opera (recombinant human BMP-7 produced in a CHO cell line)	Olympus Biotech	Posterolateral lumbar spinal fusion	2009 (EU)
Osigraft (recombinant human BMP-7 produced in a CHO cell line)	Olympus Biotech	Treatment of non-union of tibia	2001 (EU, USA)
Regranex (recombinant human PDGF produced in <i>S. cerevisiae</i> )	Ortho-McNeil	Lower extremity diabetic neuropathic ulcers	1997 (USA) 1999 (EU)
Victoza (GLP-1 analogue with attached fatty acid, produced in <i>S. cerevisiae</i> )	Novo Nordisk	Type 2 diabetes	2010 (USA) 2009 (EU)

**Box 8.6 Product case study: Increlex**

Increlex (mecasermin) is a recombinant form of human insulin-like growth factor (rhIGF)-1 produced in *E. coli*. The 7.6-kDa, 70 amino acid, single-chain polypeptide displays an amino acid sequence identical to that of the native human growth factor. It is characterized by the presence of three intrachain disulfide linkages. The product is used for the long-term treatment of growth failure in children displaying primary IGF-1 deficiency or growth hormone (GH) gene deletion who have developed neutralizing antibodies to GH (and hence cannot be treated with GH). As its name suggests, IGF bears a strong structural resemblance to insulin (or, more accurately, to proinsulin). IGF-1 is synthesized primarily in the liver, from where it is released into the bloodstream and its cell-surface receptors are found on a wide variety of cells in the body. Transcription of the IGF-1 gene is strongly initiated on binding of GH to its hepatic receptor and most of the growth-promoting effects of GH are actually mediated by IGF-1. Direct injection of IGF-1 into animals whose pituitary (and hence source of GH) has been removed stimulates longitudinal bone growth and growth of various organs/glands, including the kidneys, spleen and thymus.

Increlex manufacture is initiated by the fermentation of the producer *E. coli* cells. After initial product recovery it is subject to a number of high-resolution chromatographic steps. Formulation of the final product entails the addition of sodium acetate buffer components, sodium chloride, benzyl alcohol and polysorbate 20. After filter sterilization, the product is aseptically filled into glass vials (40 mg active protein per vial).

The product is a homodimer of the PDGF B chain (i.e. recombinant human PDGF-BB). The polypeptide chains are aligned in an antiparallel manner, linked by a single interchain disulfide

**Box 8.7 Product case study: Victoza**

Victoza (liraglutide) is a glucagon-like peptide (GLP)-1 receptor agonist produced by recombinant means in *S. cerevisiae*. It is used to treat type 2 diabetes. The product has an amino acid sequence identical to native GLP-1, with one exception: Lys<sup>34</sup> is substituted with arginine. A C<sub>16</sub> fatty acid is attached to Lys<sup>26</sup> (via a glutamic acid spacer) during downstream processing. This increases the molecule's plasma half-life from approximately 2 minutes to 13 hours, allowing once-daily administration via subcutaneous injection. The product potentiates glucose-dependent insulin secretion.

linkage. It is produced in a recombinant *Saccharomyces cerevisiae* strain containing the *PDGFB* gene. The final product is formulated in a gel and is administered topically onto the surface of the wound.

BMPs are a group of growth factors first identified in the 1960s, with in excess of 20 family members having been characterized thus far. They are dimeric glycoproteins containing seven cysteine residues, one of which participates in an interchain disulfide linkage that stabilizes the dimeric structure. As their name suggests, these proteins can induce bone formation, which underpins their therapeutic applications thus far (Table 8.11). BMPs also play an important role in the formation of cartilage, ligaments and tendons. They also regulate the proliferation and differentiation of various cell types, particularly during embryonic development.

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# Chapter 9

## Interferons, interleukins and tumour necrosis factors

### 9.1 Regulatory factors: cytokines versus hormones

For many decades hormones represented the major and only recognized group of biological regulatory factors. As a group, hormones conform to certain defining characteristics: they are biomolecules synthesized in small amounts by ductless (endocrine) glands; they travel via the bloodstream from their site of synthesis to a far-distant target tissue; and they induce characteristic regulatory effects in target tissues by binding to specific cell-surface receptors (or, in the case of steroid hormones, cytoplasmic receptors). In terms of structure, they fall into one of three categories: polypeptide, amide or steroid hormones.

During the second half of the twentieth century researchers began to identify additional regulatory factors that did not fit the classical definition of a hormone. Many of these factors were subsequently labelled 'cytokines', a term originally coined in the 1970s. Initially 'cytokine' referred to regulatory factors promoting the production, activation or regulation of cells that constitute the immune

system. Cytokines represented regulatory factors synthesized mainly by leukocytes, which generally targeted other white blood cells. Unlike classical hormones, cytokines are exclusively polypeptide-based molecules, and are generally produced by cells which are not organized into discrete anatomical glands. In addition, cytokines were seen to primarily act in a paracrine/autocrine (as opposed to endocrine) manner. (Paracrine factors target cells in the immediate vicinity of the cells synthesizing the factor. In the case of autocrine regulatory factors, the producer and target cells are the same.)

Subsequent research findings continue to blur the definition of a cytokine as well as the distinction between 'cytokines' and 'polypeptide hormones'. Cytokines display a very complex biology. They are usually synthesized by more than one cell type and also generally target several different cell types. They tend to promote a wide and complex range of responses in their target cells. It is now also clear that many cytokines can act in an endocrine as well as a paracrine/autocrine fashion and, conversely, that several hormones can display paracrine activity.

Table 9.1 presents a list of regulatory polypeptides now usually categorized as cytokines. Some,

**Table 9.1** The major polypeptides/polypeptide families that constitute the cytokine group of regulatory molecules.

Interleukins (IL-1 to IL-37)
Interferons (IFN- $\alpha$ , IFN- $\beta$ , IFN- $\gamma$ , IFN- $\lambda$ , IFN- $\tau$ , IFN- $\omega$ )
Colony-stimulating factors (G-CSF, M-CSF, GM-CSF)
Tumour necrosis factors (TNF- $\alpha$ , TNF- $\beta$ )
Neurotrophins (NGF, BDNF, NT-3, NT-4/5)
Ciliary neurotrophic factor (CNTF)
Glial cell-derived neurotrophic factor (GDNF)
Epidermal growth factor (EGF)
Erythropoietin (EPO)
Fibroblast growth factor (FGF)
Leukaemia inhibitory factor (LIF)
Macrophage inflammatory proteins (MIP-1 $\alpha$ , MIP-1 $\beta$ , MIP-2)
Platelet-derived growth factor (PDGF)
Transforming growth factors (TGF- $\alpha$ , TGF- $\beta$ )
Thrombopoietin (TPO)

G-CSF, granulocyte colony-stimulating factor; M-CSF, macrophage colony-stimulating factor. GM-CSF, granulocyte-macrophage colony-stimulating factor; NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; NT, neurotrophin.

such as erythropoietin and polypeptide growth factors, have been considered in Chapter 8. This chapter focuses on additional cytokines that have found actual therapeutic application: interferons, interleukins and tumour necrosis factor.

## 9.2 Interferons

The first cytokine to be identified and studied was interferon (IFN), which was discovered initially in 1957. It has since been demonstrated that all vertebrates produce a variety of interferons. These proteins are generally species-specific and most mammals produce at least three types of interferon: IFN- $\alpha$ , IFN- $\beta$  and IFN- $\gamma$ . Humans produce at least 12 closely related types of IFN- $\alpha$  but produce only one IFN- $\beta$  and one IFN- $\gamma$ .

IFN- $\alpha$  and IFN- $\beta$  bind the same cell-surface receptor and are both acid-stable. These are collectively termed type I interferons. IFN- $\gamma$  is acid-labile and binds to a distinct receptor molecule. This interferon has been termed type II interferon. More recently, a type III interferon subgroup has also been recognized, the IFN- $\lambda$ s, of which three members exist ( $\lambda$ 1,  $\lambda$ 2 and  $\lambda$ 3). These were originally classified as interleukins, and are also known as interleukin (IL)-29, IL-28A and IL-28B, respectively. IFN- $\lambda$ s

**Table 9.2** Human interferons and the cellular sources from which they were initially isolated.

Interferon type	Additional name	Cell type from which initially isolated/produced
Alpha interferons (at least 12 distinct but related types exist)	Leukocyte interferon, lymphoblastoid interferon	Leukocytes or lymphoblastoid cells
Beta interferon	Fibroblast interferon	Fibroblasts
Gamma interferon	Immune interferon	Activated T lymphocytes
Lambda interferon (three members)	IL-29, IL-28A, IL-28B	T lymphocytes, virus-infected cells, dendritic cells

share only limited homology (~20%) with other type I interferons and are structurally more closely aligned with the interleukins. However, they bind to a type I interferon-like receptor, signal transduction is mediated via the JAK-STAT pathway, and they display antiviral and antiproliferative activities.

Two additional members of the interferon family have been identified: IFN- $\omega$  and IFN- $\tau$ . Both are classified as type I interferons. An overview summary of the biology of interferons is presented in Tables 9.2 and 9.3.

### 9.2.1 Interferon $\alpha$

IFN- $\alpha$  is also known as leukocyte interferon or lymphoblastoid interferon, as it was initially isolated from these cell types. At least 12 different human IFN- $\alpha$ s are known to exist, all of which exhibit significant homology (14 human IFN- $\alpha$  genes exist, but one is a pseudogene and two further genes code for an identical protein). Most human IFN- $\alpha$ s are single-chain polypeptides consisting of 165–166 amino acids and are rich in leucine, glutamic acid and glutamine. They generally exhibit pI values ranging from 5.5 to 6.5. Although many IFN- $\alpha$ s are devoid of carbohydrate side chains, several are glycoproteins exhibiting varying degrees of glycosylation. The molecular masses of the IFN- $\alpha$ s may range from 16 kDa to in excess of 26 kDa, depending on the carbohydrate content of the molecule.

**Table 9.3** Summary of the biological characteristics of the interferons. Details provided relate to human interferons, except in the case of IFN- $\tau$ , which is only produced by ruminants.

Interferon	Amino acids	Molecular mass (kDa)	Glycosylated?	Major cellular sources	Major biological activities
$\alpha$ (multiple)	Usually 166	16–27	Some are	Lymphocytes, monocytes, macrophages	Confers target cells with viral resistance. Inhibits proliferation of various cell types. Regulates expression of MHC class 1 antigens
$\beta$	166	20	No	Fibroblasts, epithelial cells	Similar to those of IFN- $\alpha$ s
$\gamma$	143	40–70	Yes	T lymphocytes, NK cells	Regulates all phases of the immune and inflammatory response. Displays only weak antiviral and antiproliferative activity
$\lambda$	181 ( $\lambda$ 1) 175 ( $\lambda$ 2, $\lambda$ 3)	20	Only $\lambda$ 1	T lymphocytes	Broadly similar to type 1 IFNs, in particular antiviral activity
$\omega$	172	20	Yes	Mainly leukocytes	Similar to those of other type I interferons
$\tau$	172	19	No	Trophoblast cells	Sustains the corpus luteum during the early stages of pregnancy. In addition, displays the major biological activities of other type I interferons

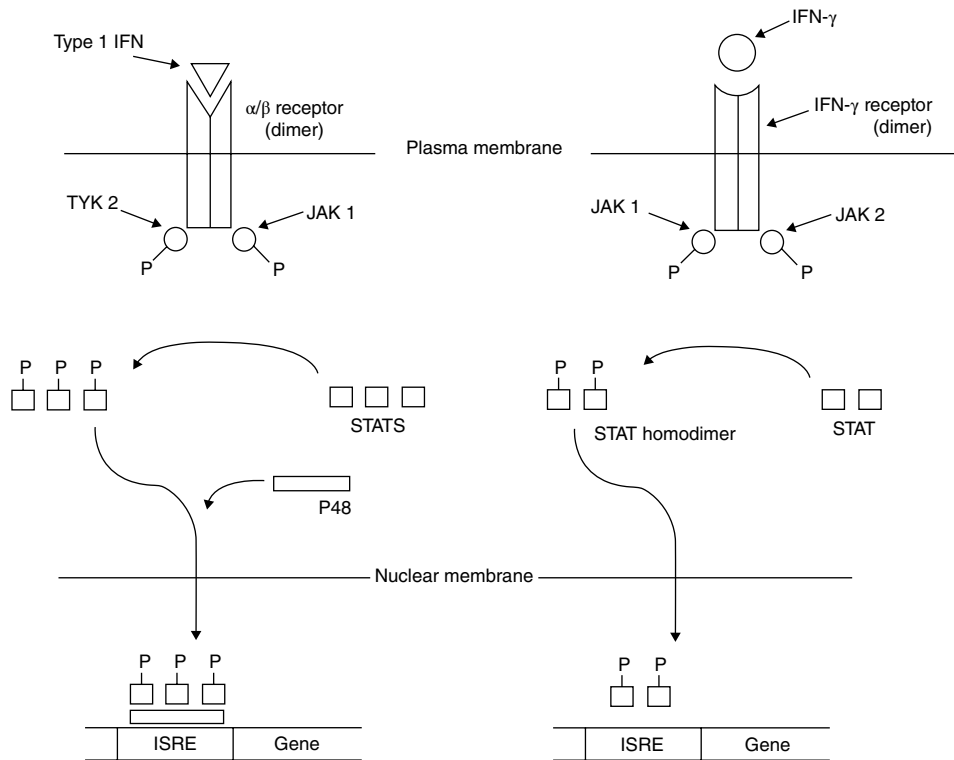
Purification of individual native IFN- $\alpha$ s initially proved difficult, due to not only their extremely low expression levels but also the similarity of their physicochemical properties. Several individual IFN- $\alpha$ s were finally purified to homogeneity in the late 1970s and early 1980s, by employing a combination of several high-resolution chromatographic techniques including immunoaffinity chromatography. Isoelectric focusing and SDS-PAGE have also been used preparatively in some purification schemes. IFN- $\alpha$ s initiate their antiviral, antiproliferative, anti-inflammatory and immunoregulatory effects by binding to specific cell-surface receptors. Two such receptors have been isolated and cloned. The IFN  $\alpha$ B receptor appears to only bind one specific IFN- $\alpha$  (called IFN- $\alpha$ B or IFN- $\alpha$ 8). The other receptor, termed the IFN  $\alpha$ / $\beta$  receptor, can apparently mediate the biological effects of all type I interferons. IFN- $\alpha$  receptors appear to be present on most cell types. They are generally present in relatively low numbers, but display high binding affinities for their ligands.

Binding of type I interferons to the IFN  $\alpha$ / $\beta$  receptor results in the activation of two intracellular receptor-associated tyrosine kinases, TYK-2 and JAK-1. These in turn phosphorylate (and hence activate) three cytoplasmic proteins known as

STATs (signal transducers and activators of transcription). The activated STATs interact with an additional cytoplasmic protein (P48), and this entire complex migrates to the nucleus, where it interacts with interferon-stimulated response elements (ISREs). ISREs are upstream regulatory elements of IFN-sensitive genes. The JAK-STAT pathway thus mediates the biological effects of type I interferons by inducing the synthesis of specific cellular gene products (Figure 9.1). Type I interferons may induce some of their biological responses by initiating additional signal transduction pathways independent of the JAK-STAT pathway. IFN- $\alpha$ s, for example, are capable of activating phospholipase C, releasing diacylglycerol and arachidonic acid.

### 9.2.2 Interferon $\beta$

Human IFN- $\beta$ , also termed fibroblast interferon, was initially derived from fibroblasts which had been exposed to certain viral particles, or to polynucleotides. It was the first interferon molecule to be purified to homogeneity. It is a glycoprotein of 166 amino acids and displays a molecular mass in the region of 20 kDa. It exhibits a pI value of approximately 8 and contains one intramolecular disulfide linkage. The



**Figure 9.1** Signal transduction mechanisms induced by type I ( $\alpha$  and  $\beta$ ) and type II ( $\gamma$ ) interferons. Binding of the interferon to the appropriate receptor results in receptor dimerization, with subsequent activation of JAK-STAT pathways. Refer to text for specific details.

human genome harbours but a single IFN- $\beta$  gene. The gene product displays 30% identity in amino acid sequence to IFN- $\alpha$ . Native IFN- $\beta$  has been purified using a combination of various chromatographic techniques. A one-step purification method employing dye affinity chromatography (Blue Sepharose) has also been developed. IFN- $\beta$  induces similar biological responses to other members of the type I interferon family, by binding to the type I interferon cell-surface receptor (Figure 9.1).

### 9.2.3 Interferon $\gamma$

IFN- $\gamma$ , often referred to as immune interferon, is produced largely by activated T lymphocytes and natural killer (NK) cells. Human IFN- $\gamma$  is a single-chain glycoprotein consisting of 143 amino acids. Its molecular mass ranges from 15 to 25 kDa, depending on the degree of glycosylation (the molecule

harbours two potential glycosylation sites). As previously mentioned, IFN- $\gamma$  differs significantly from type I interferons in terms of its physicochemical properties and biological activities. It regulates almost all aspects of the immune and inflammatory response, including the differentiation and activation of B and T lymphocytes, macrophages and NK cells. Additionally it influences the growth and activation of non-leukocytes such as endothelial cells and fibroblasts. Unlike type I interferons, IFN- $\gamma$  displays at best weak antiviral and antiproliferative activities, but does potentiate such effects when they are induced by type I interferons.

Biologically active IFN- $\gamma$  is a homodimer, formed by non-covalent, antiparallel association of two IFN- $\gamma$  monomers. Each subunit displays six major  $\alpha$ -helical regions, and is totally devoid of  $\beta$  conformation. Given its characteristic biological activities, it is not surprising that the IFN- $\gamma$  receptor is present on most white blood cell types, as well as

on endothelial and epithelial cells. Erythrocytes are devoid of such receptors. Binding of the dimeric IFN- $\gamma$  ligand promotes receptor dimerization and subsequent activation of a JAK-STAT pathway, as illustrated in Figure 9.1.

### 9.2.4 Production and medical applications of IFN- $\alpha$

Up until the 1970s the majority of interferons available for clinical use were sourced from human leukocytes (white blood cells) obtained directly from transfusion blood supplies. This leukocyte interferon consisted of a variety of related IFN- $\alpha$  molecules. The final interferon preparation obtained was only approximately 1% pure, and vast quantities of transfused blood was required to produce minute amounts of product. Although the potential clinical applications of IFN- $\alpha$  were well recognized at that time, the scarcity of this cytokine rendered impossible its widespread clinical evaluation, never mind application.

In the late 1970s bulk quantities of IFN- $\alpha$  were first produced by (non-recombinant) mammalian cell culture. A specific strain of a human lymphoblastoid cell line termed the Namalwa cell line was most often employed in this regard. After induction by Sendai virus, this cell line produces appreciable quantities of leukocyte ( $\alpha$ ) interferon. Industrial-scale production of Namalwa cell leukocyte interferon was undertaken in large culture vessels, many of which exhibit a capacity in excess of 8000 L. Subsequent downstream processing yields a partially purified product consisting of at least eight distinct molecular species of IFN- $\alpha$ .

Individual IFN- $\alpha$ s are now most conveniently produced in large quantities by recombinant DNA methodologies. Initial recombinant preparations first came on the market in the 1980s. Several recombinant human IFN- $\alpha$ s have been produced in engineered *Escherichia coli*, yeast and in some eukaryotic cells including cultured monkey cells and Chinese hamster ovary (CHO) cells. Recombinant human (rh)IFN- $\alpha$ s approved for general medical use are outlined in Table 9.4. Approved indications include the treatment of certain viral-mediated diseases (e.g. hepatitis B and C and genital

warts) as well as some cancer types (e.g. hairy cell leukaemia, renal cell carcinoma, Kaposi sarcoma and non-Hodgkin lymphoma).

The manufacture of one such IFN- $\alpha$  product (Intron A) is outlined in Figure 9.2. A wide variety of chromatographic techniques have been used in the purification of various IFN- $\alpha$  preparations. Such techniques include affinity chromatography using various immobilized ligands, including lectins, reactive dyes, concanavalin A and phenyl groups, in addition to anti-interferon monoclonal antibodies. Other techniques used have included ion-exchange, gel filtration and mineral chelate chromatography. HPLC has proven to be a particularly powerful technique in the preparation of modern high-purity interferons. The introduction of sensitive and convenient immunoassays has also facilitated the rapid purification of interferon preparations. Initial interferon assays relied on monitoring their antiviral activity in cell culture. Such bioassays were time-consuming and complex.

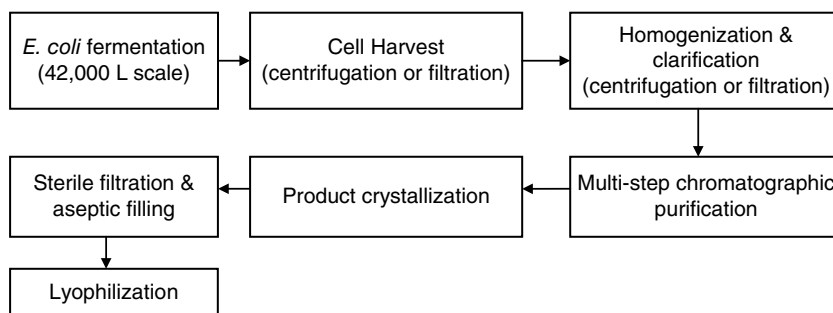
More recently, a number of chemically modified (PEGylated) recombinant interferon products have also gained marketing approval, for example PEG IntronA and Viraferon Peg (Table 9.4). The intrinsic biological activity of PEGylated and non-PEGylated interferons are essentially the same. However, the PEGylated product displays a significantly prolonged plasma half-life (13–25 hours vs. 4 hours for the un-PEGylated molecule). The prolonged half-life is mainly due to slower elimination of the molecule via the kidneys (because of its increased size), although PEGylation also appears to decrease systemic absorption from the site of injection following subcutaneous administration. As a result, PEGylated interferons are typically administered once weekly, as opposed to three times weekly for the un-PEGylated forms. One such PEGylated product is considered in Box 9.1.

### 9.2.5 Production and medical applications of IFN- $\beta$

rhIFN $\beta$ -1a is a recombinant IFN- $\beta$  produced in engineered CHO cell lines. Two such products (Avonex and Rebif) have thus far been approved for

**Table 9.4** Interferon preparations approved for general medical use. In each case the original indication for which the product was approved is listed. Additional indications have subsequently been approved for some such products.

Product	Company	Therapeutic indication	Approved
Actimmune (rhIFN- $\gamma$ -1b). Produced in <i>E. coli</i>	InterMune	Chronic granulomatous disease	1990 (USA)
Alfatronol (rhIFN- $\alpha$ -2b). Produced in <i>E. coli</i>	Merck Sharp & Dohme	Hepatitis B, C, and various cancers	2000 (EU)
Avonex (rhIFN- $\beta$ -1a). Produced in a CHO cell line	Biogen Idec	Relapsing multiple sclerosis	1997 (EU) 1996 (USA)
Betaferon/Betaseron (rhIFN- $\beta$ -1b, differs from human protein in that Cys <sup>17</sup> is replaced by Ser). Produced in <i>E. coli</i>	Bayer Pharma AG	Multiple sclerosis	1995 (EU)
Extavia (rh-IFN- $\beta$ -1B). Produced in <i>E. coli</i>	Novartis	Multiple sclerosis	2009 (USA) 2008 (EU)
IntronA (rIFN- $\alpha$ -2b). Produced in <i>E. coli</i>	Merck Sharp & Dohme	Cancer, genital warts, hepatitis	1986 (USA) 2000 (EU)
Pegasys (peginterferon $\alpha$ 2). Produced in <i>E. coli</i>	Genentech	Hepatitis C	2002 (EU, USA)
PegIntron A (PEGylated rIFN- $\alpha$ -2b). Produced in <i>E. coli</i>	Schering Plough	Chronic hepatitis C	2000 (EU) 2001 (USA)
Rebetron (combination of ribavirin and rhIFN- $\alpha$ -2b produced in <i>E. coli</i> )	Schering Plough	Chronic hepatitis C	1999 (USA)
Rebif (rhIFN- $\beta$ -1a). Produced in a CHO cell line	Merck Serono/Pfizer	Relapsing/remitting multiple sclerosis	1998 (EU) 2002 (USA)
ViraferonPeg (PEGylated rIFN- $\alpha$ -2b). Produced in <i>E. coli</i>	Schering Plough	Chronic hepatitis C	2000 (EU)



**Figure 9.2** Outline of the manufacture of the IFN- $\alpha$  product Intron A. The interferon protein is expressed intracellularly in soluble form.

general medical use (Table 9.4). The amino acid sequence of these recombinant products is identical to that of native IFN- $\beta$ , and like the native product they are glycosylated. rhIFN- $\beta$ -1b (Betaferon/Betasteron and Extavia; Table 9.4) is produced in

engineered *E. coli* cells. The resulting lack of glycosylation in itself has no significant effect on the medical efficacy of these products but shortens the product's serum half-life. In addition to lack of glycosylation, IFN- $\beta$ -1b differs from native IFN- $\beta$  by

**Box 9.1 Product case study: ViraferonPeg**

ViraferonPeg (trade name) is a PEGylated form of IFN- $\alpha$ -2b approved for medical use in the EU since 2000. It differs from native human IFN- $\alpha$ -2b only by the presence of covalently attached polyethylene glycol. ViraferonPeg is indicated for the treatment of chronic hepatitis C in adults, and is usually administered in combination with the antiviral drug ribavirin. It is produced via recombinant DNA technology in an engineered *E. coli* cell line carrying the human IFN- $\alpha$ -2b gene. After cell fermentation the interferon is purified from the bacterial culture via crystallization and multiple chromatographic steps. As part of the downstream processing, the interferon is incubated with chemically activated PEG (methoxypolyethylene glycol, mPEG), which spontaneously forms a covalent linkage via selected protein amino acid groups. The majority of interferon molecules are mono-PEGylated with minor quantities of un-PEGylated and di-PEGylated product also being produced. The product is presented in lyophilized format and contains sodium phosphate, sucrose and polysorbate as excipients.

Pharmacokinetic studies indicate a plasma half-life of 13–25 hours (compared with 4 hours for the un-PEGylated molecule), with maximum serum concentrations attained within 15–44 hours of administration. It is usually administered as once-weekly subcutaneous injections, typically for periods of 6 months.

the substitution of the cysteine residue normally found at position 17 with a serine residue. This engineered product, while retaining the characteristic biological activities of native IFN- $\beta$ , displays enhanced stability. An overview of the manufacture of one such product is presented in Figure 9.3.

Both rhIFN- $\beta$ -1a and rhIFN- $\beta$ -1b have proven effective in the treatment of multiple sclerosis

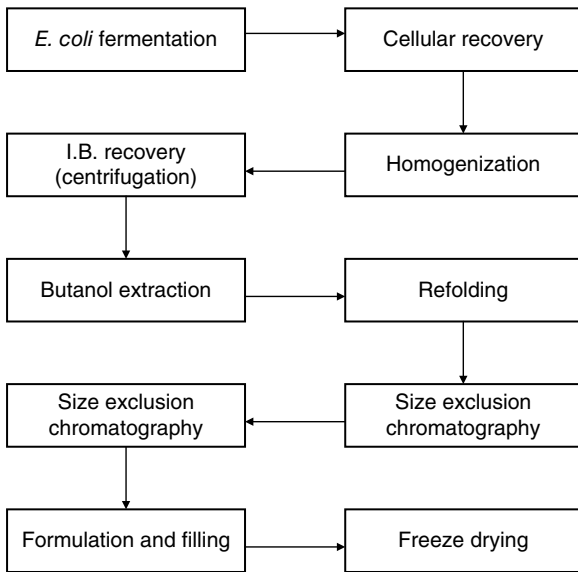
(MS). MS is a neurological condition characterized mainly by damage to the myelin sheath surrounding neurones. The ultimate cause of the disease remains unidentified, although genetic factors and viral infection are suspected of involvement. Currently there is no cure for the condition. The number of newly diagnosed cases in Europe alone stands in excess of 10,000 per year, with the majority of these individuals being in their early thirties.

MS is now predominantly seen as an immune-mediated disease, with migration of immune system cells to lesion sites in the brain. An inflammatory response follows, which is believed to contribute to neuronal damage. IFN- $\beta$  likely exerts its positive therapeutic effect at least in part by countering this inflammatory response. This cytokine is known to inhibit release of proinflammatory molecules such as IFN- $\gamma$  and tumour necrosis factor (TNF).

### 9.2.6 Production and medical uses of IFN- $\gamma$

Given its central role in promoting immunological and inflammatory responses, it is not surprising that IFN- $\gamma$  attracts clinical interest. Actimmune is the trade name given to rhIFN- $\gamma$  produced in engineered *E. coli* cells, and this product has been approved for the treatment of chronic granulomatous disease (CGD). CGD is a genetic condition characterized by the occurrence of recurrent, often life-threatening infections. The genetic defect inhibits an NADPH oxidase system active in phagocytes, thus inhibiting the production of various oxidative substances that phagocytes use to destroy microorganisms.

Clinical trials have established that administration of IFN- $\gamma$  can reduce the incidence of life-threatening infections experienced by CGD sufferers, typically by over 50%. The cytokine appears capable of maximizing production of oxidative substances in affected phagocytes. Its ability to stimulate a generalized immune response also likely contributes to its therapeutic efficacy.



**Figure 9.3** Overview of the manufacture of Betaferon, a recombinant human IFN- $\beta$  produced in *E. coli*. Fermentation is achieved using minimal salts/glucose medium and product accumulates intracellularly in inclusion body (IB) form. During downstream processing the IBs are solubilized in butanol, with subsequent removal of this denaturant to facilitate product refolding. After two consecutive gel filtration steps, excipients are added, the product is filled into glass vials and freeze-dried. It exhibits a shelf-life of 18 months when stored at 2–8°C.

## 9.3 Interleukins

The interleukins represent yet another family of cytokines. At least 37 different human interleukins have thus far been characterized (IL-1 to IL-37). The biology of some of these is summarized in Table 9.5. All are polypeptide regulatory factors that initiate their biological effects by binding to cell-surface receptors on sensitive cell types. Some are glycosylated, others are devoid of a carbohydrate moiety, and they generally display molecular masses in the range 15–30 kDa. Moreover, removal of the glycocomponent of several glycosylated interleukins (e.g. IL-2 and IL-3) has no significant effect on their biological activity, although it may shorten their serum half-life.

Interleukins exhibit many additional properties characteristic of cytokines in general. Most interleukins can be synthesized by a variety of cell types, and all show pleiotropic effects (i.e. promote multiple, distinct and usually unrelated biological effects). However, they are predominantly produced by cells of the immune system and function mainly to regulate immunity and inflammation. Some influence additional processes such as cell growth. Interleukins also exhibit redundancy: several interleukins can independently promote similar/identical effects. Most function in a paracrine/autocrine manner, although some display endocrine activity.

The signal transduction mechanisms by which most interleukins prompt their biological response are understood, in outline at least. In many cases, interleukin cell-surface receptor binding is associated with intracellular tyrosine phosphorylation events. In other cases, serine and threonine residues of specific intracellular substrates are also phosphorylated. For some interleukins, receptor binding triggers alternative signal transduction events, such as promoting an increase in intracellular calcium concentration or inducing hydrolysis of phosphatidylethanolamine with release of diacylglycerol.

Interleukin preparations and interleukin antagonists (discussed later) that have gained approval for general medical use are summarized in Table 9.6.

### 9.3.1 Interleukin-2

IL-2 is perhaps the best characterized of all the interleukins. This cytokine, which is also known as T-cell growth factor, is a single-chain glycosylated polypeptide of molecular mass 15–20 kDa. The protein consists of 133 amino acid residues arranged in four major and two minor  $\alpha$ -helical regions. These are aligned in antiparallel fashion such that their hydrophilic regions are directed towards the surrounding aqueous environment. Analysis of the crystalline structure of IL-2 preparations also reveals that the molecule is devoid of any  $\beta$  conformational sequences. IL-2 contains one intrachain disulfide bond, linking cysteine residues 58 and 105, and which is essential for biological activity. The molecule exhibits an isoelectric point between 6.5 and



**Table 9.5** Some human interleukins: summary of size, sources and major biological activities.

Name	Molecular mass (kDa)	Major cellular producers	Major biological activities
IL-1 $\alpha$	17.5	Monocytes, macrophages, lymphocytes,	Activation of T cells, endothelial cells
IL-1 $\beta$	17.3	NK cells, vascular endothelial cells, fibroblasts, smooth muscle cells, keratinocytes	and monocytes. Promotes antibody synthesis, induces fibroblast proliferation and promotes acute-phase protein synthesis
IL-2	15–20	T lymphocytes	Promotes growth and differentiation of T and B lymphocytes, NK cells, monocytes, macrophages and oligodendrocytes
IL-3	14–30	Activated T cells, mast cells, eosinophils	Haemopoietic growth factor
IL-4	15–19	T cells, mast cells	T and B cell growth and differentiation, promotes synthesis of IgE
IL-5	45 (homodimer)	Mast cells, T cells, eosinophils	Eosinophil differentiation
IL-6	26	T and B cells, macrophages, fibroblasts, keratinocytes, astrocytes, endothelial cells	Regulates B- and T-cell function, haemopoiesis and acute-phase reactions
IL-7	20–28	Bone marrow, spleen cells, thymic stromal cells	Stimulates proliferation and differentiation of immature B cells, immature and mature T cells
IL-8	6–8	Multiple cell types including monocytes, lymphocytes, hepatocytes, keratinocytes, fibroblasts, endothelial cells, among others	Proinflammatory cytokine. Neutrophil chemoattractant and activating factor
IL-9	32–39	Activated lymphocytes, fibroblasts, hepatocytes	Enhances T lymphocyte and mast cell proliferation
IL-10	35–40	Activated T lymphocytes	Stimulates B-cell proliferation, thymocytes and mast cells. Stimulates IgA production by B cells
IL-11	23	IL-1 stimulated fibroblasts	Haemopoietic growth factor
IL-12	75–80	B cells, monocytes, macrophages	Co-stimulates lymphocyte proliferation, enhances NK cell activity, induces IFN- $\gamma$ production by T cells and NK cells
IL-13	17	Activated T cells	Promotes B-cell proliferation, inhibits production of proinflammatory cytokines
IL-14	60	T lymphocytes	Enhances proliferation of activated B cells, inhibits immunoglobulin synthesis
IL-15	14–15	Epithelial cells, monocytes, skeletal muscle, placenta, lung, liver, heart	Shares many of the biological activities of IL-12

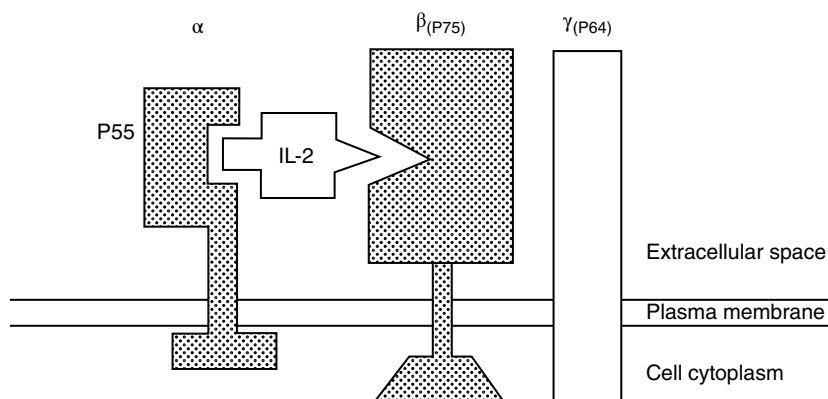
6.8. The human gene coding for IL-2 comprises four exons separated by three introns.

The IL-2 receptor has also been characterized in detail. The high-affinity receptor consists of three distinct transmembrane polypeptide chains which interact with each other non-covalently (Figure 9.4). The smaller of the receptor subunits (the  $\alpha$  subunit) has a molecular mass of 55 kDa and

consists of three segments: a large extracellular domain capable of binding IL-2, a transmembrane domain, and an intracellular domain consisting of 13 amino acids. The larger polypeptide component of the IL-2 receptor (the  $\beta$  subunit) has a molecular mass of 75 kDa. Again, this polypeptide contains extracellular, transmembrane and intracellular domains. In this case, however, the intracellular

**Table 9.6** Interleukins and interleukin antagonists approved for general medical use.

Product	Marketing authorization	Therapeutic indication	Approved
Arcalyst/Riloncept (riloncept; dimeric fusion protein with each monomer consisting of the ligand-binding domains of the hIL-1 receptor and the IL-1 receptor accessory protein, and the Fc region of human IgG1). Produced in CHO cells	Regeneron	Treatment of cryopyrin-associated periodic syndromes (CAPS), prevention of gout flares	2009 (EU) 2008 (USA)
Kineret (anakinra; rIL-1 receptor antagonist). Produced in <i>E. coli</i>	Amgen	Rheumatoid arthritis	2001 (USA)
Neumega (rIL-11, lacks N-terminal Pro of native human molecule). Produced in <i>E. coli</i>	Pfizer	Prevention of chemotherapy-induced thrombocytopenia	1997 (USA)
Ontak (rIL-2-diphtheria toxin fusion protein which targets cells displaying a surface IL-2 receptor). Produced in <i>E. coli</i>	Eisai Inc.	Cutaneous T-cell lymphoma	1999 (USA)
Proleukin (rIL-2, differs from human molecule in that it is devoid of an N-terminal Ala, and Cys <sup>125</sup> has been replaced by a Ser). Produced in <i>E. coli</i>	Prometheus Laboratories	Renal cell carcinoma, metastatic melanoma	1992 (USA)

**Figure 9.4** Schematic representation of the IL-2 receptor. Refer to text for details.

domain is relatively large, consisting of 286 amino acids. It is this portion of the molecule that initiates the intracellular response on binding of IL-2. The third element of the intact receptor, the  $\gamma$  chain, is a 64-kDa transmembrane glycoprotein that does not interact directly with IL-2 but is necessary for signal transduction. This  $\gamma$  polypeptide also appears to form part of several interleukin receptors including receptors for IL-4, IL-7, IL-9, IL-13 and IL-15.

The exact mechanism by which signal transduction is accomplished has not been elucidated,

although tyrosine kinase activity appears to play a role in this process. The IL-2 molecule contains two distinct receptor-binding sites on its surface. One site recognizes and binds the  $\alpha$  receptor subunit while the other binds the  $\beta$  subunit, as illustrated in Figure 9.4. Various studies have revealed substantial differences in the affinity and kinetics of IL-2 binding to the two distinct receptor subunits. IL-2 binds the  $\alpha$  (55 kDa) subunit rapidly, with association/disassociation rates almost comparable to diffusional values. In contrast, IL-2 binds the  $\beta$  receptor subunit (P75) with greater affinity and with

decreased association/disassociation rates. The combination of these two binding characteristics in the intact heterodimeric species yields receptors of very high affinity.

IL-2 plays a pivotal role in normal immunological functioning, regulating many aspects of both innate and acquired immunity (Box 9.2). It is synthesized and secreted by T lymphocytes on their activation by either an antigen or a mitogen. IL-2 then stimulates the further growth and differentiation of activated T (and B) lymphocytes (Figure 9.5). It also potentiates the activity of NK cells, monocytes and macrophages.

Together with antigen, IL-2 is one of the primary molecular effectors capable of inducing an antigen-specific immune response, as proposed initially by Sir Frank MacFarlane Burnet in his clonal selection theory. MacFarlane Burnet rightly predicted that an extensive repertoire of lymphocytes was present naturally in the body, at least one member of which would specifically recognize virtually any antigen that the body might encounter. The presentation of antigen to such a specific lymphocyte would induce the clonal expansion of that particular cell and, in this way, the immune system would mount an immediate and specific response against the offending antigen.

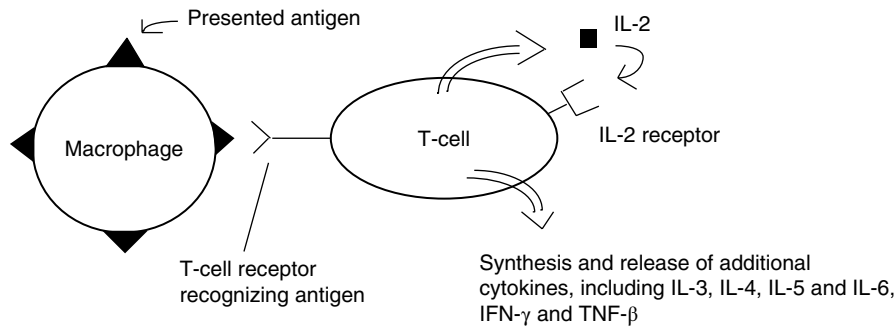
When an antigen gains entry into the body, some of the antigenic material is invariably ingested by macrophages. These cells then 'present' fragments of the ingested material on their surface, hence the origin of the term 'antigen-presenting cell'. A small proportion of circulatory T lymphocytes recognize and bind presented antigen via a specific cell-surface receptor (Figure 9.5). These antigen-specific cells then undergo selective clonal expansion while other circulating lymphocytes, which do not recognize the antigen, remain quiescent. This growth and division of such T cells depends on the presence of not only antigen but also IL-1. IL-1 is produced by activated macrophages. Activated T-helper cells in turn produce IL-2, which stimulates further T-cell differentiation. Along with antigen and IL-6, IL-2 also co-stimulates the proliferation of B lymphocytes (capable of producing antibodies which bind selectively to the foreign moiety). Activated B and T cells also begin to express IL-2 receptors on their

### Box 9.2 Innate and acquired immunity

Higher animals protect themselves against infection by microorganisms and other foreign substances by a variety of means. In general such protective mechanisms may be divided into two types: innate immunity (also termed natural or native immunity) and acquired immunity. Innate immunity is a constitutive form of immunity, being present from birth. It is non-specific (i.e. is not pathogen or antigen specific, but operates against almost any substance which threatens the body). Furthermore, it is not enhanced or amplified by exposure to the foreign substance. Elements of innate immunity include the following.

1. *Physicochemical barriers*: the skin and mucous membranes, stomach acid, digestive enzymes, bile in the small intestine and the low pH of the vagina. These barriers function by preventing entry of the pathogen into the body or by destroying the pathogen before it can colonize within the body.
2. *Phagocytosis*: the ingestion and destruction of microbes and other foreign particles by phagocytes, a special class of defence cells. Phagocytes include macrophages, monocytes, polymorphonuclear leukocytes (PMNs) and natural killer cells (see also Box 9.3).

Acquired immunity is an inducible form of immunity, found only in vertebrates. Acquired immunity is induced by an encounter with a foreign substance or antigen. This type of immune response is antigen-specific and increases in magnitude on continued/recurrent exposure to the inducing antigen. It is largely mediated by T lymphocytes ('cellular immunity') and antibody-producing B lymphocytes (humoral immunity, see also Box 9.3).



**Figure 9.5** Activation of T cells by interaction with macrophage-displayed antigen. Activation results in IL-2 production, which acts in an autocrine manner to stimulate further T-cell growth and division. IL-2 thus represents the major regulatory molecule responsible for stimulation of cell-mediated immunity.

surface. Quiescent lymphocytes are normally devoid of such receptors.

In contrast to most lymphocytes, NK cells constitutively express IL-2 receptors on their surface. Binding of IL-2 to such receptors induces the immediate proliferation of NK cells. IL-2 also stimulates increased production and secretion of various NK-cell products such as a variety of additional cytokines, including TNF, IFN- $\gamma$  and colony-stimulating factors. These cytokines serve to further potentiate the overall immunological response. NK cells comprise approximately 10% of circulating lymphocytes. These cells are believed to play a particularly important role in immunological reactions against cancer cells and virally infected cells (Box 9.3).

### 9.3.1.1 Production and medical applications

IL-2 is secreted in small quantities by activated T-helper cells. Several tumour cell lines, such as the Jurkat leukaemia line, produce increased quantities of this cytokine. Cell lines such as these provided much of the IL-2 used for its initial characterization. Such a source would, however, be hopelessly inadequate in terms of producing clinically significant quantities of this interleukin. Recombinant DNA technology now facilitates the large-scale production of virtually unlimited amounts of IL-2. Both the native human IL-2 gene and cDNA have been used in recombinant protein production. The resultant protein products exhibit a range of biological activities identical to those of the native IL-2 molecule. Although IL-2 is normally glycosylated, most commercialized

### Box 9.3 Some cell types whose biological function is modulated by interleukins or other cytokines

- Astrocytes: cells found in the central nervous system whose likely function is to provide nutrients for neurones
- Eosinophils: a type of polymorphonuclear leukocyte (white blood cell) capable of ingesting particulate matter, and which is also involved in allergic reactions
- Epidermal cells: cells found in the outer layer of skin
- Fibroblasts: cells found in connective tissue that function to produce the ground substance of connective tissue, as well as the precursors of collagen and elastin fibres
- Haemopoietic cells: bone marrow stem cells from which all classes of blood cells are derived
- Keratinocytes: any epidermal cell that produces keratin
- Leukocyte: any white blood cell, i.e. any blood cell that contains a nucleus
- B lymphocyte: antibody-producing white blood cells which mediate humoral immunity
- T lymphocyte: white blood cells responsible for cell-mediated immunity
- Macrophages: phagocytic cells present mainly in connective tissue and organs, with functions similar to those of monocytes

**Mast cells:** cells of connective tissue housing granules containing histamine, heparin and serotonin, released during inflammation/allergic reactions

**Monocytes:** white blood cell which ingests and destroys foreign particles such as bacteria and tissue debris

**Natural killer (NK) cells:** white blood cells which can kill cancer cells and virally infected cells

**Smooth muscle cells:** muscle cells that produce slow long-term contractions of which an individual is unaware. Found mainly in hollow organs (intestine, stomach, bladder and blood vessels)

**Vascular endothelial cells:** single layer of cells that line blood vessels

recombinant products are produced in *E. coli* and hence are devoid of carbohydrate side chains.

IL-2 has been used for a number of years in the treatment of some forms of cancer (see Table 9.6). The effectiveness of IL-2 in treating such conditions lies in its ability not only to enhance B- and T-cell responses but also to mobilize NK cells. Experiments in animal models carried out in the early 1980s illustrated that lung tumours regressed if NK cells were removed from the body, activated by incubation with IL-2 and then reintroduced into the body along with additional IL-2.

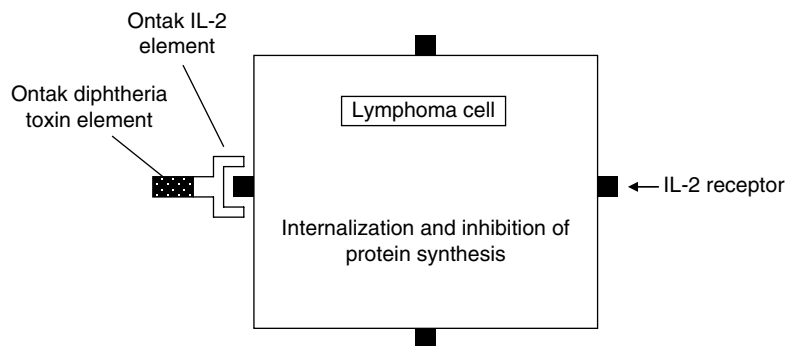
Some cancer types are known to constitutively express high levels of IL-2 receptors on their surface. This provides an IL-2-based targeting mechanism of such cancer cells, as exemplified by the product Ontak (Box 9.4).

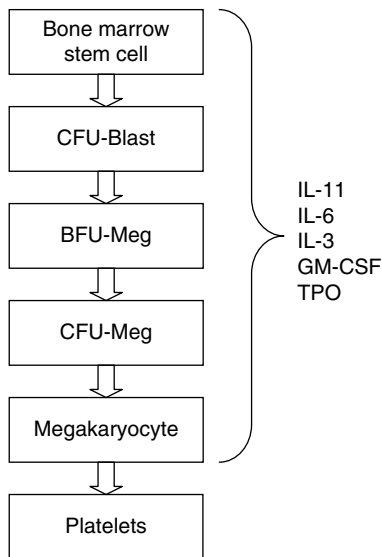
#### Box 9.4 Product case study: Ontak

Ontak (trade name, also known as denileukin diftitox) is an engineered fusion protein produced by recombinant means in *E. coli*. It gained approval for general medical use in the USA in 1999 and is indicated for the treatment of patients with cutaneous T-cell lymphoma (CTCL). The fusion product is composed of diphtheria toxin fragments A and B directly linked to IL-2. It displays a molecular mass of 58 kDa and is purified using reverse phase chromatography and diafiltration. The product is formulated as a sterile frozen solution containing citric acid, EDTA and polysorbate as excipients. It is presented in

single-use vials. The product is normally administered intravenously daily for five consecutive days every 3 weeks over the treatment period.

The IL-2 portion of the fusion protein facilitates product interaction with cells displaying cell-surface IL-2 receptors, found in high levels on some leukaemia and lymphoma cells, including CTCL cells. Binding appears to trigger internalization of the receptor–fusion protein complex. Sufficient quantities of the latter escape immediate cellular destruction to allow diphtheria toxin-mediated inhibition of cellular protein synthesis. Cell death usually results within hours.





**Figure 9.6** The production of platelets from bone marrow stem cells. Stem cells undergo differentiation under the influence of various combinations of cytokines, thereby producing red blood cells (erythrocytes), white blood cells (leukocytes) or platelets. This process is known as haemopoiesis. Thrombopoiesis (the production of platelets) is described here. Megakaryocytes are the immediate progenitor cells from which platelets are derived. Platelet production entails the budding off of small membrane-bound vesicles from the megakaryocyte surface. These vesicles (platelets) then enter circulation. A combination of cytokines serve to promote platelet production. The interleukins and granulocyte-macrophage colony stimulating factor (GM-CSF) appear to promote differentiation of earlier cells in the sequence, whereas thrombopoietin (TPO) mainly stimulates the final stages of platelet production. Characteristic cytokine redundancy likely results in several of these regulatory factors displaying overlapping activity in the context of platelet production. The scheme, as presented is likely an oversimplification of the actual process. IL, interleukin; CFU, colony-forming unit; BFU, burst-forming unit.

### 9.3.2 Interleukin-11

Human IL-11 is a 23-kDa polypeptide predominantly produced by fibroblasts and bone marrow stromal cells. It is a haemopoietic growth factor that stimulates thrombopoiesis (Figure 9.6). cDNA coding for human IL-11 was first isolated in 1991,

and from this its amino acid sequence was deduced. The cDNA has been expressed in a number of recombinant expression systems and IL-11's tertiary structure consists of four  $\alpha$ -helical bundles with two stretches of  $\beta$ -sheet.

rhIL-11 produced in an engineered *E. coli* strain is used for the treatment/prevention of chemotherapy-induced thrombocytopenia, a condition characterized by a low blood platelet count. Chemotherapy often induces thrombocytopenia as stem cells are particularly sensitive to many chemotherapeutic agents. Prior to the approval of IL-11 for this indication, the only treatment available was direct platelet transfusions. This carried with it the low but real risk of accidental transmission of disease via infected platelet preparations.

### 9.3.3 Interleukin-1

IL-1 is produced by a number of cell types (see Table 9.5), predominantly by phagocytic cells such as macrophages and monocytes. There are actually two distinct IL-1 molecules, termed IL-1 $\alpha$  and IL-1 $\beta$ . Although these interleukins are the products of two distinct genes and exhibit only limited homology, they bind to the same receptor and induce identical biological responses. Both IL-1 $\alpha$  and IL-1 $\beta$  are initially synthesized as precursor molecules with molecular masses of 31 kDa. These precursors are subsequently processed proteolytically, yielding active biological molecules with a molecular mass of approximately 17.5 kDa. Human IL-1 $\alpha$  has an isoelectric point in the region of 5.5 while that of IL-1 $\beta$  is close to 7.0.

Two molecular forms of the IL-1 receptor have been identified. These have been termed type I and type II receptors. The type I receptor is found to be present mainly on the surface of T cells and fibroblasts. The type II receptor is present on B lymphocytes. Both IL-1 $\alpha$  and IL-1 $\beta$  can bind to both receptors.

IL-1 exhibits a wide range of biological activities, such as:

- promotes inflammation (appears to be its major biological activity);

- promotes proliferation of various cell types including lymphocytes, fibroblasts and thymocytes;
- acts as a co-stimulator of haematopoietic cell growth and differentiation;
- stimulates release of additional cytokines from various cells;
- helps promote wound healing;
- induces hepatic acute-phase protein synthesis;
- displays antiproliferative effects on some cancer cell lines.

IL-1 promotes the release of IL-2 from activated T lymphocytes, and many biological activities attributed to IL-1 may be mediated, at least partly, by IL-2. IL-1 has also been implicated in the progression of a number of disease states, most notably proinflammatory conditions.

IL-1 itself has not been approved for any medical purpose. Because of its role in mediating acute/chronic inflammation, downward modulation of IL-1 activity may prove effective in ameliorating the clinical severity of these conditions. Again, several approaches may prove useful in this regard, such as:

- administration of anti-IL-1 antibodies;
- administration of soluble forms of the IL-1 receptor;
- administration of the native IL-1 receptor antagonist.

Kineret is the trade name given to a recently approved product based on the latter strategy. Indicated in the treatment of rheumatoid arthritis, the product consists of a recombinant form of the native human IL-1 receptor antagonist. The 17.3-kDa, 153 amino acid product is produced in engineered *E. coli* and differs from the native human molecule in that it is non-glycosylated and contains an additional N-terminal methionine residue (a consequence of the expression system used). A daily (subcutaneous) injection of 100 mg is recommended for patients with rheumatoid arthritis. This inflammatory condition is (not surprisingly) characterized by the presence of high levels of IL-1 in the synovial fluid of affected joints. In addition to its proinflammatory properties, IL-1 also mediates

additional negative influences on joint/bone, including promoting cartilage degradation and stimulation of bone resorption.

Arcalyst (also known as rilonacept) is an additional IL-1 blocker, approved for the treatment of cryopyrin-associated periodic syndromes (CAPS), a group of rare autoinflammatory conditions characterized by the overproduction of IL-1 $\beta$  in the body, driving inflammation. General features of these conditions include recurrent fevers, rash and joint pain. Arcalyst is an engineered dimeric fusion protein, with each of its two constituent polypeptides consisting of the ligand-binding domains of the human IL-1 receptor (IL-R1) and IL-1 receptor accessory protein (IL-1RacP), linked to the constant (Fc) portion of human IgG1. The 251-kDa glycosylated product is expressed in an engineered CHO cell line. Arcalyst brings about its therapeutic effect by acting as a decoy receptor, binding excess free IL-1 $\beta$  and hence preventing it from interacting with cell-surface receptors. The product's antibody Fc portion confers on it an extended half-life, supporting once-weekly injections.

## 9.4 Tumour necrosis factors

TNFs are additional members of the cytokine family of regulatory proteins. Two forms of TNF are recognized, TNF- $\alpha$  (cachectin) and TNF- $\beta$  (lymphotoxin). Although both proteins bind the same receptors and elicit broadly similar biological responses, they are distinct molecules and share less than 30% identity. A summary of the physicochemical characteristics of TNF- $\alpha$  and TNF- $\beta$  is provided in Table 9.7.

### 9.4.1 TNF- $\alpha$

The eventual discovery of TNF- $\alpha$  stems from observations made at the turn of the century by an American surgeon called William Coley. Coley observed that the tumours of some cancer patients regressed or disappeared after they suffered a severe bacterial infection. Coley thus attempted to treat cancer patients by administering live bacteria.

**Table 9.7** Summary of the physicochemical characteristics of human TNF- $\alpha$  and TNF- $\beta$ .

Property	TNF- $\alpha$	TNF- $\beta$
Amino acid content	157	171
Molecular mass (kDa)	52*	25
Glycosylation	No	Yes
Disulfide bonds	1	0
pI	5.6	5.8

\* Biologically active human TNF- $\alpha$  is a homotrimer. The monomer, which is biologically inactive, displays a molecular mass of 17.3 kDa.

However, this approach suffered from several disadvantages, not least of which was the inability to control the ensuing infection in those pre-antibiotic days. In an effort to overcome such difficulties, Coley subsequently developed a vaccine consisting of dead bacterial suspensions, which became known as Coley's toxins. Some clinical successes were recorded when cancer patients were treated with such toxins. Consistent results were never attained and this method of treatment fell out of medical fashion.

The active component of Coley's toxins was later shown to be a complex biomolecule termed lipopolysaccharide (LPS), also known as endotoxin (see Chapter 5). LPS itself is devoid of any antitumour activity. The serum of animals injected with LPS was found to contain a factor toxic to such cancerous cells. This factor, which was produced by specific cells in response to LPS, was termed 'tumour necrosis factor' (necrosis refers to cellular death). LPS represents the most potent known stimulant of TNF- $\alpha$  production.

TNF- $\alpha$  is synthesized by a wide variety of cell types, most notably activated macrophages, monocytes, certain T lymphocytes, NK cells in addition to the brain and liver cells. Although LPS represents the most potent inducer of TNF- $\alpha$  synthesis, various other agents such as some viruses, fungi and parasites also stimulate the synthesis and release of this cytokine. Furthermore, TNF- $\alpha$  may act in an autocrine manner, stimulating its own production.

Native human TNF- $\alpha$  is a homotrimer, consisting of three identical polypeptide subunits tightly associated about a threefold axis of symmetry. This arrange-



**Figure 9.7** Three-dimensional structure of TNF- $\alpha$ . Image from the RCSB PDB ([www.pdb.org](http://www.pdb.org)) of PDB ID: 1TNF (M.J. Eck, S.R. Sprang (1989) The structure of tumor necrosis factor-alpha at 2.6 Å resolution. Implications for receptor binding. *J.Biol.Chem.*264: 17595-17605).

ment resembles the assembly of protein subunits in many viral capsids. The individual polypeptide subunits of human TNF- $\alpha$  are non-glycosylated and consist of 157 amino acids. The molecule has a molecular mass of 17.3 kDa and contains one intrachain disulfide linkage. X-ray crystallographic studies of the TNF- $\alpha$  monomer have shown that the molecule is elongated (Figure 9.7). Much of its amino acid sequence forms two  $\beta$ -pleated sheets, each of which contains five antiparallel  $\beta$  strands. Human TNF- $\alpha$  is synthesized initially as a 233 amino acid precursor molecule. Proteolytic cleavage of a 76 amino acid signal sequence releases native TNF- $\alpha$ . TNF- $\alpha$  may also exist in a 26-kDa membrane-bound form.



#### 9.4.1.1 Biological effects

TNF- $\alpha$  induces its biological effects by binding specific receptors present on the surface of susceptible cells. Two distinct TNF- $\alpha$  receptors have been identified. One receptor (TNF-R55) has a molecular mass of 55 kDa whereas the second receptor (TNF-R75) has a molecular mass in the region of 75 kDa. These two distinct receptor types show no more than 25% sequence identity. TNF-R55 is present on a wide range of cell types whereas the distribution of the type II receptor is more limited.

Both receptor types are members of the nerve growth factor receptor superfamily and exhibit the characteristic four (cysteine-rich) repeat units in their extracellular domain. The extracellular domains of TNF-R55 and TNF-R75 exhibit only 28% identity, while their intracellular domains are devoid of any homology, indicating the likely existence of distinct signalling mechanisms.

It appears that TNF-R55 is capable of mediating most TNF activities, while the biological activities induced via the TNF-R75 receptor are more limited. For example, TNF's cytotoxic activity, as well as its ability to induce synthesis of various cytokines and prostaglandins, are all mediated mainly or exclusively by TNF-R55. TNF-R75 appears to play a more prominent role in the induction of synthesis of T lymphocytes. All the biological activities mediated by TNF-R75 can also be triggered via TNF-R55, and usually at much lower densities of receptors. TNF-R75 thus appears to play more of an accessory role, mainly to enhance effects mediated via TNF-R55.

The exact molecular mechanisms by which TNF- $\alpha$  induces its biological effects remain to be determined. Binding of this cytokine to its receptor results in receptor oligomerization. This in turn triggers a variety of events, mediated by G-proteins in addition to the activation of adenylate cyclase, phospholipase A<sub>2</sub> and protein kinases. The exact biological actions induced by TNF- $\alpha$  may vary from cell to cell. Additional factors, such as the presence of other cytokines, further modulate the observed effects attributed to TNF- $\alpha$  action on sensitive cells. The major biological activities of TNF- $\alpha$  include:

- promoting an inflammatory response, both directly and indirectly;

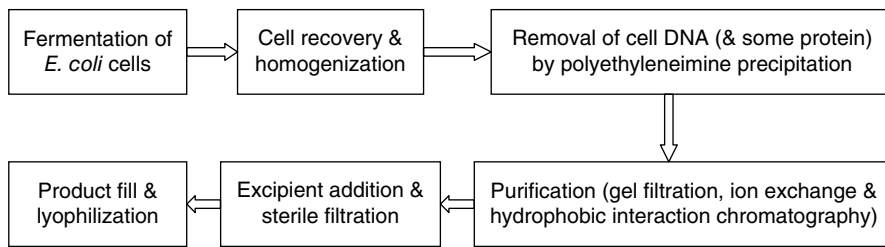
- activating a range of phagocytic cells;
- displaying cytotoxicity against a range of transformed cells;
- inducing synthesis and release of a range of additional cytokines, including IL-1, IL-6, IL-8 and colony-stimulating factors;
- elevated systemic levels mediate septic shock, cachexia and possibly (in part) various autoimmune conditions.

Under normal conditions TNF- $\alpha$  is produced in relatively low levels and exerts its influences in a paracrine/autocrine manner. It plays a central role in several physiological processes such as inflammation and the immune response. It upregulates non-specific immunity directly by activating a range of phagocytic cells. It also indirectly influences additional elements of the immune response by promoting the synthesis and release of a range of additional cytokines. It promotes an inflammatory response both directly (e.g. by activating neutrophils and other proinflammatory leukocytes) and indirectly (e.g. by promoting synthesis of proinflammatory cytokines). Indeed the use of anti-TNF antibody-based products or TNF antagonists represents a major therapeutic approach for treating inflammatory conditions including rheumatoid arthritis and Crohn's disease (see Chapter 7).

Its proinflammatory activities, and particularly its stimulation of various elements of innate and acquired immunity, also form the basis of its cytotoxicity towards various transformed cells. However, the ability of TNF- $\alpha$  to induce tumour cell necrosis (death) is now known to be a secondary biological activity of the molecule.

Various conditions (e.g. serious microbial infections) can trigger overproduction of TNF- $\alpha$ . Under such circumstances this cytokine enters the general circulation and acts in an endocrine-like manner. The majority of such systemic (i.e. whole body as opposed to localized) effects have negative health implications.

Septic shock is a serious medical condition usually associated with Gram-negative bacterial infection. LPS present in the outer membrane of such bacteria plays a central role in the development of this condition. LPS, as previously mentioned, is the



**Figure 9.8** Schematic overview of the production of Beromun, a recombinant human TNF- $\alpha$  product produced intracellularly in engineered *E. coli* cells.

most potent known inducer of TNF synthesis and it has been demonstrated clinically that administration of exogenous TNF- $\alpha$  can induce symptoms identical to those seen in patients suffering from septic shock. Furthermore, it has been shown that pretreatment with anti-TNF- $\alpha$  antibodies exerts a protective effect on animals subsequently challenged with potentially lethal doses of LPS.

Prolonged production of inappropriately elevated levels of TNF- $\alpha$  has also been implicated in the development of cachexia, the wasting syndrome often associated with chronic parasitic or other infections, and with cancer.

#### 9.4.1.2 Medical applications

The TNF- $\alpha$  gene has been cloned and expressed in recombinant *E. coli*. The resultant availability of large quantities of purified, biologically active TNF has facilitated clinical evaluation in a number of disease states, most notably cancer. Many such trials either employing TNF, alone or in combination with IFN- $\gamma$ , have yielded disappointing results.

Such trials usually employed rhTNF- $\alpha$  administered systemically. At therapeutically relevant doses this induced a wide range of unacceptable side effects. However, high-dose TNF- $\alpha$  administration in combination with chemotherapy has proven effective in certain cancers affecting the limbs. In this context, the root of the limb is surgically isolated and the limb is then perfused with TNF- $\alpha$ . This so-called isolated limb perfusion technique facilitates administration of high local concentrations of TNF- $\alpha$  while preventing leakage into the general circulation. This in turn prevents the occurrence of negative systemic effects. Beromun is the

trade name given to rhTNF- $\alpha$  produced in *E. coli* which has been approved for this purpose. An overview of its industrial production is presented in Figure 9.8.

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# Chapter 10

## Proteins used for analytical purposes

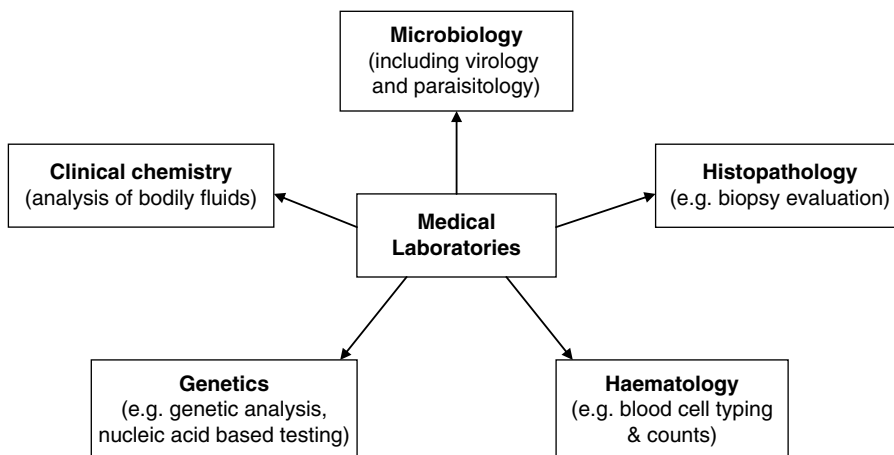
A number of protein types, most notably enzymes and antibodies, have found widespread application as analytical reagents. Because of their biospecificity, these proteins are capable of detecting and quantifying specific analytes of interest, often in complex mixtures. While protein-based analytical tests are applied to areas such as environmental and food analysis, they are most commonly applied in the medical/healthcare sector, where they are classified as a type of medical device known as an *in vitro* diagnostic agent (IVD). Enzyme and antibody-based IVDs are particularly critical to the practice of clinical chemistry, the scientific discipline charged with detecting, monitoring and quantifying a broad variety of marker substances present in bodily fluids, primarily blood and urine (Figure 10.1).

An understanding of normal and abnormal metabolic activity allows clinicians to link changes in the concentration of various biomolecules ('markers') to disease states or to impending medical events. Marker detection and quantification can greatly assist doctors in the accurate prediction, diagnosis or monitoring of diseases and medical conditions, thus allow-

ing them to formulate the most appropriate therapeutic responses.

A wide range of biomolecules are of potentially significant diagnostic value. Such substances include low-molecular-mass metabolic products such as urea, glucose, cholesterol or steroid hormones. Many substances of higher molecular weight, such as specific proteins that may be released from damaged tissue or whose normal concentration is altered due to a particular metabolic aberration, are also of diagnostic value. Some illustrative examples of diagnostic markers routinely analysed in clinical chemistry are provided in Table 10.1 and specific examples of such tests are provided over the course of this chapter.

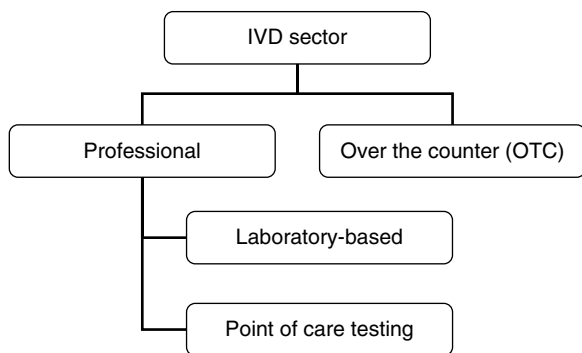
Most disease conditions are dynamic rather than static in nature. The value of many diagnostic results are often closely correlated with not only the sensitivity and specificity of the test, but also the speed with which results may be obtained. Indeed the progression of many disease states is often monitored by performing repeat tests on samples obtained from the patient at appropriate time intervals.



**Figure 10.1** Some of the areas of speciality that constitute modern medical laboratories. Such laboratories may be located within a hospital or may be stand-alone, private testing companies. The exact organization and profile of testing undertaken will vary from laboratory to laboratory.

**Table 10.1** Some analytes routinely measured in clinical chemistry laboratories, along with their diagnostic significance.

Analyte	Condition(s) associated with increased concentration of analyte	Condition(s) associated with decreased concentration of analyte
Alanine aminotransferase	Hepatitis, cirrhosis, hepatomas	
Albumin	Blood-brain barrier damage	Malnutrition, liver cirrhosis, severe burns, inflammation, renal disease
Amylase	Pancreatitis	Liver disease
Bilirubin, total	Hepatitis, haemolytic disease, cirrhosis	
Blood globulins	Infections, multiple myeloma	Leukaemia, immunosuppression
Calcium (Ca <sup>2+</sup> )	Hyperparathyroidism, some cancers	Hypoparathyroidism, kidney disease, pancreatitis
Carcinoembryonic antigen (CEA)	Cancers (bladder, breast, colorectal, liver, pancreas)	
Cholesterol, total	Hypothyroidism, poorly managed diabetes, kidney disease	Anaemia, liver diseases, starvation
C-reactive protein	Inflammation, tumours, tissue destruction, active rheumatoid arthritis	
Creatinine	Kidney dysfunction	
Creatine kinase	Muscle damage	
Glucose	Diabetes mellitus, Cushing disease	Insulin excess, adrenal insufficiency
Human chorionic gonadotrophin (hCG)	Pregnancy	
Lactate dehydrogenase	Heart attack, liver disease, lung disease	
Potassium (K <sup>+</sup> )	Shock, renal disease, circulatory failure	Vomiting/diarrhoea, some cancers
Prostate-specific antigen	Prostate cancer	
Rheumatoid factor	Rheumatoid arthritis, mixed connective tissue disease, viral and bacterial disorders	
Thyroid-stimulating hormone	Thyroid disorders	Thyroid disorders
Transferrin	Iron deficiency, pregnancy, acute hepatitis, oral contraceptive use	Inflammation
Troponin I and T	Heart attack	
Uric acid	Gout, kidney disease, leukaemia	
Urine protein, total	Kidney failure, diabetes	



**Figure 10.2** IVD sectors. Refer to text for specific details.

## 10.1 The IVD sector

The IVD sector may be subdivided into several main segments (Figure 10.2). Professional diagnostics are those undertaken by trained health-care staff. This sector can be further subdivided into laboratory-based and point-of-care testing. Laboratory-based tests are undertaken in laboratory settings, often using automatic multisample analysers (autoanalyzers; Box 10.1) which increases the speed, efficiency, throughput and economy with which diagnostic assays are carried out. Point-of-care tests are undertaken directly by medical personnel (e.g. at a hospital bedside or in a doctor's surgery), allowing for real-time medical decisions following the diagnostic result. Over-the-counter (OTC) tests are those IVDs which are available for purchase by the general public, allowing an element of self-diagnosis, usually in a home setting. Several tests (e.g. blood glucose monitoring and pregnancy tests) are undertaken in all sectoral segments. The science behind any one test in these different segments is usually the same, with only the format of the test being different, as will become obvious later in the chapter. Global IVD sales reached an estimated \$44 billion in 2011, and is projected to reach \$60 billion by the middle of the decade. The major multinational IVD companies include Roche, Abbott and Siemens (Figure 10.3).

### Box 10.1 Autoanalyzers

When first developed, immunoassays and enzyme-based analytical assays were invariably undertaken manually. This entailed physical transfer of sample and reagents by laboratory personnel, generally followed by manual operation of an appropriate detector such as a spectrophotometer. Over the years these procedures have been automated and in most cases miniaturized in the form of an analytical instrument known as an autoanalyzer. The major diagnostic companies have developed their own autoanalyzer range. Roche market their own Cobas® analyzer series while Abbot autoanalyzers are marketed under the 'Architect' brand name. The various members of any one autoanalyzer series vary in terms of testing capacity as well as the exact profile of tests undertaken. Originally, clinical chemistry and immunoassay-based analysers were separate instruments. Modern integrated systems are often modular based, with capacity to undertake both assay types. For example, Beckman Coulter's UniCel® Dx C880i system (see photograph below) has the capacity to undertake up to 120 different clinical chemistry/immunoassay-based tests at any one time, with an hourly throughput rate of up to 1440 chemistry and up to 400 immunoassay tests.



Image courtesy of Beckman Coulter, Inc.

Most assays require in the range of 1–100  $\mu\text{L}$  of analyte sample/reagents and take no more than a few minutes to complete. Generally, each manufacturer develops a series of groups or ‘panels’ of tests, each based on a particular medical theme (e.g. cancer, cardiac, thyroid function). For example, among its tests, Roche manufacture a series of 10 cardiac marker tests (including for total, HDL and LDL cholesterol, and triglycerides) while Abbot immunoassay tests include a panel of tests for six cardiac markers, including troponin I, myoglobin and creatine kinase.

The main components ‘under the bonnet’ of an autoanalyzer include reagent reservoirs, an automated robotic sample handling system and a detection system (e.g. a photometric instrument). The reagent reservoir is usually refrigerated and most reagents are usually supplied in liquid form and typically display on-board stabilities of 1–2 months.

The autoanalyzer itself is classified as a medical device and hence must be approved for use in most world regions by regulatory authorities such as the FDA. The instruments are not inexpensive, with the larger-capacity ones displaying a price tag in the region of \$500,000.

## 10.2 The basis of analyte detection and quantification

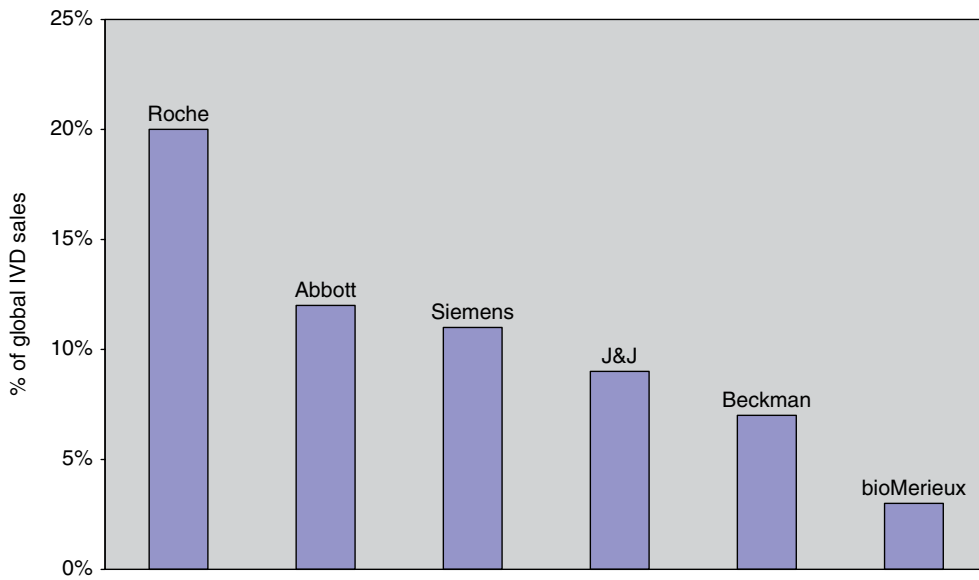
The vast majority of analytes measured in clinical chemistry do not inherently possess unique physicochemical properties that allow their direct measurement in solution, certainly when present in complex analyte mixtures characteristic of biological samples. Analytical reagents such as enzymes or antibodies are thus applied to transform or otherwise modify or label the target analyte in a way that does facilitate its detection and quantification.

Many specific such examples are outlined in the remaining sections of this chapter but all are typically are based on linking analyte concentration to either optical or electrochemical methods (Figure 10.4).

Optical (photometric) methods rely on passing light (usually of a specific wavelength) through the sample to be analysed, with subsequent measurement of the light emanating from the sample using a photodetector (Figure 10.4). In the case of absorbance photometry, the analyte absorbs some of the incident light. The amount of light absorbed, which is directly proportional to the analyte concentration of the sample, is quantified by the photodetector. Some other tests are based on the formation of analyte-based insoluble particles which remain suspended in solution, hence making the solution turbid (e.g. the addition of analyte-specific antibodies can potentially achieve this). This forms the basis of turbidimetric tests, in which such suspended particles interfere with the passage of incident light in a straight line through the sample (by blocking or reflecting/scattering it in different directions). The photodetector is positioned to detect only light passing straight through the sample which, in this case, is inversely proportional to the amount of analyte present.

Nephelometric methods are based on a variation of the turbidimetric approach, in which the photodetector is placed at an angle to the incident light path. In this case, rather than measuring the decrease in light passing straight through the sample, it detects and quantifies some of the light scattered by the particles. The amount of light falling on the photodetector is therefore directly proportional to the amount of analyte present. Finally, fluorescence-based photometric approaches employ fluorescent reagents capable of binding specifically and only to the target analyte (thereby effectively making the target analyte fluorescent). Fluorescent molecules (fluorophores) are those capable of absorbing light of a specific wavelength and then emitting light of a slightly longer (i.e. lower energy) wavelength. The photodetector is set specifically to measure this emitted light of longer wavelength, hence the amount of light





IVD Company	Web address
Roche	<a href="http://www.roche.com/">http://www.roche.com/</a>
Abbott	<a href="http://www.abbottdiagnostics.com/home.cfm">http://www.abbottdiagnostics.com/home.cfm</a>
Siemens	<a href="http://healthcare.siemens.com/laboratory-diagnostics">http://healthcare.siemens.com/laboratory-diagnostics</a>
Johnson & Johnson	<a href="http://www.jnj.com/connect/healthcare-products/medical-technologies">http://www.jnj.com/connect/healthcare-products/medical-technologies</a>
Beckman coulter	<a href="https://www.beckmancoulter.com">https://www.beckmancoulter.com</a>
bioMerieux	<a href="http://www.biomerieux.com">http://www.biomerieux.com</a>

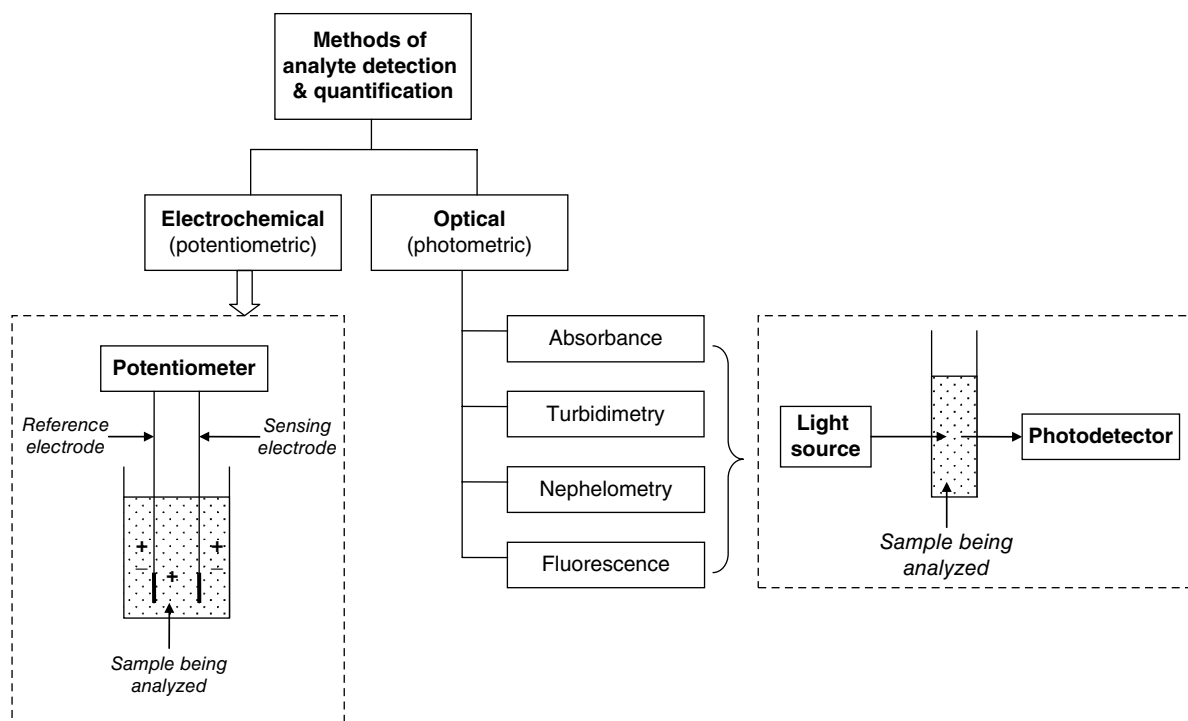
**Figure 10.3** Major global IVD companies who, between them, account for almost two-thirds of global IVD sales. In terms of global market, the USA accounts for in the region of 45% of all sales, with Europe accounting for a further 30% of sales. Additional major IVD companies include Bayer Healthcare, LifeScan Inc. and Becton Dickinson.

detected is directly proportional to the analyte concentration.

Potentiometric techniques are based on measurement of the electrical potential between two electrodes (the reference and sensing electrodes; Figure 10.4). The electrical potential between the two electrodes is affected by the concentration of specific ions in solution and specific ion-selective electrodes are usually used (a pH meter is an example of an ion-selective electrode; it detects and quantifies  $H^+$  ion concentration in solution). Such an approach is particularly convenient if measuring concentration of electrolytes such as  $K^+$ ,  $Na^+$  or  $Cl^-$ .

## 10.3 Enzymes as diagnostic/analytical reagents

A variety of enzymes have been used as diagnostic reagents for many years. Enzymes may be used to detect and estimate the levels of specific analytes present in biological samples. In certain situations, enzymes may also be used to catalytically remove specific compounds present in such samples, which may interfere with the assay of a particular metabolite. Enzymes are also widely used as labels in enzyme immunoassays (EIAs, see later). Their high degree of selectivity, coupled with their catalytic



**Figure 10.4** Overview of optical and electrochemical approaches to analyte detection. Refer to text for details.

efficiency, render many enzymatic preparations ideal diagnostic reagents. Currently in the region of 70 enzymes used for analytical purposes are produced commercially, with combined global sales of over \$500 million annually.

A number of criteria determine if an enzyme is suited to analytical application.

- *Specificity of reaction catalysed:* the enzyme must display high substrate specificity for the analyte to be measured. It must be devoid of side activities towards additional substances potentially present in the sample to be analysed if such side reactions could in any way interfere with the detection system.
- *Kinetic properties:* the enzyme should display suitable  $K_m$  and  $K_{cat}$  values, and not be subject to inhibition by any substance likely or potentially present in the samples to be assayed.
- *Source availability and cost:* the enzyme should be produced in modest/high quantities by its (native or recombinant) producer. The cost of

upstream and downstream processing should be competitive.

- *pH and temperature vs. activity profiles:* should be conducive to significant catalytic activity under the assay conditions employed.
- *Enzyme stability:* the enzyme should be stable under the storage conditions specified for several months, and it should be stable under the conditions that the assay is performed.

Enzymes used for analytical purposes are obtained from a variety of plant, animal and microbial sources (Table 10.2). Many are still produced by direct extraction from native producer sources, although others (e.g. alkaline phosphatase and cholesterol oxidase) are now largely produced via recombinant DNA technology. Recombinant systems used for commercial production include *Streptomyces* and *Pichia* species, although a whole range of analytical enzymes have been expressed in recombinant *Escherichia coli* as well as in various fungal systems.

**Table 10.2** Some enzymes used directly or indirectly as diagnostic reagents. The original enzyme source and likely applications are also listed. Many are now produced via recombinant means.

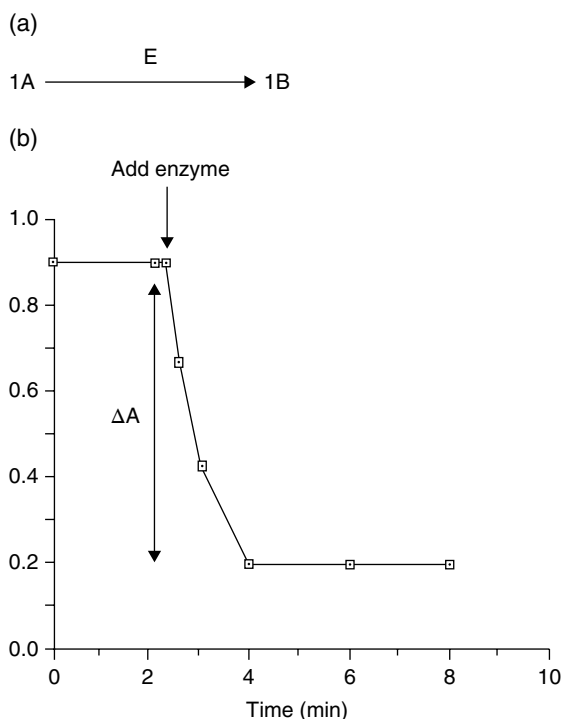
Enzyme	Original source	Application
Acetylcholinesterase	Bovine erythrocytes	Detection of organophosphorus compounds such as pesticides
Alcohol dehydrogenase	Yeast	Determination of alcohol levels in biological fluids
Alkaline phosphatase	Calf intestine and kidney	Conjugation to antibodies allows its use as an indicator in ELISA systems
Arginase	Beef liver	Determination of L-arginine levels in plasma and urine
Ascorbate oxidase	<i>Cucurbita</i> species	Determination of ascorbic acid levels; eliminates interference by ascorbic acid
Cholesterol esterase	Pig/beef pancreas, <i>Pseudomonas</i> sp.	Determination of serum cholesterol levels
Creatine kinase	Rabbit muscle, beef heart, pig heart	Diagnosis of cardiac and skeletal malfunction
Glucose 6-phosphate dehydrogenase	Yeast, <i>Leuconostoc mesenteroides</i>	Determination of glucose and ATP in conjunction with hexokinase
Glucose oxidase	<i>Aspergillus niger</i>	Determination of glucose in biological samples in conjunction with peroxidase; a marker for ELISA systems
Glutamate dehydrogenase	Beef liver	Determination of blood urea nitrogen in conjunction with urease
Glycerol kinase	<i>Candida mycoderma</i> , <i>Arthrobacter</i> sp.	Determination of triglyceride levels in blood in conjunction with lipase
Glycerol 3-phosphate dehydrogenase	Rabbit muscle	Determination of serum triglycerides
Hexokinase	Yeast	Determination of glucose in body fluids
Peroxidase	Horseradish	Indicator enzyme for reactions in which peroxide is produced
Phosphoenolpyruvate carboxylase	Maize leaves	Determination of CO <sub>2</sub> in body fluids
Urease	Jack bean	Determination of blood urea nitrogen; marker enzyme for ELISA systems
Uricase	Porcine liver	Determination of uric acid
Xanthine oxidase	Buttermilk	Determination of xanthine and hypoxanthine in biological fluids

Most commercial enzymes used for analytical purposes are required in relative modest quantities (<10 kg) annually. A few such as glucose oxidase are produced at much higher levels (100 kg or more per year). Most enzyme preparations used for diagnostic purposes have been subjected to at least partial purification (see Chapter 5).

Most enzymatic preparations used in the detection of various molecules of diagnostic significance are free in solution. However, for some applications, the enzyme is used in immobilized format. Examples of the latter format are detailed later.

### 10.3.1 End-point versus kinetic methods

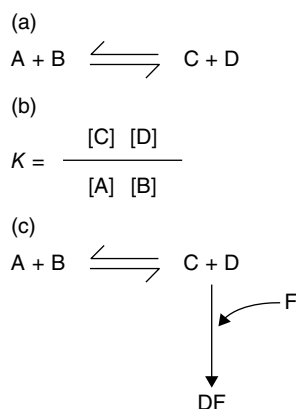
Enzymes used in the detection and quantification of specific analytes normally employ the analyte in question as a substrate. Changes in the concentration of one or other of the co-reactants, cofactors or products of the reaction must be readily monitored. In practice this is most often achieved by spectrophotometry, fluorimetry or (in the case of many biosensors) electrochemical



**Figure 10.5** Detection and quantification of a specific analyte, A, by an enzyme-based end-point method. The enzyme, when incubated under appropriate assay conditions, completely or virtually completely converts A into B in a stoichiometric fashion. In this case, one molecule of A is converted into one molecule of B (a). The amount of substrate consumed or product formed is quantified (b). In this case, A absorbs (at the wavelength chosen) whereas B does not. The change in absorbance recorded can be directly related to analyte concentration by Beer's law.

measurement. As the magnitude of signal generated is proportional to the analyte concentration present, quantitative tests may be developed. For example, in the case of spectrophotometric tests, the quantity of light absorbed is related to the substrate concentration by Beer's law ( $A = \epsilon cl$ , where  $A$  represents absorbency measurement obtained,  $\epsilon$  molar absorption coefficient,  $c$  concentration of analyte and  $l$  path length). Enzyme-based analyte quantification is carried out by either end-point or kinetic methods.

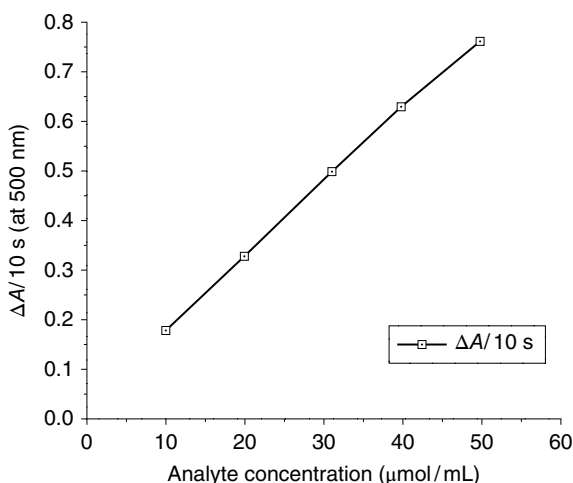
End-point methods may be developed if an enzyme of appropriate characteristics (e.g. substrate



**Figure 10.6** Generalized reaction in which an enzyme interconverts A + B and C + D. The equilibrium constant ( $K$ ) value for the reaction is calculated according to the formula presented in (b). A reaction which, on reaching equilibrium, has almost completely converted A + B into C + D will display a high  $K$  value. If the reaction does not go to near completion, it can essentially be pulled to completion by addition of a chemical which reacts with one of the products (C or D). This is represented as F in diagram (c). In this example, D is removed almost as soon as it is formed, so its concentration continually remains low.

specificity and kinetic properties) is identified. Essentially the enzyme must be capable of selectively, speedily and completely converting the target analyte into product in a stoichiometric fashion. In such instances prespecified quantities of enzyme and test solution are co-incubated under appropriate conditions (of temperature and pH), with subsequent quantitative determination of the signal generated (e.g. change in absorbency in the case of spectrophotometric tests; Figure 10.5). Some enzymes will achieve incomplete analyte conversion into product if the reaction displays an unfavourable equilibrium constant. End-point analyte quantification is still possible as long as the assay is carried out under conditions which 'shove' or 'pull' the reaction to completion. In practice this can be achieved by increasing the initial concentration of co-substrate (if one exists) or by adding a substance which reacts with one of the reaction products thereby trapping it (Figure 10.6).

An alternative approach to enzyme-based detection and quantification of a specific analyte is



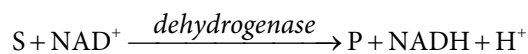
**Figure 10.7** Generalized example of a calibration curve used to correlate reaction rate with analyte (i.e. substrate) concentration. In this case the analyte absorbs at 500 nm, whereas the reaction product does not. Assaying a sample containing an unknown concentration of analyte will yield a  $\Delta A/10$  s value, which can then be correlated with the analyte concentration by reference to the standard curve.

termed the kinetic method. Basic enzyme kinetics illustrate that, for any reaction, if the substrate concentration is much lower than the enzyme's Michaelis constant (i.e.  $S \ll K_m$ ), then the observed reaction rate is linearly proportional to the substrate concentration. The kinetic approach therefore measures the rate at which substrate is being converted (i.e.  $-dS/dt$ ) or the rate at which product is being formed (i.e.  $+dP/dt$ ). This approach requires construction of a calibration curve in which varying known substrate concentrations are used (Figure 10.7). Kinetic-based assays can invariably be carried out much faster than end-point assays, although an enzyme displaying a high  $K_m$  towards the analyte must generally be used.

### 10.3.2 Some common enzyme-based diagnostic tests

Two of the most commonly employed enzyme types in diagnostic systems are dehydrogenases and oxidases. In the case of dehydrogenases, progression

of the reaction may be followed by monitoring the conversion of NADH to NAD<sup>+</sup> or vice versa, as illustrated below (S represents reaction substrate, P reaction product).



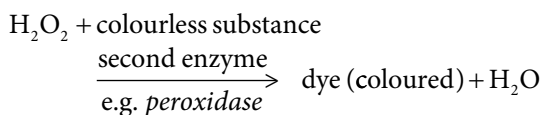
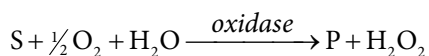
Oxidation or reduction of the cofactor may be readily monitored spectrophotometrically, as NADH absorbs strongly at 340 nm, whereas NAD<sup>+</sup> does not absorb at this wavelength. Progression of the reaction may also be followed by fluorescence or luminescence. From the above generalized example, it is clear that one molecule of NADH is formed for each molecule of substrate (S) present, the analyte whose concentration is to be determined. Thus, in such a system, the total quantity of NADH formed reflects the quantity of substrate present in the sample analysed.

When considering this example, it becomes evident that all assay reagents should be present in excess, such that it is the concentration of substrate which dictates the final quantity of NADH formed. If insufficient NAD<sup>+</sup> were present, for example, the reaction would cease once all the cofactor was converted to NADH, although significant quantities of the substrate S might still remain. In such circumstances, the quantity of substrate present in the sample analysed would be underestimated.

Oxidases, which yield hydrogen peroxide as a reaction product, are also commonly employed in diagnostic systems. However, in most such instances none of the final products of this primary reaction (i.e. 'P' or H<sub>2</sub>O<sub>2</sub> in the equation below) are readily quantified. A second enzymatic step is therefore included in the assay system. The second 'linker' enzyme utilizes one of the products of the initial enzymatic step as one of its substrates, H<sub>2</sub>O<sub>2</sub> in the generalized example below. Unlike the primary enzymatic step, one or other of the products of this second reaction is easily measured by use of an appropriate assay. In the example cited, the second enzyme utilizes the H<sub>2</sub>O<sub>2</sub> produced in the initial reaction, together with a second colourless substance as substrates, yielding H<sub>2</sub>O and a

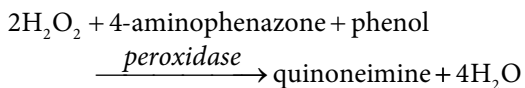
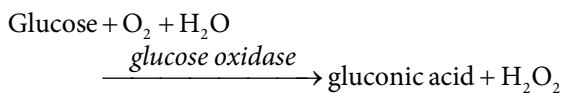
coloured dye as products. The quantity of dye produced can be determined by monitoring the increase in absorbance at the appropriate wavelength. As long as all the reagents present in the assay system are in excess, the quantity of dye produced by such a coupled assay is directly related to the quantity of substrate present in the sample analysed.

The concepts outlined in the generalized examples discussed are illustrated more clearly by the specific examples discussed below. Several alternative enzymatic methods have been developed to assay many biological substances of diagnostic interest.



### 10.3.3 Assay of blood glucose

The concentration of glucose present in blood is an important diagnostic marker for several disease states, especially diabetes. Blood glucose determinations constitute one of the most common assays carried out in clinical chemistry laboratories. Most such determinations are based on specific assays, of which there are several available. A system using glucose oxidase was one of the first enzyme-based analytical tests developed. The reaction principle is as follows:

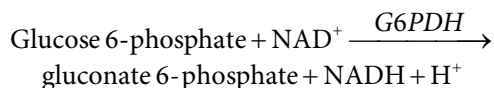
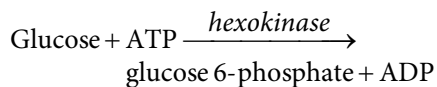


In this coupled assay, the level of glucose in the initial sample is directly related to the quantity

of quinoneimine formed. Unlike the other reaction products, quinoneimine is a red-violet dye whose concentration can easily be determined by measuring its absorbance at a wavelength of 500 nm.

In this example the reagents present in the assay system consist of the enzymes glucose oxidase and peroxidase, as well as phenol and 4-aminophenazone. A specified volume of this reagent cocktail would be incubated with a specific volume of the serum sample to be assayed. The reaction would be allowed to proceed for an appropriate time, typically several minutes, at an appropriate temperature, typically 20–37°C, until all the glucose present in the serum sample has been enzymatically converted to gluconic acid. Several additional assays in which serum samples have been replaced with standard glucose solutions are normally run concurrently. The glucose concentration present in the serum sample can be calculated by comparison of unknown with standard absorbance values.

A second assay system commonly used to determine blood glucose levels employs the enzymes hexokinase and glucose 6-phosphate dehydrogenase (G6PDH). The reaction principle is outlined below.



The concentration of glucose present in the sample is related to the quantity of NADH formed by the coupled assay. This may be easily monitored by measuring the resultant absorbance at 340 nm. The above assay systems are capable of rapidly, accurately and conveniently determining the concentration of blood glucose present in any sample tested. Such a result, however, solely reflects the concentration of glucose present at the time the blood sample was taken.

Diagnostic tests which monitor average long-term blood glucose levels are also available.

Such tests are invariably based on assessing the level of glycosylated haemoglobin present in a serum sample. Elevated blood glucose concentrations promote the glycosylation of haemoglobin molecules by covalent linkage of a glucose residue to the terminal valine residue of the haemoglobin  $\beta$ -chain. This reaction may occur progressively in the erythrocyte throughout its normal 120-day lifespan. Thus the level of glycosylated haemoglobin present in a blood sample reflects blood glucose levels over a period of several weeks. This assay is invaluable in assessing the long-term effectiveness of therapeutic approaches as applied to diabetic patients. Separation of glycosylated haemoglobin from native haemoglobin is normally achieved by electrophoretic or chromatographic procedures.

Determination of the presence of glucose in urine also constitutes an important diagnostic test. Such a system is often employed as an OTC or point-of-care test for the initial detection of diabetes.

### 10.3.4 Assay of blood cholesterol and triglycerides

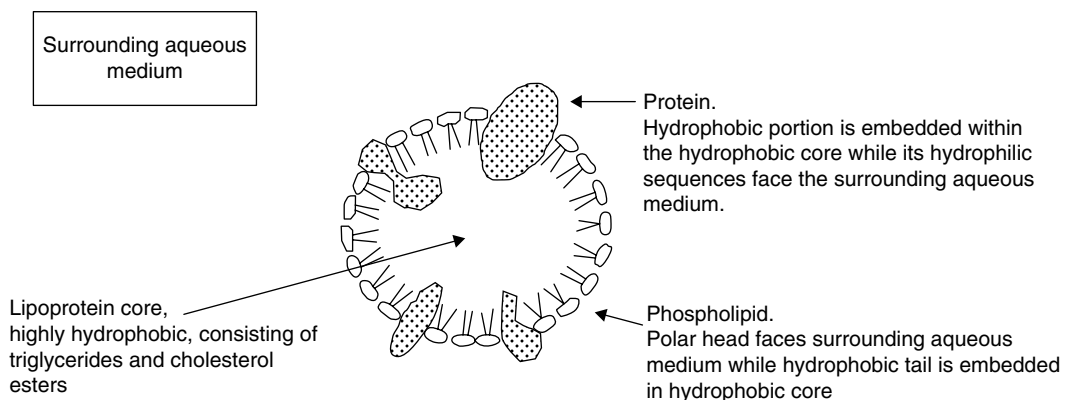
There exists a well-established link between atherosclerosis and elevated plasma cholesterol levels. This association relates in particular to levels

of cholesterol associated with low-density lipoproteins (LDL-cholesterol). The condition of atherosclerosis is responsible for the deaths of one in two individuals in most Western societies.

Because of their largely hydrophobic nature, both cholesterol and triglycerides are transported through the bloodstream in the form of soluble complexes termed lipoproteins (Figure 10.8). The outer layer of lipoprotein particles consists largely of phospholipids and proteins termed apoproteins. The hydrophilic portions of these molecules are thus oriented towards the surrounding aqueous environment. The internal portion of such lipoproteins is composed predominantly of triglycerides and cholesterol covalently linked to long-chain fatty acids via ester bonds (i.e. cholesterol esters).

Based on differences in physicochemical properties, lipoproteins may be classified as high-density lipoproteins (HDL), low-density lipoproteins (LDL) or very low density lipoproteins (VLDL) (Table 10.3). Chylomicrons, another group of lipoproteins, are found in plasma, particularly after the ingestion of a lipid-rich meal. Chylomicrons are the largest lipoproteins and consist almost exclusively of triglycerides. They serve to transport dietary fats.

Increases in the circulating concentration of LDL-cholesterol in particular is linked to the development of atherosclerosis. Elevated levels of HDL-cholesterol, on the other hand, appear to exert a protective effect



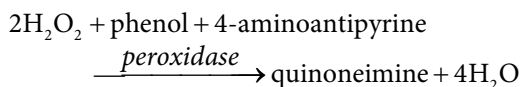
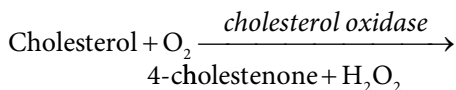
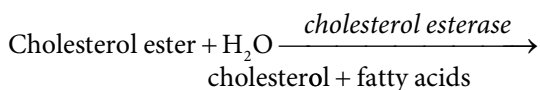
**Figure 10.8** Generalized structure of a plasma lipoprotein.

**Table 10.3** Approximate content of triglycerides and cholesterol in various lipoproteins.

	Chylomicrons	VLDL	LDL	HDL
Triglycerides (%)	85-90	50-60	10	3-4
Cholesterol (%)	2-4	15	45	17-18

against the development of fatty deposits on the inner walls of arteries characteristic of atherosclerosis. This may be due to the fact that HDL functions to transport cholesterol back from peripheral tissues to the liver where it may be converted to bile salts.

Several diagnostic tests capable of measuring serum concentrations of cholesterol are available. Total serum cholesterol levels may be estimated by employing the three-step coupled enzymatic system illustrated below.

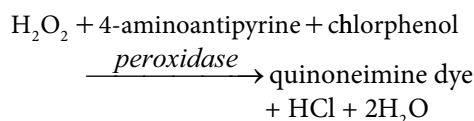
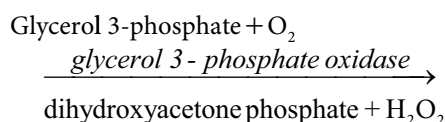
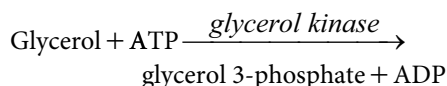
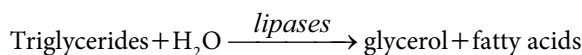


In the initial reaction, cholesterol esterase catalyses the hydrolytic cleavage of cholesterol esters, yielding free cholesterol, which may then be oxidized by cholesterol oxidase. The resultant  $\text{H}_2\text{O}_2$  can be quantified by the peroxidase system. The end product, quinoneimine, is a red dye which may be easily quantified by measuring its absorbance at 500 nm.

Total serum HDL-cholesterol may be specifically quantified by using the above assay method. In this case it is necessary to firstly remove the other lipoproteins from the serum sample. This may be achieved by the addition of phosphotungstic acid and magnesium ions, which promote the precipitation of LDL, VLDL and chylomicrons. Following a centrifugal step, the cholesterol content of the HDL-containing supernatant may be assayed by the above procedure. More recently, variant technical procedures have been developed that can distinguish

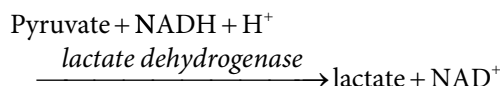
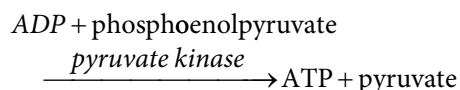
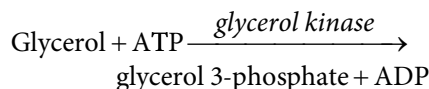
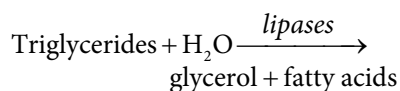
between the various fractions above, rendering initial separation unnecessary.

Elevated levels of serum triglycerides have been linked to atherosclerosis and coronary artery disease. Various enzymatic systems have been developed that facilitate appraisal of triglyceride concentration in serum. One commonly used system which is based on four sequential enzymatic reactions is illustrated below.



The concentration of triglycerides in the serum sample provided is obviously proportional to the quantity of dye produced. Quinoneimine levels may be determined by measuring absorbance at 500 nm.

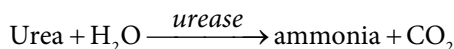
A variety of additional enzymatic systems, such as the one shown below, have been developed to quantify triglyceride levels in biological samples.





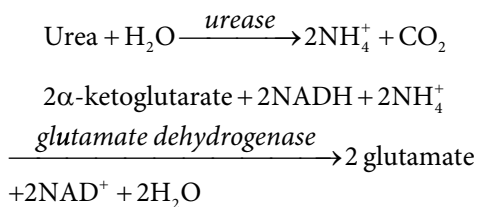
### 10.3.5 Assay of blood urea and uric acid

Determination of urea and uric acid levels are frequently carried out in most clinical laboratories. Elevated levels of these metabolites are often indicative of a variety of metabolic disorders, including diseases of the kidney. Most methods used to estimate urea levels include the enzyme urease, which catalyses the hydrolytic cleavage of urea as shown below.



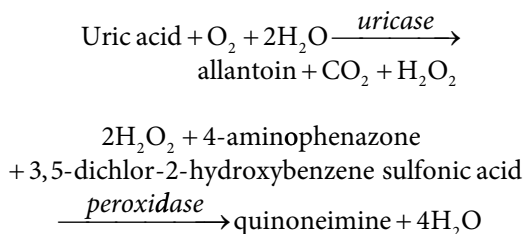
A variety of methods may then be used to detect and quantify the amount of ammonia formed by this reaction. One such method relies on chemical detection of the ammonia formed. Ammonium ions react with phenol and hypochlorite in the presence of sodium nitroprusside, forming a blue coloured complex that absorbs light strongly at 640 nm.

An alternative enzymatic method used to quantify the ammonia produced utilizes the enzyme glutamate dehydrogenase, as illustrated below.

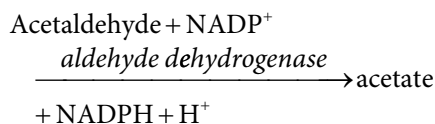
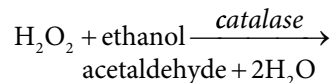
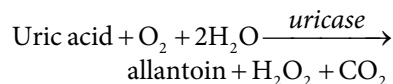


The  $\text{NAD}^+$  formed by the second reaction may readily be monitored, and it is of course proportional to the quantity of urea present in the initial sample.

Uric acid may also be quantified by a number of enzymatic systems. One of the more common methods uses a coupled assay system consisting of uricase and peroxidase, as shown below. The end product, the red dye quinoneimine, has maximal absorbance at 520 nm.



An alternative method for the quantification of uric acid is as follows:



The increase in absorbance at 340 nm due to the formation of NADPH is proportional to the quantity of uric acid present in the sample.

### 10.3.6 Immobilized enzymes as diagnostic reagents

There has been a steady increase in the development and use of diagnostic reagents in immobilized form. Enzymes immobilized on reagent strips are used for selected diagnostic purposes in clinical laboratories, directly by doctors and indeed by patients themselves. One of the most commonly employed such reagent strips are those designed to detect glucose in body fluids (Box 10.2). Immobilized enzymes also often represent the biological component of biosensors, as described below.

## 10.4 Biosensors

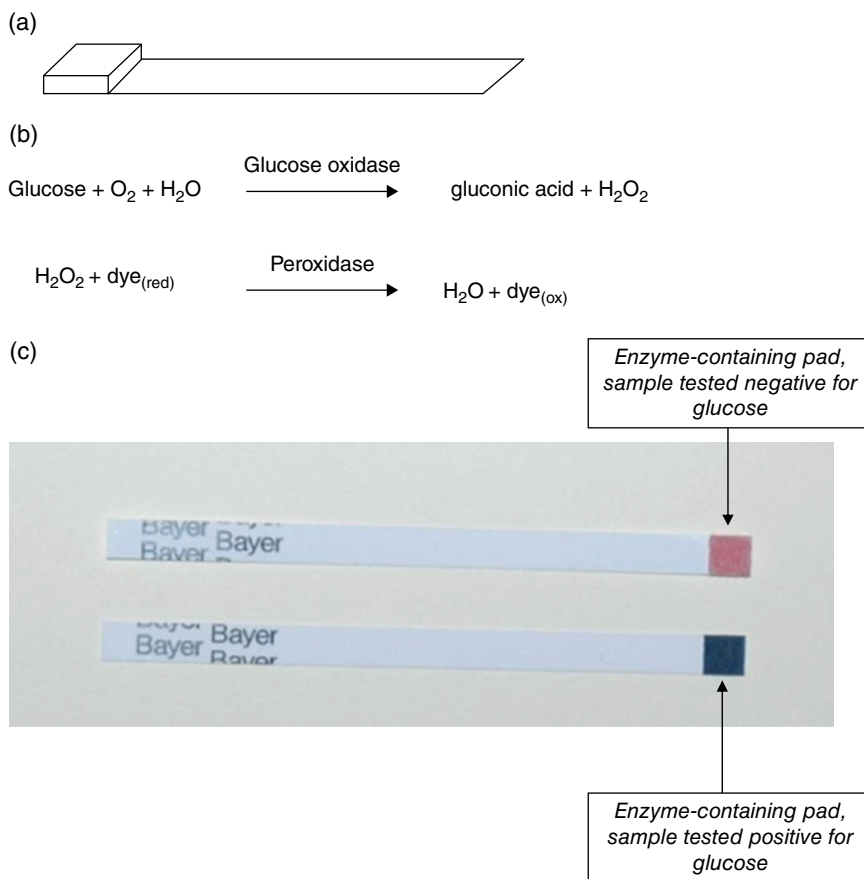
Biosensors are a class of analytical device that can detect and quantify specific analytes in complex samples. They consist of a biological component, which selectively binds the target analyte in a quantitative fashion; a transducer, which quantitatively converts the biological signal into a physical or chemical one; and a detector, which gives a quantitative readout of the transduced signal to the end user (Figure 10.9).

The biological component must obviously exhibit extreme specificity for the target analyte and binding

**Box 10.2 Product case study: glucose test strips**

Test strip technology allows the rapid detection and semi-quantification of glucose in urine. As such it is often used by medical practitioners as a fast, convenient and inexpensive initial diagnostic test in suspected cases of diabetes. The device consists of a thin strip of plastic to which an absorbent pad has been attached at one end, on to which sample is applied (see part (a) in diagram below). Immobilized in the pad are enzyme(s) capable of yielding a coloured product in the

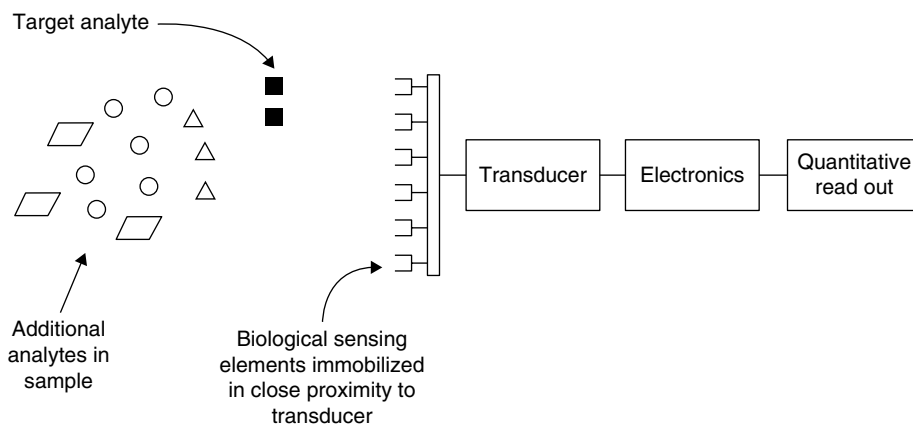
presence of glucose. A combination of glucose oxidase, peroxidase and a chromogen that changes colour/develops colour on its oxidation (e.g. potassium iodide) are most commonly employed (part (b) in diagram). The change in colour visualized is evident (part (c) in diagram). The intensity of the colour produced reflects the concentration of the metabolite (glucose) in the sample applied. Such kits may therefore be employed in a semi-quantitative fashion.



Some manufacturers market test strips housing multiple pads, each capable of detecting a specific analyte. Common multi-analyte test strips can detect not only glucose but also bilirubin, ketone, haemoglobin (i.e. presence of blood), pH, protein and nitrite. Apart from

glucose, however, the detection mechanism for these additional analytes is based on chemical as opposed to enzymatic reagents.

Prominent manufacturers of diagnostic test strips include Bayer (under the trade name Diastix) and Roche (under the trade name Diabier-test).



**Figure 10.9** Generalized schematic diagram of a biosensor. Refer to text for specific details.

of the analyte must produce a detectable response capable of being transduced (converted) into a physical or chemical signal. For the majority of biosensors, the biological component is an enzyme. Most biosensors use electrochemical-based systems as signal transducers, although alternative optical, mass and thermometric systems have been developed. Biosensors display a number of advantages as analyte detection/quantification systems, including the following.

- Reusability, which has favourable cost implications.
- No additional reagents are required to make measurements.
- The assay sample need not be pretreated to remove coloured compounds or particulate matter for example.
- Flexibility: portable biosensors can be used in the field. Biosensors can be used to run individual or multiple samples whenever required (it is uneconomic/impractical to run single assay samples on a multianalyte analyser).
- Ease of use: biosensors are uncomplicated and can usually be used by personnel with little technical training.

Most biosensors have found application in a diagnostics/clinical setting (Table 10.4), although some are used for food analysis (Table 10.5), as well as in environmental monitoring and in bioprocess control.

**Table 10.4** Some analytes of diagnostic importance for which specific quantitative enzyme electrodes have been developed. The enzymes used in these test systems are also listed.

Analyte	Enzyme used in its detection
Glucose	Glucose oxidase
Urea	Urease
Uric acid	Uricase
Amino acids	L-Amino acid oxidase
Cholesterol	Cholesterol oxidase
Alcohols	Alcohol oxidase
Penicillin	Penicillinase

### 10.4.1 Enzyme-based biosensors

A wide variety of immobilized enzymes have been used in the construction of enzyme-based biosensors. These biosensors usually employ electrochemical-based detection systems. The principles adopted in the design and construction of an enzyme biosensor are relatively straightforward (Figure 10.10). The enzyme may for example be immobilized by housing it within a membranous structure which surrounds the chosen electrode. The pore size of the outer membrane that faces the surrounding aqueous environment of the test sample must be sufficiently large to allow free penetration of all the substrates necessary for the immobilized enzyme reaction.

**Table 10.5** Some analytes of importance in the food industry for which biosensors have been developed (although not all have been commercialized).

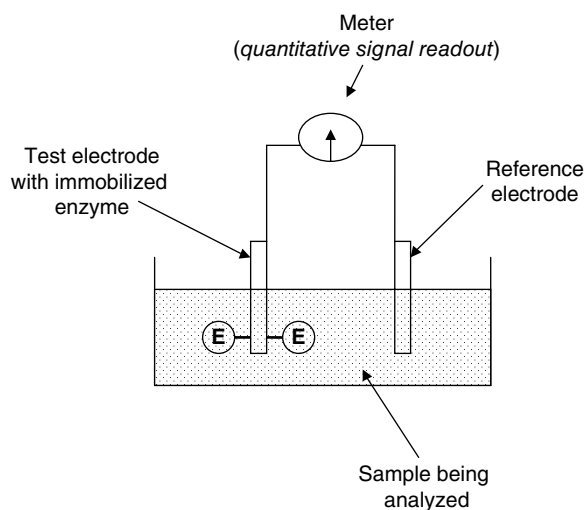
Analyte	Enzyme
Glucose	Glucose oxidase
Fructose	Fructose dehydrogenase
Sucrose	Invertase and glucose oxidase
Lactose	$\beta$ -Galactosidase and glucose oxidase
Lactic acid	Lactate oxidase or lactate dehydrogenase
Ascorbic acid	Ascorbate oxidase
Citric acid	Citrate lyase and oxaloacetate decarboxylase
Ethanol	Alcohol oxidase or alcohol dehydrogenase
Aspartame	APBC and aspartate aminotransferase and glutamate oxidase
Sulfite	Sulfite oxidase

*Note:* In most cases a single enzyme in the biosensor is sufficient. However, in some cases a combination of two or more enzymes are required to generate an easily detectable reaction product. Invertase, for example, hydrolyses the disaccharide sucrose, yielding glucose and fructose. Glucose oxidase then generates a measurable electrochemical signal by oxidizing the glucose produced. In the case of aspartame, aspartate peptide bond-cleaving enzyme (APBC) cleaves the peptide bond of this dipeptide, yielding free aspartate and phenylalanine methyl ester. Aspartate aminotransferase then converts aspartate to glutamate, and glutamate oxidase in turn generates the electrochemical signal.

One of these substrates is obviously the target analyte. In order to protect the immobilized enzyme this outer membrane, which is often made from polycarbonate, must exclude entry of proteins, particulate matter and whole cells. The inner membrane, resting between the immobilized enzyme and the electrode surface, must in no way interfere with the diffusion of reaction substrates/products. These molecules must freely come into contact with the electrode surface. This inner membrane is often made from cellulose acetate.

Additional modalities of enzyme immobilization have also been used (see also Chapter 11, section 11.2). The enzyme can be entrapped within a gel made from polyacrylamide or gelatin for example. Alternatively, the enzyme can be covalently linked to a support material in close proximity to the detecting electrode, or directly to the electrode itself. Direct covalent linkage of enzyme generally yields biosensors with the longest operational lifetimes.

Several electrode types may be used in constructing the enzyme electrode. Electrodes that



**Figure 10.10** Diagrammatic representation of an enzyme electrode.

specifically detect gases such as  $O_2$ ,  $CO_2$  and  $NH_3$ , as well as various ionic species, are all commercially available. The electrode chosen will depend on what products are produced by the enzymatic reaction. If for example oxygen is evolved, then the oxygen electrode may be used. The quantity of oxygen molecules impinging on the electrode surface is then converted into some form of detectable signal, such as a flow of electrical current. This signal can then be monitored. Readings obtained when the electrode is immersed in solutions containing known concentrations of the analyte allows a standard curve to be constructed.

In the construction of an enzyme electrode an enzyme is normally chosen which (i) utilizes as substrate the analyte whose concentration is sought, and (ii) one or other of whose additional substrates or reaction products may be detected and quantified by the actual electrode. By far the most commonly employed enzyme-based biosensors are those designed to measure blood glucose levels (Box 10.3).

First-generation enzyme biosensors as described above suffer from a number of drawbacks, including the generation of high background values, due to the presence of interfering ions in assay samples. The incorporation of electron-transferring mediators in such biosensor systems largely overcomes

**Box 10.3 Product case study: enzyme-based blood glucose biosensors**

The diagnosis and in particular the management of diabetes requires regular quantification of blood glucose levels. Traditionally, this required analysis of blood in a clinical chemistry laboratory. However, the advent of glucose biosensors has transformed this, allowing convenient point-of-care/home blood glucose monitoring to become the norm. Commercialized glucose biosensors are sold under various trade names, including Accu-Chek (Roche), FreeStyle (Abbott), Contour (Bayer) and OneTouch (Life Scan, which is owned by Johnson & Johnson). Typically, testing requires no more than a few microlitres of blood, usually obtained from a fingertip using a lancet, and test results are obtained in as little as 5 seconds.

Blood glucose biosensor technology is based on the immobilization onto an electrode of an appropriate oxidoreductase enzyme (glucose oxidase or more commonly glucose dehydrogenase, for example the Accu-Chek system utilizes glucose dehydrogenase from *Acinetobacter calcoaceticus* but produced via recombinant means in *E. coli*). Catalytic conversion of glucose therefore results in an electron transfer process at the electrode surface, resulting in the generation of an electrical current (amperometric detection) proportional to the concentration of glucose present in the sample. This allows a digital read-out of the glucose concentration in the blood sample. The detecting electrode is usually moulded into a flat plastic-coated disposable (i.e. single-use) test strip which fits into a hand-held digital reader (see photograph below).

While the blood glucose biosensor is the best-known commercialized product, similar biosensor systems for home/point-of-care detection of additional diagnostic analytes including cholesterol and lactate have been developed and commercialized. For example,



Abbotts Precision Xtra blood glucose monitor. Source: © Abbott Laboratories.

Roche's Accutrend cholesterol system is used for total blood cholesterol determination. In this case, the electrode-immobilized enzyme is cholesterol oxidase, which converts cholesterol to 4-cholesten-3-one and hydrogen peroxide. The hydrogen peroxide reacts with an indicator substance (TMB) via a peroxidase enzyme (also present) thereby oxidizing it. The oxidized form of TMB has a blue colour, which is detected and quantified colorimetrically in the hand-held meter.

such difficulties. Such mediators are low-molecular-weight substances such as quinones and ferrocenes, which can be covalently linked to the enzyme. These facilitate electron transfer between the (redox-based) enzyme employed and the electrode. The mediator operates at low redox potential allowing operation of the system at low electrode potential. Under such conditions the electrode detects only the transfer from the mediator, which will be directly proportional to the concentration of the target analyte present in the sample. Indeed research continues into the development of novel

materials that could increase still further the efficiency and speed of electron transfer from the redox centre of the enzyme and the electrode. Much work has focused on the use of graphene and carbon nanotubes, which display excellent electrical conductivity and mechanical strength, making them suitable as a scaffold for enzyme immobilization and for relaying electrons to the actual electrode surface.

For most enzyme electrodes the target analyte is a (co)substrate of the enzyme used. A variation is the inhibited enzyme electrode. In this case the enzyme is chosen on the basis that it is inhibited by the target analyte. The presence of the analyte in a sample therefore results in a reduction of enzyme activity, which is quantitatively measured by an electrochemical (or other) detection system. Various pesticides, for example, can be detected via their inhibition of immobilized acetylcholinesterase.

### 10.4.2 Non-enzyme-based biosensors

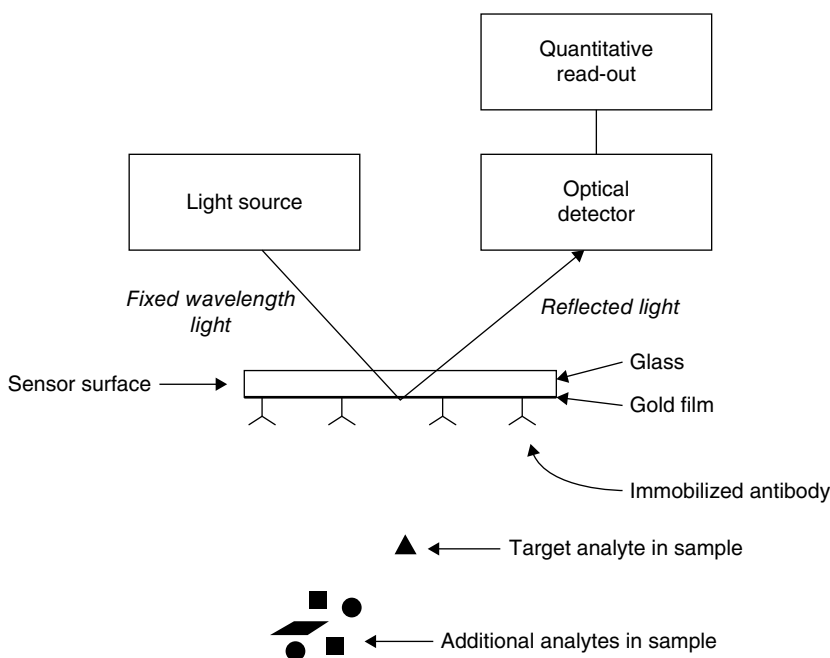
A range of biosensors utilizing a biological component other than an enzyme have also been developed. Alternative biological components include (non-catalytic) proteins, DNA and whole cells. Proteins such as glutathione *S*-transferases have been engineered such that they selectively bind specific heavy metals. The engineered proteins have been immobilized directly onto gold electrodes which act as signal transducers. Binding of a heavy metal to the engineered protein results in a change in protein conformation, which in turn alters system capacitance in a manner proportional to the concentration of the target metal.

Antibody-based biosensors have also been developed. An obvious strength of this approach is that antibodies can be raised against most antigens of interest. Predictably the major technical hurdle has been the development of suitable transduction systems capable of detecting antigen–antibody binding. The tip of an atomic force microscope may be used to monitor biospecific molecular interactions such as antibody–antigen binding. However, cost considerations alone renders commercial exploitation of

this approach unlikely. Optical techniques such as surface plasmon resonance can also directly measure antibody–antigen interactions occurring at a surface–solution interface (Figure 10.11), and some systems based on this approach have been developed. An alternative antibody-based biosensor system is represented by the digital pregnancy test, as discussed in section 10.5.4.

The general strategy pursued in developing DNA-based biosensors entails the immobilization of single-stranded DNA sequences. Hybridization with complementary target-sensitive sequences could be linked to a system giving suitable optical or electrical signals. Such biosensors display obvious potential in clinical, environmental and forensic science. Biosensors using whole cells have also been developed, and in some instances commercialized. The cells used are invariably microbial, which are more robust under *in vitro* conditions than plant or animal cells. The cells are generally entrapped in a gel or membrane held in close association with an electrode. One of the first such systems to be commercialized was a biosensor for determining the biological oxygen demand of environmental samples. Organic pollutants present in the sample will be metabolized by the immobilized microbial (*Trichosporon cutaneum*) cells. The increase in associated cellular respiration results in increased consumption of dissolved oxygen, which can be measured quantitatively by an oxygen electrode.

While the biosensors that have been commercialized to date are predominantly single analyte biosensors, efforts continue to develop array sensors capable of simultaneously detecting and quantifying multiple analytes. For example, hand-held point-of-care sensors capable of detecting up to six analytes have been developed. Another research objective, largely relying on nanotechnology, is to miniaturize appropriate single-use biosensors/biosensor arrays and develop their biocompatibility (e.g. by coating with chitosan) such that they could be implanted into the body, thereby providing continuous monitoring of analytes of interest. Such an approach in the context of glucose monitoring, if coupled to insulin delivery by a linked pump, could bring the concept of an artificial pancreas far closer to reality.



**Figure 10.11** Simplified overview of surface plasmon resonance (SPR)-mediated detection of antibody–antigen binding. Light of a fixed wavelength is directed at (and reflected from) a thin metal film as shown. Binding of the target analyte to the metal surface (via the immobilized antibody) slightly changes the refractive index of the surface layer. This in turn causes a reduction in intensity of the reflected light, which can be measured quantitatively by an appropriate detector.

## 10.5 Antibodies as analytical reagents

Assays that employ antibodies to detect and quantify specific substances are generally termed immunoassays. Antibodies are favoured as analytical reagents because they exhibit extreme specificity in their recognition of a particular ligand – the antigen which stimulated their production. Antibodies thus find widespread analytical use in clinical laboratories (Table 10.6), but are also used in other sectors, including the food industry and for environmental monitoring. While all immunoassays rely on antibody–analyte (antigen) binding, the format of the immunoassay test can vary considerably (Figure 10.12).

Initially, polyclonal antibodies produced by injecting antigen into animals such as horses were used in immunoassays. Monoclonal antibodies produced via classical hybridoma technology as well as

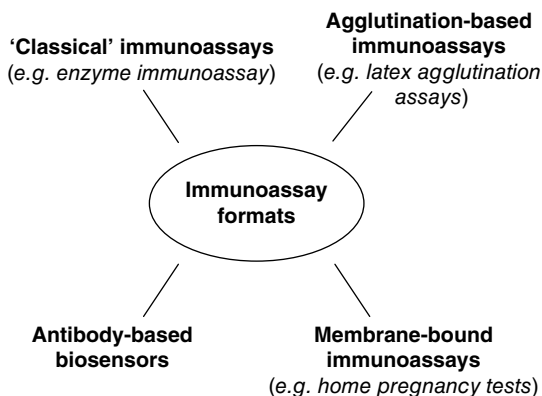
via recombinant DNA technology, including antigen-binding antibody fragments, are more commonly used nowadays. The production of antibodies was considered in Chapter 7.

Antibody molecules themselves have no inherent characteristics that facilitate their direct detection in immunoassay systems. With very few exceptions (e.g. surface plasmon resonance-mediated detection systems; Figure 10.11), a second important step of immunoassay design therefore involves the use of a suitable detection label. The label is usually covalently attached to the antibody, although in some assay formats it can be attached to antigen (Table 10.7 and Figure 10.13). The label serves to facilitate the rapid detection of antibody–antigen binding. Immunoassay systems that use radioactive labels as markers are termed radioimmunoassays (RIA), whereas systems using enzymes are termed enzyme immunoassays (EIA).

Classical immunoassays can be categorized as heterogeneous or homogeneous systems. Heterogeneous

**Table 10.6** Some specific examples of analytes of diagnostic importance for which immunoassays are commercially available.

Analyte	Diagnostic application
Thyroid-stimulating hormone (TSH), thyroxine ( $T_4$ ), triiodothyronine ( $T_3$ )	Thyroid function
Cortisol	Metabolic function
Follicle-stimulating hormone (FSH), luteinizing hormone (LH), estrogen, progesterone	Fertility
Human chorionic gonadotrophin (hCG)	Pregnancy
Troponin, creatine kinase MB	Cardiovascular damage
Apolipoprotein A1 (Apo A1), apolipoprotein B (Apo B)	Cardiovascular disease
Rheumatoid factor (RF)	Rheumatoid arthritis
Erythropoietin, ferritin	Anaemia
Total IgE	Allergy
Insulin, glucose	Diabetes
C-reactive protein (CRP)	Inflammation
Prostate-specific antigen (PSA)	Prostate cancer
Carcinoembryonic antigen (CEA)	Cancer (colorectal, lung, breast, gastrointestinal)

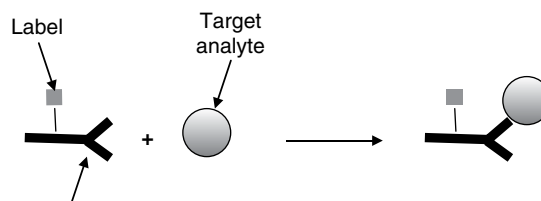


**Figure 10.12** The main immunoassay formats currently in use. Classical immunoassays, and to a limited extent agglutination-based assays, are typically undertaken in clinical chemistry laboratories. Membrane-based systems and biosensors tend to be used mainly for point-of-care testing or home use. Examples of all such systems/formats are considered in this chapter.

assays require the separation of bound and free label after the antibody–analyte incubation step, in order to allow accurate quantification of bound label

**Table 10.7** Labels usually used in immunoassay systems. Refer to text for additional details.

Antibody label	Detection principle
Radioactive tag (e.g. $^{125}\text{I}$ )	Detection of radioactivity emitted from the label
Enzyme (e.g. alkaline phosphatase)	Detection of enzymatic activity, usually through use of a chromogenic substrate
Fluorescent tag (e.g. fluorescein)	Detection of fluorescence (e.g. fluorescein absorbs light at 490 nm and emits light at 520 nm)
Chemiluminescent tag (e.g. luminol)	Detection of chemiluminescence (e.g. oxidized luminol emits light at 445 nm)
Particle-based tag (e.g. coloured latex)	Direct detection of colour



Antibody raised against the target analyte

**Figure 10.13** Generalized diagrammatic representation of label use in immunoassay systems. In the scenario presented, the label is attached to the antibody and therefore a direct correlation will exist between the amount of analyte present in the sample being assayed and the amount of bound label.

(which is proportional to the amount of target analyte present in the sample; Figure 10.13). Assay systems that do not require such separation of bound and free label are termed homogeneous systems.

### 10.5.1 Classical immunoassay systems

Classical immunoassay is an assay undertaken in sample container(s) such as a test tube, a microtitre plate well or an autoanalyzer sample well. The antibody (or, for some formats, the antigen) is labelled. The label is invariably a radioisotope (RIA), an enzyme (EIA), fluorophore (fluorescence



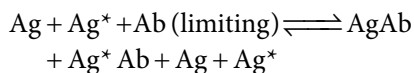
immunoassay) or is chemiluminescent (chemiluminescence immunoassay). The assays may be either heterogeneous or homogeneous in design. If heterogeneous, either the antibody or antigen is immobilized so as to facilitate separation of bound from free label (see examples later). This usually entails immobilization either to the walls of the well in which the immunoassay is undertaken, or to magnetic beads.

### 10.5.1.1 Radioimmunoassay

RIA was among the first immunoassay systems to be developed (in the late 1950s). Such systems have been successfully employed in the quantification of a large variety of biomolecules present in the body at very low concentrations.

Two basic reagents are required when initially developing an RIA system: (i) antibodies which have been raised against the antigen of interest and (ii) highly purified antigen that has been labelled with a radioactive tag such as  $^{125}\text{I}$ . In all assay systems the concentration of antibody added must be limiting, while the concentration of radiolabelled antigen must be in excess.

In a typical RIA, appropriate volumes of antibody, labelled antigen and sample supplied for assay are incubated together. As the antibody concentration is limiting, the antigen (Ag) present in the sample will compete with the labelled antigen ( $\text{Ag}^*$ ) for binding to antibody (Ab):



In all such systems, the quantity of both antibody and labelled antigen used remain constant. Thus, when equilibrium is reached, the greater the quantity of antigen present in the assay sample, the less labelled antigen that will be bound by antibody.

The next step in the assay involves separation of bound from free antigen (i.e. a heterogeneous assay). Separation is normally achieved by using techniques which precipitate the antibody–antigen complex while leaving unbound antigen, both labelled and unlabelled, in solution. Physical separation may

then be achieved by decanting the supernatant from the assay tube, such that only the precipitate remains. In practice, precipitation of antigen–antibody complex from solution is most often achieved by adding to the reaction mixture a second antibody, which has been raised against the first antigen-binding antibody.

On separation of free from bound, the level of radioactivity associated with bound antigen is measured. As previously mentioned, the more unlabelled antigen present in the assay sample, the lower the proportion of labelled antigen bound and hence the lower the radioactive count obtained. A standard curve that relates antigen concentration to levels of radioactivity bound may be constructed by assaying several samples containing known concentrations of unlabelled antigen. The concentration of antigen present in the samples may thus be calculated by reference to the standard curve.

The development of RIAs has revolutionized many areas of clinical and other biological sciences over the past half century or so. However, there are a number of disadvantages associated with the use of radioactive elements in such systems:

- the need for radiological protection;
- the generation of radioactive waste;
- the short shelf-life of some assays due to the short half-life of some radioactive compounds;
- a requirement for expensive analytical equipment, and often dedicated areas in which to perform the assays.

While RIAs find some application, such disadvantages have led to the development of a variety of additional immunoassay systems that use alternative labels, of which enzymes remain among the most prominent.

### 10.5.1.2 Enzyme immunoassay

EIA systems take advantage of (i) the extreme specificity and affinity with which antibodies bind antigens that stimulated their initial production and (ii) the catalytic efficiency of enzymes, which facilitates signal amplification as well as straightforward detection and quantification. In general, many

EIAs exhibit similar sensitivities to RIAs but are free from most of the disadvantages associated with RIAs.

Some EIAs have been developed in which the activity of the enzyme label is significantly altered by binding of antibody to antigen. In such cases, there is no requirement to separate free from bound. Assay systems of this design are therefore homogeneous systems. The enzyme-multiplied immunoassay technique (EMIT) is an example of a homogeneous immunoassay that has found widespread commercial application (Figure 10.14).

EIAs were first introduced in the early 1970s. In most such systems, the antibody was immobilized on a solid surface, such as the internal walls of the wells in a microtitre plate (Figure 10.15). A more recently developed format for heterogeneous immunoassay formats is immobilization on magnetic bead particles, which allows convenient separation of bound from free by the simple application of a magnetic field. As in the case of classical RIAs, the immobilized antibody is incubated with a known amount of labelled antigen, in addition to unknown quantities of antigen present in the samples being assayed. As the level of antibody present is limiting, labelled and unlabelled antigen compete with each other for binding. The greater the quantity of unlabelled antigen present in the assay sample, the less labelled antigen will be retained by the immobilized antibody. After allowing antibody–antigen binding to reach equilibrium, unbound antigen is removed by a washing step. The amount of enzyme-labelled antigen retained is assayed for enzymatic activity.

An alternative variation of this EIA involves immobilizing the antigen and employing an antibody–enzyme conjugate. The principle involved is outlined in Figure 10.16. In this case, the immobilized antigen and the free antigen present in the assay sample compete for binding to a limited amount of enzyme-labelled antibody. The more free antigen present in the assay sample, the less enzyme–antibody conjugate that will bind the immobilized antigen. A washing step removes all unbound material. A subsequent enzyme assay allows accurate estimation of the quantity of bound enzyme.

A standard curve of antigen concentration versus bound enzymatic activity may be constructed by

assaying several samples containing known quantities of antigen. Readings for unknowns may therefore be quantified by reference to this standard curve.

#### *Enzyme-linked immunosorbent assay*

Since their initial introduction over 30 years ago, many variations on the basic EIA concept have been designed. One of the most popular EIA systems in use is that of the enzyme-linked immunosorbent assay (ELISA). The basic principle on which the ELISA system is based is illustrated in Figure 10.17. In this form it is also often referred to as the double-antibody sandwich technique.

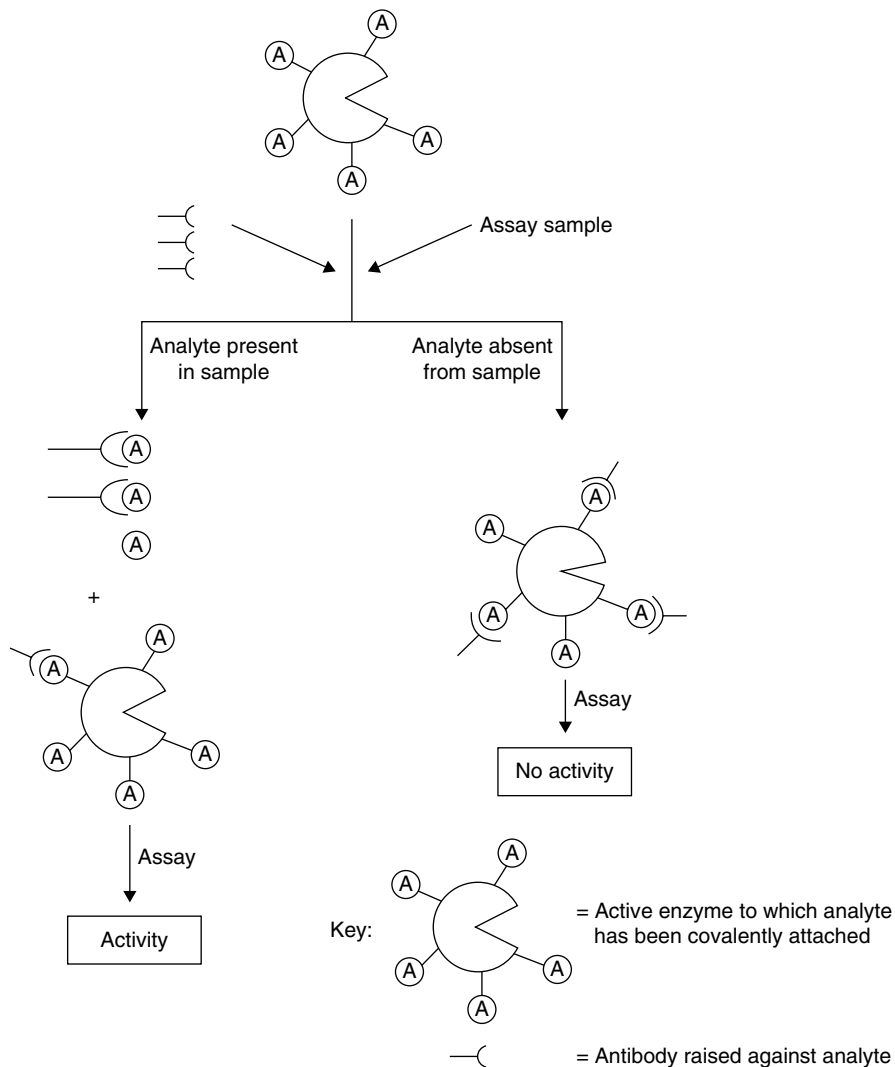
In the basic ELISA system, antibodies raised against the antigen of interest are adsorbed onto a solid surface, again usually the internal walls of microtitre plate wells. The sample to be assayed is then incubated in the wells. Antigen present will bind to the immobilized antibodies. After an appropriate period to allow antibody–antigen binding to reach equilibrium, the wells are washed.

A preparation containing a second antibody, which also recognizes the antigen, is then added. If monoclonal antibodies are used, this second monoclonal antibody must recognize an epitope on the antigen surface that differs from the epitope recognized by the primary or immobilized monoclonal antibody. The second antibody will also bind to the retained antigen and the enzyme label is conjugated to this second antibody.

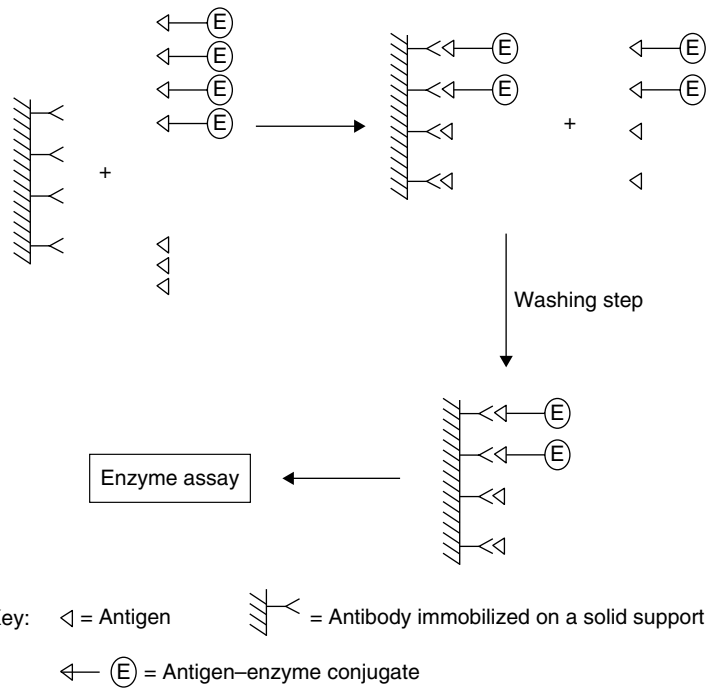
Subsequent to a further washing step to remove any unbound antibody–enzyme conjugate, the activity of the enzyme retained is assayed. The activity recorded is proportional to the quantity of antigen present in the sample assayed. A series of standard antigen concentrations may be assayed to allow construction of a standard curve. The standard curve facilitates calculation of antigen quantities present in unknown samples.

#### *Enzymes used in EIAs*

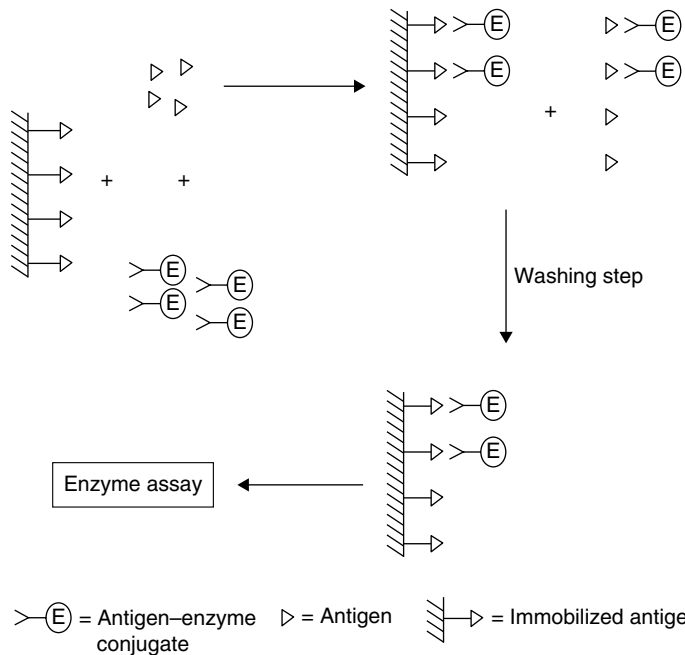
A wide variety of enzymes have been used as labels in various ELISA systems. Suitable enzymes may be chosen to fit a number of criteria. Apart from its catalytic properties, one obvious criterion is that the activity of the enzyme chosen be easily monitored. Many of the enzymes used produce a coloured



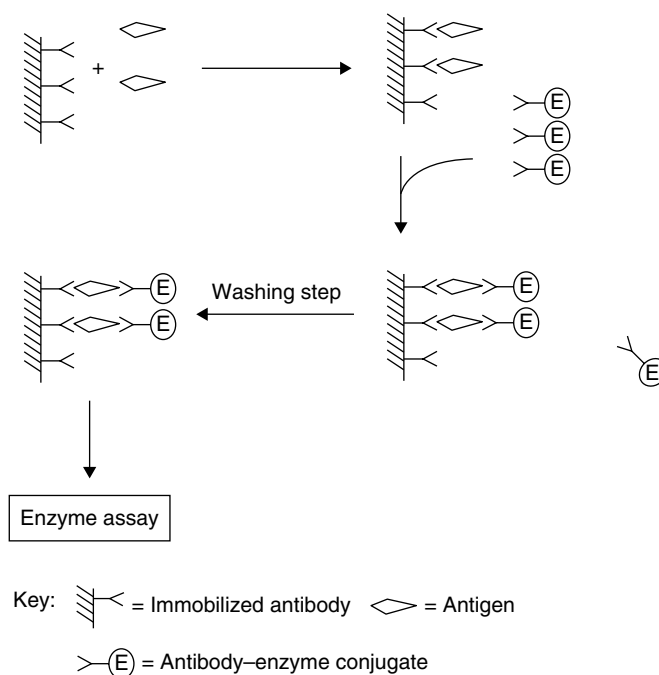
**Figure 10.14** Basic principle of the enzyme-multiplied immunoassay technique (EMIT). Purified preparations of the analyte to be detected are covalently linked to a chosen enzyme. This process must not result in enzyme inactivation. Antibody that specifically binds the target analyte is also required. When running the test the antibody is added in limiting quantities to the enzyme-analyte conjugate, and the sample to be analysed is also added. If the analyte is present in the sample, it will compete with the conjugated analyte for antibody binding, and thus the enzyme will remain maximally or near-maximally active. Activity is quantified by the use of a chromogenic substrate. However, if no analyte is present in the assay sample, the antibody binds exclusively to the enzyme-analyte conjugate, thereby significantly or totally inactivating the enzyme. A standard curve can be constructed by assaying samples of known analyte concentration, and thus analyte concentration will be proportional to activity levels recorded. Note that binding of antibody to the analyte-enzyme conjugate likely decreases enzyme activity by a twofold mechanism: (i) it induces a change in enzyme conformation and (ii) because of its high molecular mass, the antibody may sterically hinder substrate access to the active site. For any given EMIT system the level of inhibition achievable will depend on the exact enzyme and analyte in question, the level of analyte substitution and the mode of attaching the analyte to the enzyme.



**Figure 10.15** Principle of competitive solid-phase enzyme immunoassay. The bound enzymatic activity is inversely proportional to the quantity of unlabelled antigen present in the sample assayed.



**Figure 10.16** Enzyme immunoassay system using immobilized antigen. The higher the level of antigen present in the sample for assay, the lower the level of enzyme-antibody conjugate retained on the immobilized phase, and hence the lower the level of enzymatic activity recorded.



**Figure 10.17** Principle of non-competitive ELISA.

product that may be easily monitored by colorimetric methods. Enzymes most often used as labels include alkaline phosphatase and horseradish peroxidase, in addition to  $\beta$ -galactosidase, glucose oxidase and urease. All such enzymes can utilize a suitable chromogenic substrate.

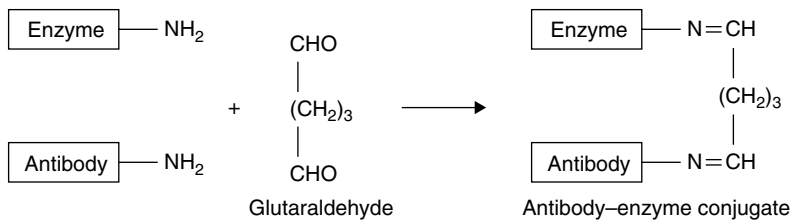
Alkaline phosphatase isolated from calf intestine was one of the first enzymes to be used in ELISA systems. The substrate normally utilized is *para*-nitrophenylphosphate (PNPP). PNPP is enzymatically hydrolysed by alkaline phosphatase, releasing inorganic phosphate and *para*-nitrophenol (PNP), which is yellow and absorbs light maximally at 405 nm. The substrate used with  $\beta$ -galactosidase is normally *O*-nitrophenyl- $\beta$ -D-galactopyranoside, with colour development being measured at 420 nm.

The covalent coupling or conjugation of the chosen enzyme to the second antibody used in ELISA systems may be achieved by a number of chemical methods. Perhaps the simplest of such methods involves chemical conjugation with glutaraldehyde (Figure 10.18), which is a homobifunctional reagent (its two reactive groups that link two proteins together are identical). Glutaraldehyde reacts irreversibly with the  $\epsilon$ -amino group of lysine,

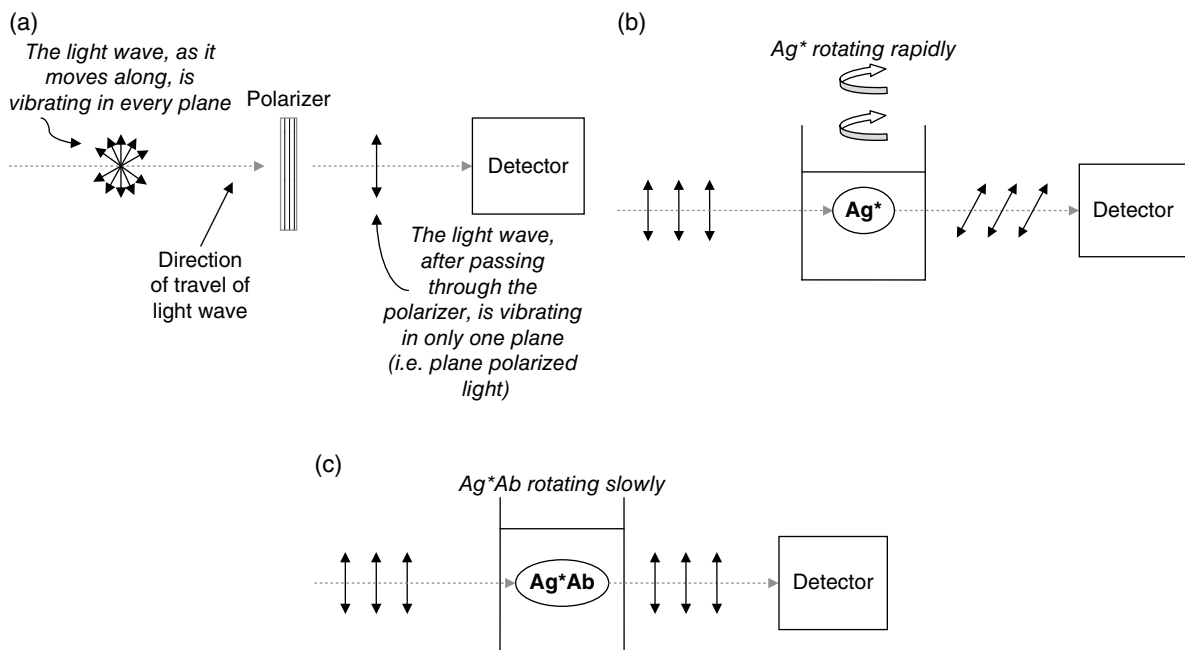
forming covalent linkages. The glutaraldehyde method is popular due to its simplicity and inexpensiveness. All conjugation methods, however, are likely to result in the inactivation of a proportion of coupled enzyme molecules and indeed antibodies.

### 10.5.2 Fluorescent and chemiluminescent-based immunoassay labels

As mentioned earlier in the chapter, fluorescent labels are those capable of absorbing light of a specific wavelength and then emitting light of a slightly longer (i.e. lower energy) wavelength. Fluorescein is a commonly used fluorophore that absorbs light at 490 nm and emits light at 520 nm, and can be conjugated to an antigen or an antibody. A commonly employed format of fluorescence-based assay is termed fluorescence polarization immunoassay (FPIA). FPIA is a homogeneous competitive assay often used for the detection and quantification of low-molecular-mass toxicology analytes, such as drugs of abuse, therapeutic drugs and some hormones.



**Figure 10.18** Chemical coupling of enzyme to antibody species using glutaraldehyde. Amino groups shown on the free enzyme and free antibody are  $\epsilon$ -amino groups of lysine residues. The reactants are incubated for a period of 1 hour or more. Excess free lysine may be added for efficient termination of the coupling procedure. Unreacted glutaraldehyde may be removed by dialysis for example. The procedure will inevitably yield a proportion of antibody-antibody and enzyme-enzyme conjugates in addition to the enzyme-antibody conjugate. Separation of these if required can subsequently be undertaken by gel filtration chromatography for example.



**Figure 10.19** Light waves, as they travel along, vibrate in every plane. If these light waves are passed through a polarizer, only light waves vibrating in a single plane will emerge (a). All molecules rotate in solution, with larger molecules rotating more slowly than smaller molecules. Consider therefore the scenario outlined in (b): plane polarized light passes through a sample containing a fluorescently labelled low-molecular-mass antigen molecule ( $\text{Ag}^*$ , a few hundred daltons), which is rotating relatively rapidly in solution. The fluorescent label absorbs the incident light but, before it releases the absorbed light as fluorescence, the label has rotated a little. As a result the plane of polarized light emitted as fluorescence is altered relative to the incident polarized light. Compare this to the scenario presented in (c): the incident plane polarized light is absorbed by the fluorescent tag attached to the antigen molecule. However, bound to this in turn is IgG (antibody), a very large molecule of molecular mass 150 kDa. In effect the fluorescent tag is therefore part of a very large structure that is thus rotating much more slowly in solution. As a result the plane of polarized light emitted as fluorescence is unaltered relative to the plane of the incident polarized light because the structure has not rotated in solution in the time between absorbing and emitting the light. Therefore FPIA distinguishes between free  $\text{Ag}^*$  and antibody-bound  $\text{Ag}^*$  by their different fluorescence polarization properties when exposed to polarized light.

Assay procedure entails co-incubation of sample (containing the analyte to be detected, i.e. the antigen, Ag), purified fluorescent-labelled antigen ( $\text{Ag}^*$ ) and antibody (Ab). As the antibody concentration is limiting, the antigen present in the sample supplied will compete with the labelled antigen for binding to antibody (see equilibrium equation in section 10.5.1.1). In all such systems, the quantity of both antibody and labelled antigen used remain constant. Thus, when equilibrium is reached, the greater the quantity of antigen present in the assay sample, the less labelled antigen that will be bound by antibody.

Both free and bound label fluoresces, so one would imagine that the next step in such an assay would be separation of bound from free (i.e. a heterogeneous assay in format). However, the fact that all molecules rotate in solution, with larger molecules rotating more slowly than smaller ones, along with the use of polarized light (wavelength 490 nm), allows assay development in homogeneous format as outlined in Figure 10.19.

Chemiluminescent substances are ones which, on participation in a chemical reaction, emit light as a reaction product. Luminol exemplifies one such molecule. When it is oxidized (e.g. by  $\text{H}_2\text{O}_2$ ) it emits light (maximum wavelength 445 nm). Additional chemiluminescent molecules include isoluminol and acridinium esters. Chemiluminescent labels are popular immunoassay labels as the light emitted can easily be quantified by photometric means. Moreover, chemiluminescent-based immunoassays tend to be extremely sensitive, allowing the detection and accurate quantification of target protein analytes down to the  $10^{-12}$  level. Chemiluminescent immunoassays can be designed in various formats, in which the chemiluminescent label can be conjugated to the antibody, to purified antigen (i.e. the target analyte) or can be unbound. Some such potential formats are outlined in Figure 10.20.

### 10.5.3 Latex agglutination-based immunoassay formats

Latex agglutination-based assays represent an alternative format to the immunoassays described

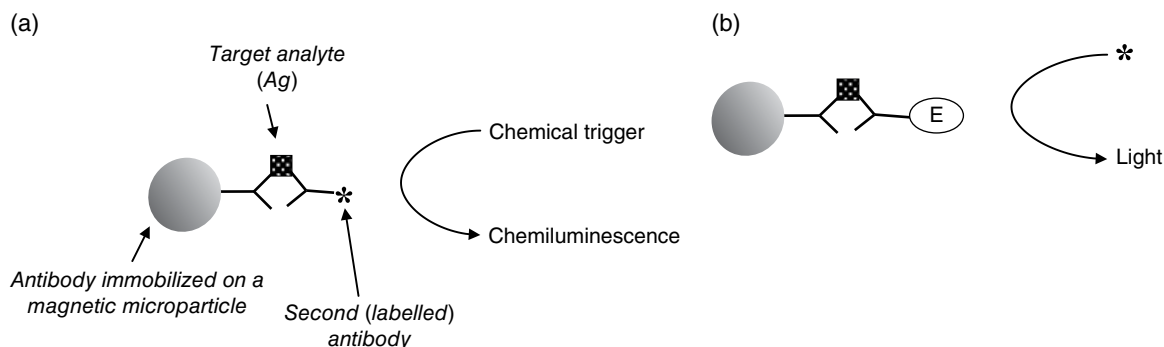
thus far. Historically, they found application particularly in the detection of pregnancy (Box 10.4) as well as for detecting the presence of various pathogens in biological samples. While popular for several decades, their commercial application in more recent times has decreased significantly, for various reasons:

- additional assay formats often offer greater assay sensitivity;
- additional formats are either more amenable to automation (e.g. classical immunoassay) or are more convenient to use (e.g. membrane-based tests, see section 10.5.4).

Most latexes used are manufactured from polystyrene as spherical particles. Particle diameters generally range between 0.1 and 1.1  $\mu\text{m}$ . Individual latex particles are therefore not visible to the unaided human eye. Incubation of latex with a protein solution leads to adsorption of the protein molecules onto the surface of the latex particle. Alternatively, various reactive groups may be initially introduced into the latex. This permits the covalent linkage of proteins to such particles.

Latex particles coated with antibody can be utilized to detect the presence of a specific antigen in a biological sample. Conversely, latex particles coated with a specific protein antigen may be used to detect the presence of antibodies that recognize that specific antigen. Latex-based diagnostic systems rely on the inherent specificity of an antibody-antigen reaction, and the bifunctional nature of antibody binding, to promote agglutination of the latex particles.

As illustrated in Figure 10.21, when latex particles coated with antibody that has been raised against a specific antigen are incubated with a biological sample containing that antigen, the ensuing antigen-antibody reaction results in the formation of large aggregates of latex particles, i.e. the latex agglutinates. The antigen effectively acts as a bridge between adjacent latex particles. This process of agglutination is visibly evident as the newly aggregated particles are clearly seen by the naked eye.



**Figure 10.20** Two example formats in which chemiluminescent-based immunoassays may be designed. (a) An antibody raised against the target analyte (antigen) is immobilized onto magnetic microparticles. These are then incubated with the test sample containing the analyte of interest. Target analyte will bind as indicated. Subsequent to this a second analyte-binding antibody to which a chemiluminescent label is attached is added. A washing step is undertaken to remove any unbound antibody-label conjugate. Application of a magnetic field keeps the magnetic particles in the assay tube during this step. Finally, a chemical capable of reacting with the chemiluminescent label (thereby generating light) is added. The amount of light generated (chemiluminescence) is directly proportional to the amount of analyte present in the sample being tested. The format shown in (b) is substantially similar to that in (a), except in this case conjugated to the second antibody is an enzyme as opposed to a chemiluminescent label. In this format the final immunoassay step entails the addition of a chemiluminescent molecule that the enzyme is capable of transforming into the chemiluminescent form that actually emits light. Very often a co-reactant is also required. For example, a popular chemiluminescent-based immunoassay format entails the use of horseradish peroxidase as the conjugated enzyme. The final step undertaken is the addition of a cocktail of luminol and  $\text{H}_2\text{O}_2$ . The horseradish peroxidase catalyses the reduction of  $\text{H}_2\text{O}_2$  (to  $\text{H}_2\text{O}$ ), with the simultaneous oxidation of luminol. The oxidized form of luminol emits light.

#### Box 10.4 The basis of pregnancy diagnosis

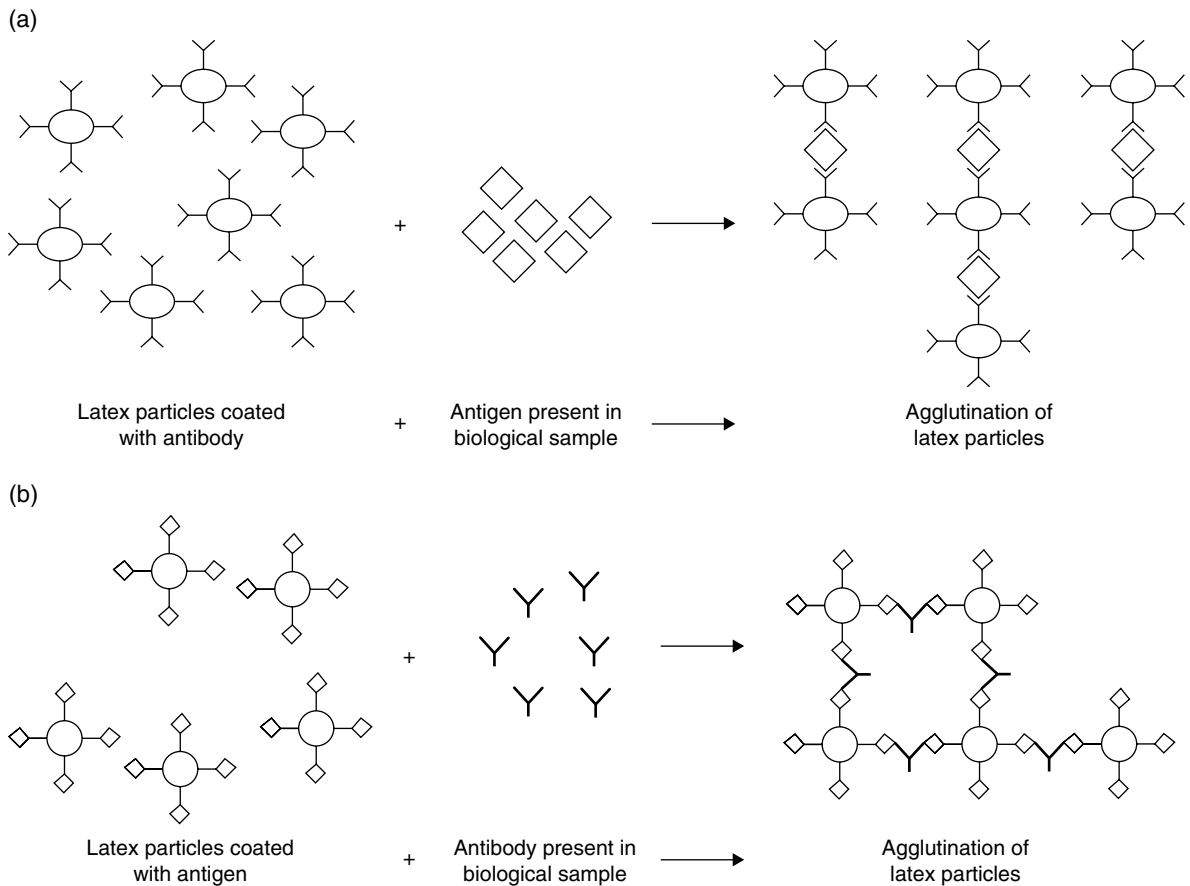
Shortly after implantation of a fertilized egg, the human placenta begins to synthesize and secrete human chorionic gonadotrophin (hCG; see Chapter 8). hCG is not normally synthesized in healthy non-pregnant females (although hCG may be produced by several cancer types). However, this hormone is found in both the serum and urine of pregnant women. hCG therefore represents a good diagnostic marker for pregnancy. The fact that it may be detected in the urine of pregnant females makes it all the more desirable as urine samples may be conveniently collected.

Many of the initial hCG-based pregnancy detection systems were based on bioassays. This involved injection of urine samples to immature female rabbits or rats. Growth and ripening of the ovarian follicles in such animals indicated the presence of hCG and

hence pregnancy. Modern pregnancy diagnostic systems are virtually all based on immunological detection of hCG in urine (or serum) samples. Classical and, to a limited extent in some world regions, latex-based systems are typically employed in clinical chemistry laboratories, while membrane-bound systems (see main text) are used for point-of-care testing and home use.

In practice, such assay systems are initiated by mixing a sample of antibody- or antigen-coated latex with the biological sample to be analysed. If the sample contains the appropriate antigen or antibody, the latex will agglutinate. The agglutination process is perceived visually as the transformation of a homogeneous milk-like latex mixture into a more granular form. The agglutinated latex particles are similar in appearance to discrete grains of sea sand.

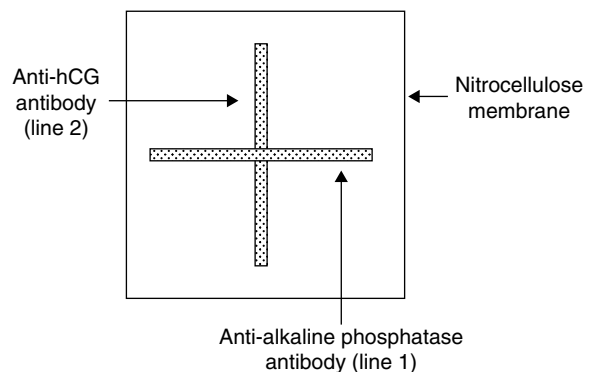




**Figure 10.21** Latex agglutination assay system. (a) Latex particles have been coated with antibody. The presence of appropriate antigen in the test sample results in agglutination of the latex particles. The antigen present effectively acts as a bridge between adjacent latex particles. (b) The latex particles have been coated with antigen. In this case the presence of appropriate antibody in the assay sample results in agglutination of the latex, i.e. the antibody acts as a bridge between adjacent latex particles. Agglutination can easily be detected visually.

### 10.5.4 Membrane-bound diagnostic systems

Antibodies immobilized on membranes such as nitrocellulose may also be used as diagnostic reagents. Thoughtful design of such systems, in particular those designed to detect pregnancy, has contributed greatly to their ease of use. For example, in one such early system, two thin lines of antibody are sprayed in the shape of a cross on the surface of the nitrocellulose membrane (Figure 10.22). This process is achieved by specialized industrial spraying equipment. The antibody species applied along one line specifically binds



**Figure 10.22** A membrane-bound pregnancy (hCG) detection system.

human chorionic gonadotrophin (hCG). The antibody applied along the second line binds alkaline phosphatase. At this stage, of course, both such antibody lines are invisible to the human eye.

A second component employed in such systems is a freeze-dried preparation of anti-hCG antibody conjugated to alkaline phosphatase (AP). The urine sample obtained for testing is used to reconstitute this antibody–enzyme conjugate. The reconstituted conjugate is then allowed to come into contact with the membrane surface.

A urine sample from a non-pregnant female will contain no hCG. Therefore, the hCG-binding sites of the antibody–enzyme conjugate reconstituted by the urine sample remains unoccupied. When this sample comes into contact with the membrane surface, the conjugate is bound via the AP to the anti-AP antibody line (line 1 in Figure 10.22). Nothing binds to the anti-hCG antibody line (line 2, Figure 10.22) as no hCG is present in the urine.

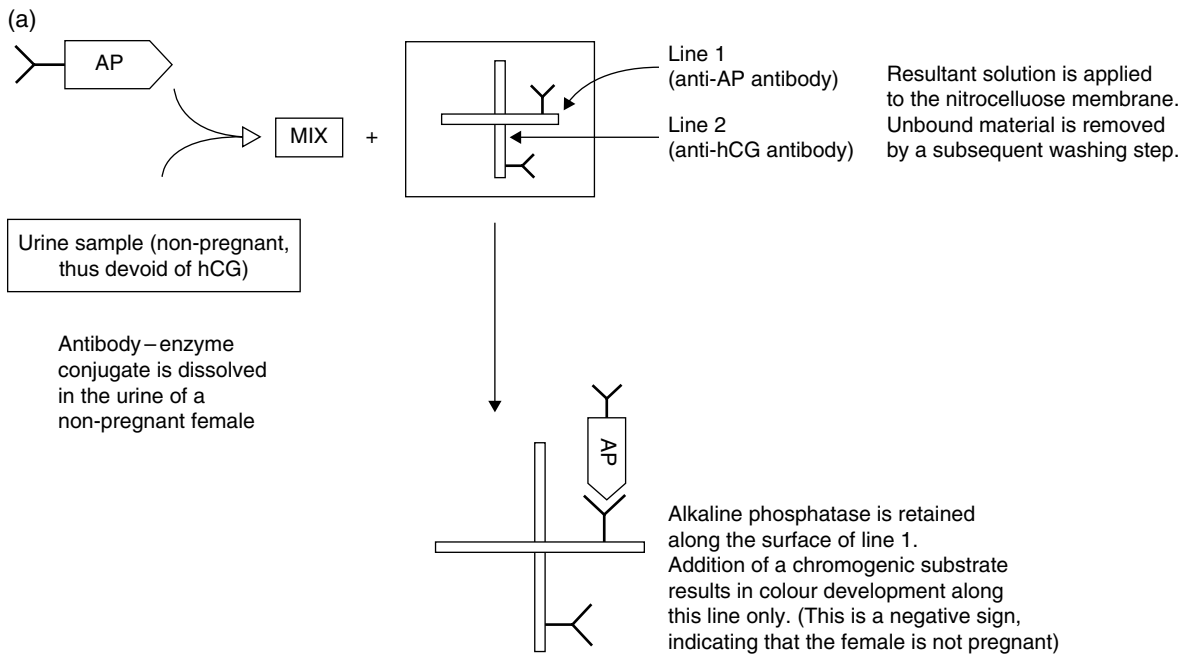
After rinsing the membrane in order to remove unbound material, it is briefly immersed in a solution containing an AP chromogenic substrate such as PNPP. Colour development is therefore witnessed all along the anti-AP antibody line due to the presence of bound AP (Figure 10.23a). No colour development occurs along line 2 as no hCG is present. Thus a minus sign (–) is produced indicating that the female is not pregnant.

On the other hand, the urine of a pregnant female does contain hCG and this hCG is bound by the antibody–enzyme conjugate on its reconstitution with the urine sample. In this case, the conjugate is bound by both antibody lines on coming in contact with the membrane surface. The conjugate binds line 1 via the AP moiety. Binding to line 2 (e.g. anti-hCG antibody line) occurs because the hCG acts as a bridge (Figure 10.23b). In this case, immersion of the membrane in a developing solution results in colour development along both lines, so that a plus (+) sign is obtained indicating pregnancy.

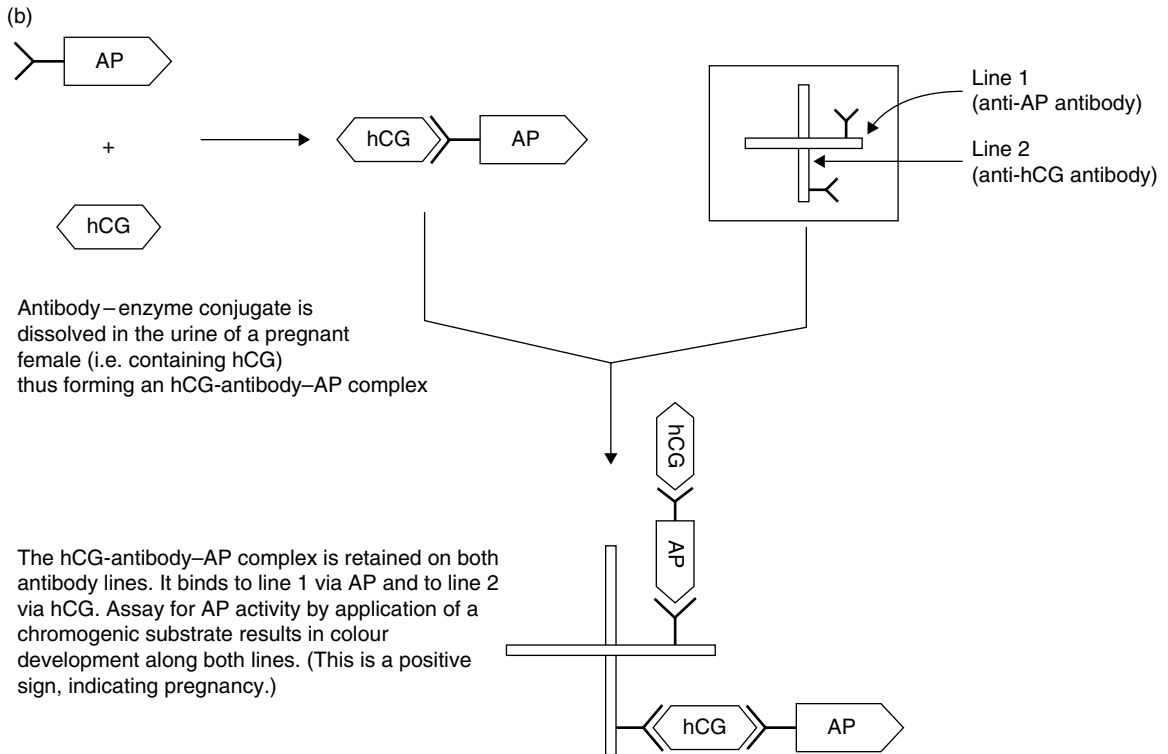
Most modern membrane-bound pregnancy detection systems are also membrane-based, but are design variants of the above approach that require no operator input post urine sample application (Figure 10.24). These so-called lateral flow tests (lateral flow immunochromatographic assays) require

the user to initially hold the sample pad of the test device in a flow of urine for a few seconds (alternatively it can be dipped in a recently collected urine sample). Inside in the test device's moulded outer plastic casing is a test strip (membrane) attached to an absorbent sample pad at one end and an absorbent soak pad at the other end (Figure 10.24). The sample pad soaks up some of the urine to be tested. The urine sample travels along the length of the porous membrane via capillary action, collecting the detector reagent on its way (Figure 10.24b), eventually being soaked up at the other end by the soak pad. The urine flow dissolves the detector antibody conjugate, carrying it along the full length of the test membrane over a period of 2–3 minutes. The test membrane itself contains two areas (bands) in which antibodies have been immobilized. In the first or test band, an anti-hCG antibody has been immobilized (this recognizes a different epitope of the hCG than does the detector antibody). In the second (control) band area, an antibody raised against the actual detector antibody has been immobilized. If the urine contains hCG (i.e. is from a pregnant female), the coloured label conjugate will be retained at the site of both the test band and the control band. This will be visualized as two coloured lines developing on the test membrane (Figure 10.24a,b,d). On the other hand, if no hCG is present in the urine sample (i.e. the female is not pregnant), the coloured label conjugate will be retained only at the control band. This will be visualized as a single coloured line developing on the test membrane (Figure 10.24e). The use of a control band is also important in that it validates that the assay has been successfully undertaken.

Various labels have been conjugated to detector antibody in such lateral flow systems. However, the most commonly employed ones are coloured (blue) latex particles or gold particles with diameters in the 40–nm range, which generally yield strong (and hence easily observed) blue or red lines, respectively (gold particles in the nanometre diameter range are red in colour due to localized surface plasmon resonance). Recent advances in this area have led to the development of digital-based quantitative lateral flow test devices (biosensors) that can indicate not only pregnancy but also the approximate duration of pregnancy (Box 10.5).

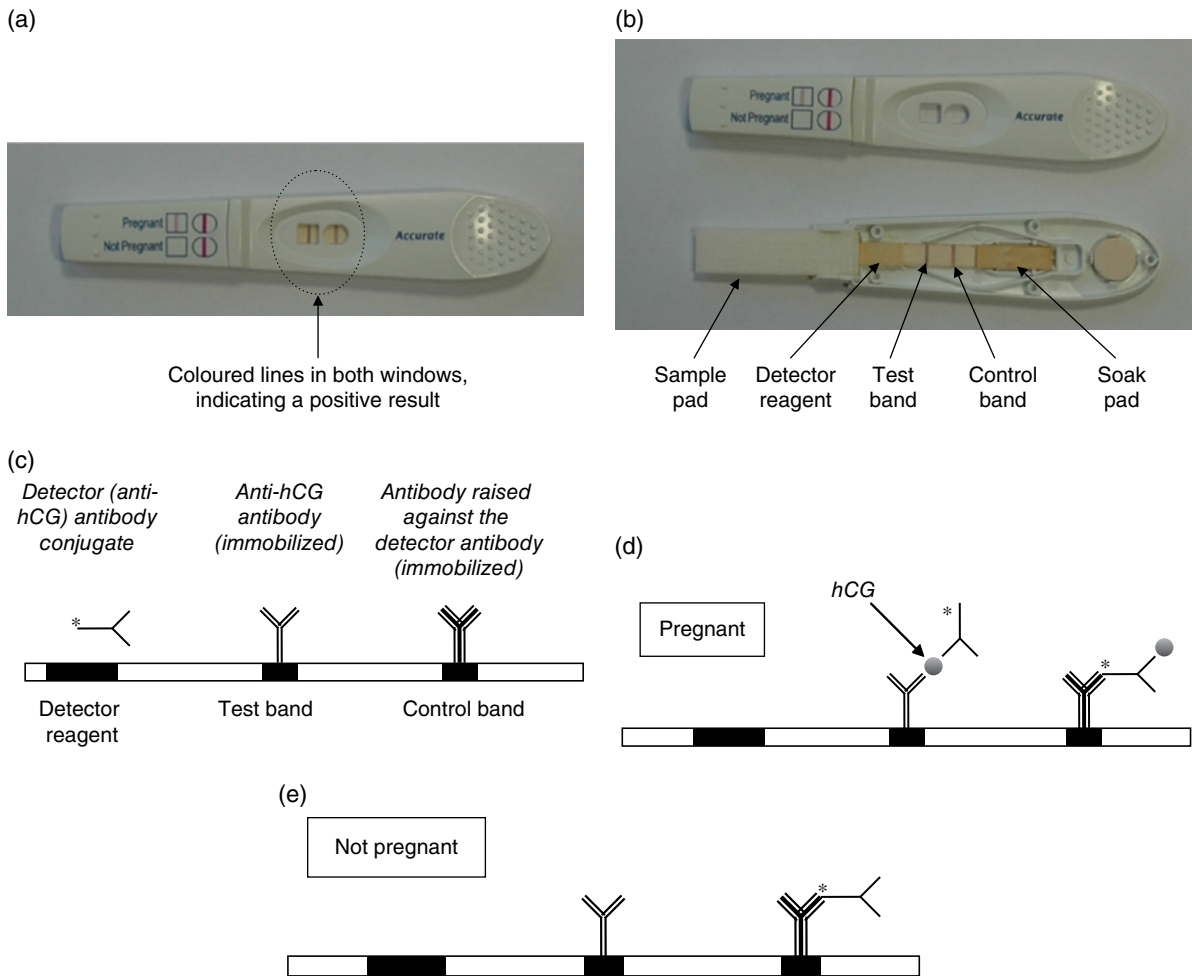


Key: = Alkaline phosphatase–anti-hCG antibody conjugate



Key: = Alkaline phosphatase–anti-hCG antibody conjugate  
 = hCG

**Figure 10.23** Principle of membrane-bound hCG (pregnancy) detection kit: (a) events occurring if the female is not pregnant; (b) events occurring if the female is pregnant.



**Figure 10.24** (a) Photo of a typical lateral flow pregnancy detection device. (b) Device with its internal test strip revealed. (c) Diagrammatic representation of the test strip itself. (d) Diagrammatic representation of the test strip on completion of a test on a pregnant female. (e) Diagrammatic representation of the test strip on completion of a test on a non-pregnant female. Refer to text for details.

### Box 10.5 Product case study: Clearblue digital pregnancy test with conception indicator

Membrane-bound pregnancy detection devices are qualitative, essentially giving a 'yes' or 'no' answer. hCG levels can be detected in the blood as little as 24 hours after implantation of the fertilized egg, and is present in the urine within another few days (usually 4–5 days before the onset of the next menstrual cycle is expected). Moreover, the concentration of hCG in the serum/urine doubles

approximately every 2 days over the first several weeks of pregnancy. Thus the quantitative amount of hCG in the urine can also serve as a quantitative indication of approximately how long the test female has been pregnant. Miniaturized optical readers have been incorporated into lateral flow test devices, such as the Clearblue digital pregnancy test device. The optical reader can quantify

the intensity of the test line produced. This quantitatively reflects the amount of hCG present in the sample, providing an indication of how long the female has been pregnant. The optical reader generates a digital read-out indication if the female is pregnant and, if so, for approximately how many weeks.



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Lateral flow systems have been developed for a range of additional diagnostic applications, including for monitoring fertility, detection of viruses (hepatitis

A and B), detection of various pathogens and toxins, detection of specific therapeutic drugs and illicit drugs, as well as for various disease biomarkers.

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# Chapter 11

## Industrial enzymes: an introduction

The previous five chapters have focused on proteins used for therapeutic and diagnostic/analytical purposes. Most such proteins are produced in relatively small quantities (tens to hundreds of kilograms annually) and are highly purified. As with many healthcare products they are often expensive, and economic considerations are often secondary to functional excellence. This chapter (along with the subsequent two) focuses on a different group of proteins, that of 'industrial' or 'bulk' enzymes. This group includes amylases, lignocellulose-degrading enzymes, pectinases, proteases, lipases, phytases, penicillin acylases and cyclodextrin glycosyltransferases. The majority of these enzymes are hydrolytic depolymerases. In contrast to enzymes used for therapeutic or diagnostic purposes, industrial enzymes are produced in large quantities, in the order of thousands to hundreds of thousands of kilograms annually, and are purified to at most a limited degree (see Chapter 5). Furthermore, in most instances economic considerations such as production costs are of critical importance to their commercial success (Table 11.1).

**Table 11.1** Selected attributes of industrial enzymes compared with those of proteins used for healthcare purposes.

Industrial enzymes	Medical/diagnostic proteins
Produced in larger quantities	Produced in smaller quantities
Partially purified at best	Extensively purified
Economic considerations critical	Economic considerations are of secondary importance to functional excellence of product
Function: catalytic	Function: various (hormones, growth factors, cytokines, other regulatory factors, blood factors, vaccines, antibodies, enzymes)
Source: mainly microbial and recombinant	Source: mainly human/animal and recombinant products
Mainly secreted into the extracellular medium by producer strains	May be intracellular or extracellular

**Table 11.2** Some industrially important enzymes, their traditional sources and sample industrial applications.

Enzyme	Traditional source	Sample applications
$\alpha$ -Amylase	<i>Bacillus amyloliquefaciens</i> <i>Bacillus licheniformis</i> <i>Bacillus subtilis</i> <i>Aspergillus oryzae</i>	Hydrolyses $\alpha 1 \rightarrow 4$ linkages in starch. Used to liquefy starch and reduce its viscosity
$\beta$ -Amylase	<i>Bacillus polymyxa</i> <i>Bacillus circulans</i> Barley	Enzymatic degradation of starch yielding the disaccharide maltose
Glucoamylase (amyloglucosidase)	<i>Aspergillus niger</i> <i>Rhizopus</i> spp.	Hydrolysis of starch, yielding dextrose
Pullulanase	<i>Bacillus</i> spp. <i>Aerobacter aerogenes</i> <i>Klebsiella</i> spp.	Debranching of starch by hydrolysis of $\alpha 1 \rightarrow 6$ glycosidic linkages
Glucose isomerase	<i>Bacillus coagulans</i> <i>Bacillus stearothermophilus</i> <i>Streptomyces</i> spp. <i>Arthrobacter</i>	Production of high-fructose syrup by conversion of glucose to fructose
$\beta$ -Galactosidase (lactase)	<i>Bacillus coagulans</i> <i>Streptomyces</i> spp. <i>Saccharomyces</i> spp. <i>Aspergillus</i> spp.	Hydrolysis of milk lactose yielding glucose and galactose
Invertase (sucrase)	<i>Saccharomyces</i> spp.	Hydrolysis of sucrose, yielding glucose and fructose
Cellulase and hemicellulase	<i>Trichoderma</i> spp. <i>Sporotrichum cellulophilum</i> <i>Actinomyces</i> spp. <i>Aeromonas</i> spp. <i>Aspergillus niger</i>	Enzymatic hydrolysis of cellulose-containing material
Pectinases	<i>Aspergillus niger</i> <i>Fusarium</i> spp.	Enzymatic hydrolysis of pectin
Proteases	Various <i>Bacillus</i> spp. <i>Bacillus amyloliquefaciens</i> <i>Bacillus subtilis</i> <i>Streptomyces</i> spp. <i>Aspergillus oryzae</i> Some animal sources such as calf stomach	Enzymatic hydrolysis of proteins widely used in detergents and in brewing, baking and meat tenderization
Lipases	<i>Mucor</i> spp. <i>Myriococcum</i> spp. Animal pancreas	Enzymatic hydrolysis of lipids. Used in dairy industry for flavour development in foods and also used in detergents

Bulk enzymes are used in a great many biotechnological processes. Many technologies such as brewing, wine-making and cheese manufacture may be traced to the very dawn of history. In such instances individuals unknowingly employed microorganisms as a source of the enzymes required to transform initial substrates into products such as ethanol or cheese. A greater

understanding of the molecular basis by which these conversions occur facilitated the subsequent utilization of isolated enzymes for specific industrial purposes.

Enzymatic preparations currently find application in the brewing, breadmaking, starch processing and cheesemaking industries, among others (Table 11.2). The availability of such enzymes



has also facilitated the development of numerous additional biotechnological processes that produce a wide range of industrially important commodities. Enzymes are thus also used in the production of sweeteners and in modification of the flavour, texture and appearance of many food-stuffs. They are employed to tenderize meat, clarify beer, wine and fruit juices, and are included in many detergent preparations.

## 11.1 Sales value and manufacturers

By 2013 the annual worldwide sales value of industrial enzymes stood in the region of US\$3.7 billion, approximately double the figure recorded a decade earlier. Proteases accounted for almost 50% of the market share, while carbohydrate-degrading enzymes accounted for much of the remainder.

Some major developers and manufacturers of industrial enzymes are listed in Table 11.3.

**Table 11.3** Some enzyme-producing companies, as well as technical associations representing enzyme producers.

### Enzyme-producing companies

AB Enzymes (<http://www.abenzymes.com>)

Amano Enzymes (<http://www.amano-enzyme.co.jp>)

DSM (Gist-Brocades) (<http://www.dsm.com>)

DuPont (Danisco/Genencor) (<http://biosciences.dupont.com>)

Novozymes (<http://www.novozymes.com>)

Shin Nihon (<http://www.takabio.com>)

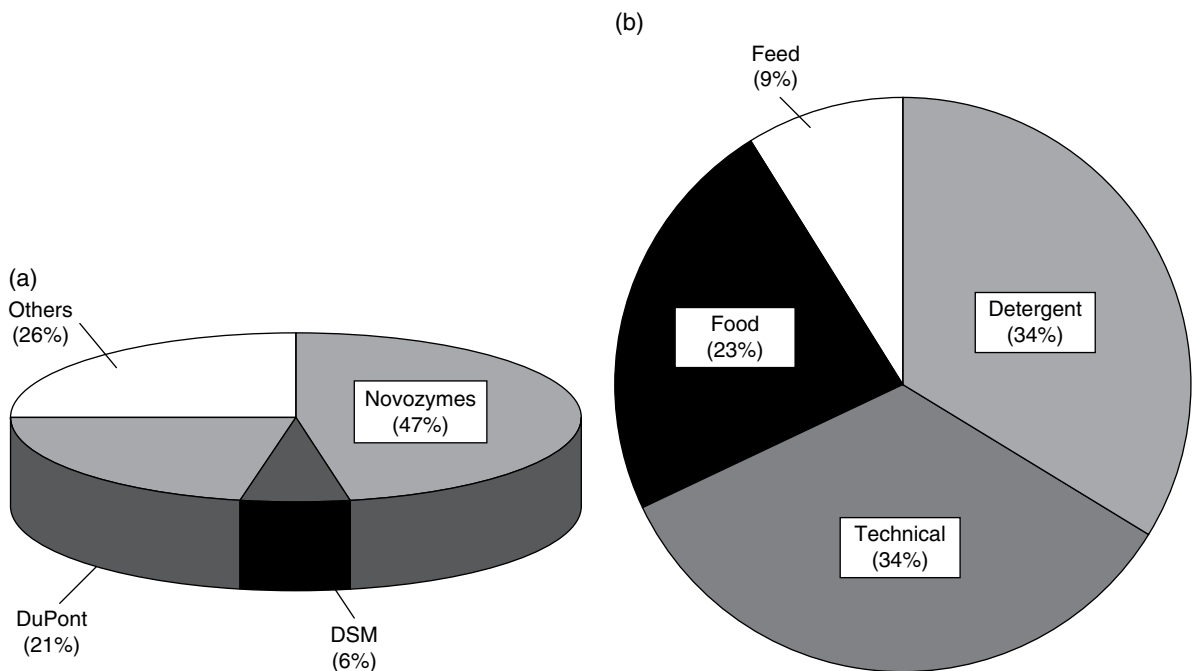
Verenium (<http://www.verenium.com>)

### Technical representative associations

AMFEP (<http://www.amfep.org>)

ETA (<http://www.enzymetechnicalassoc.org>)

Novozymes remains the world's largest supplier of such enzyme products, accounting for almost 50% of the global enzyme market. The company markets several hundred enzyme preparations, used in the detergent, animal feed, alcohol, wine, brewing, baking, juice, leather, pulp, paper, food, textile and biofuel industries (Figure 11.1). DuPont and DSM represent the other two major global enzyme players. Both of these companies



**Figure 11.1** (a) Market share of major global enzyme producers. (b) Sales of Novozymes enzymes by major application. Note that technical enzymes include those used to degrade starch and cellulose to glucose and those used in non-food processing industries such as the textiles industry.

**Table 11.4** Some industrial enzymes produced by recombinant DNA technology. Several of these are considered in more details in subsequent chapters.

Product name	Description	Use	Company	Original gene source	Gene expressed in
Maxiren	Chymosin	Cheesemaking	DSM	Calf stomach cells	<i>Kluyveromyces lactis</i>
Distillase	Glucoamylase	Starch processing	DuPont	<i>Trichoderma reesei</i>	<i>Trichoderma reesei</i>
Natuphos	Phytase	Animal feed additive	DSM	<i>Aspergillus niger</i>	<i>Aspergillus niger</i>
Lipolase	Lipase	Detergent additive	Novozymes	<i>Humicola lanuginosa</i>	<i>Aspergillus oryzae</i>
Novozyme 26062	Pullulanase	Starch processing	Novozymes	<i>Bacillus acidopullulyticus</i>	<i>Bacillus subtilis</i>
Lumafast	Lipase	Detergent additive	DuPont	<i>Pseudomonas mendocina</i>	<i>Bacillus</i> sp.
Celustar CL	Cellulase	Various	Dyadic Intl	<i>Myceliophthora thermophila</i>	<i>Myceliophthora thermophila</i>
Rheozyme	Pectin esterase	Fruit processing	Novozymes	<i>Aspergillus aculeatus</i>	<i>Aspergillus oryzae</i>
Carezyme	Cellulase	Detergent additive	Novozymes	<i>Humicola insolens</i>	<i>Aspergillus oryzae</i>

developed a global presence in part by purchasing enzyme-producing companies. The food and enzyme biotechnology company Gist Brocades, for example, merged with DSM in 1998, while the enzyme company Genencor was acquired by Danisco in 2005, which itself was acquired by DuPont in 2011. The Japanese company Shin Nihon represents yet another notable enzyme company. It produces its food enzyme products largely via solid-state fermentation. Shin Nihon enzymes are marketed internationally by Takabio (Table 11.3).

## 11.2 Sources and engineering

The majority of industrial enzymes are traditionally obtained from microorganisms. The producer strains are usually members of a family of microbes classified as GRAS (generally recognized as safe). Such bulk enzymes are produced primarily by bacteria and fungi, most notably by members of the genera *Bacillus* and *Aspergillus* (Table 11.2). Today the majority of industrial enzymes are produced by recombinant means (Table 11.4). Moreover, many are engineered. The initial focus of enzyme engineering in this context centred on the development of oxidation-resistant detergent enzymes (Table 11.5 and Chapter 12), while additional engineering aims include the development of amylases displaying enhanced thermal stability for application in the

**Table 11.5** Selected examples of commercialized detergent enzymes whose amino acid sequence was logically altered by site-directed mutagenesis in order to enhance their industrial utility by rendering them oxidation-resistant.

Enzyme (trade name)	Company	Description
Purafect OxAm	DuPont	Oxidation-resistant $\alpha$ -amylase
Duramyl	Novozymes	Oxidation-resistant $\alpha$ -amylase
Lipomax	DSM	Oxidation-resistant lipase
Lipolase ultra	Novozymes	Oxidation-resistant lipase
Maxapem	DSM	Oxidation-resistant protease
Everlase	Novozymes	Oxidation-resistant protease

starch processing industry, development of proteases with more relaxed substrate specificity (for detergent use), and development of enzymes displaying enhanced stability in the presence of surfactants (for detergent use). Specific examples of recombinant and engineered enzymes are considered in subsequent chapters.

A number of databases that provide selected information specifically relating to enzymes are listed in Table 11.6. The Brenda database, for example, contains almost 6000 different enzyme entries. Enzyme information available includes

**Table 11.6** Some databases providing specific information relating to enzymes.

Database name	Description	Address
BRENDA	Comprehensive information on several thousand enzymes	<a href="http://www.brenda-enzymes.info">http://www.brenda-enzymes.info</a>
Swissprot enzyme	Enzyme nomenclature site	<a href="http://enzyme.expasy.org">http://enzyme.expasy.org</a>
PDB enzyme structures database	Enzyme structures deposited with the protein databank	<a href="http://www.ebi.ac.uk/thornton-srv/databases/enzymes">http://www.ebi.ac.uk/thornton-srv/databases/enzymes</a>
Catalytic site atlas	Catalytic sites and residues identified in enzymes using structural data	<a href="http://www.ebi.ac.uk/thornton-srv/databases/CSA">http://www.ebi.ac.uk/thornton-srv/databases/CSA</a>
Enzyme portal	Integrates information (e.g. sequence, genome and gene expression information) available from various sources	<a href="http://www.ebi.ac.uk/enzymeportal/about">http://www.ebi.ac.uk/enzymeportal/about</a>
MEROPS	Information on proteases and proteolytic inhibitors	<a href="http://merops.sanger.ac.uk">http://merops.sanger.ac.uk</a>
Cazy	Information on carbohydrases	<a href="http://www.cazy.org">http://www.cazy.org</a>
Lipase engineering database	Information on the sequence and structure of lipases	<a href="http://www.led.uni-stuttgart.de">http://www.led.uni-stuttgart.de</a>
Rebase	Information on restriction enzymes	<a href="http://rebase.neb.com/rebase/rebase.html">http://rebase.neb.com/rebase/rebase.html</a>

enzyme sources, reaction catalysed, nomenclature, functional parameters (e.g. kinetic data and the effect of temperature and pH on enzyme activity/stability), structural information as well as some application and engineering information.

## 11.3 Environmental benefits

In some instances industrial enzymes are developed to achieve a specific environmental-related objective. Examples include the application of lignocellulases and lipases to biofuel production and the use of phytase in animal feed to reduce environmental phosphate pollution (see Chapters 12 and 13). However, the application of enzymes in many other processes can indirectly benefit the environment and renders processes more sustainable. This can happen when enzyme use reduces or eliminates the use of chemical constituents such as acid/alkali/organic solvents, or reduces energy input required in the process. Specific examples include:

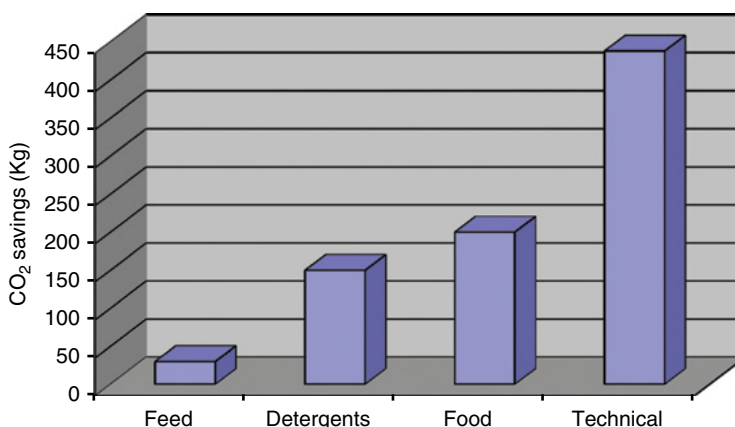
- the use of enzymes in detergents, which can help reduce surfactant levels and washing temperature required to clean clothing satisfactorily (see Chapters 12 and 13);

- the use of enzymes to clean biotech processing equipment, which could reduce the use of alkali and energy input required significantly (see Box 12.1);
- the use of enzymes in leather processing, which reduces the quantities of other chemicals required (see Chapter 12).

The contribution of enzymes to 'green chemistry' is a point often highlighted by the enzyme producers themselves, and Novozymes for example have calculated that the many such enzyme applications can very significantly reduce CO<sub>2</sub> emission levels characteristic of many industrial processes (Figure 11.2).

## 11.4 Enzyme detection and quantification

Enzymes are always sold on an activity basis, as opposed to weight. An essential prerequisite to working with enzymes is the availability of an accurate and reliable method by which enzyme activity can be detected and quantified. Enzyme activity is invariably assayed (measured) by incubating it with its substrate and quantifying the amount of substrate consumed or the amount of product generated over a specified time frame (usually several



**Figure 11.2** Industry (Novozymes) estimates of average CO<sub>2</sub> emission savings (kg CO<sub>2</sub> saved per kg enzyme used) achieved by the use of enzymes in the indicated application area.

minutes) under specified conditions (e.g. of pH and temperature, which can obviously affect enzyme activity). Enzyme activity is usually expressed in units (U). A common definition of a unit of enzyme activity is the amount of enzyme that will catalyse the transformation of 1  $\mu\text{mol}$  of substrate per minute under the defined reaction conditions used. An alternative definition is that of the katal (kat), where 1 katal is the amount of enzyme that will raise the rate of reaction by 1 mol/s in the defined assay system.

A wide range of approaches can be taken to assay enzymes, and in many cases enzymes can be assayed using several different assays. In such instances the most appropriate industrial assay to use is the one which most accurately reflects the desired functional outcome of the enzyme application. Spectrophotometric-based assays can be used if enzyme activity is associated with a change in absorbance in the UV/visible range (e.g. if either the substrate or product absorbs UV/visible light). Such assays are popular as the approach is straightforward, fast and accurate.

## 11.5 Immobilized enzymes

The vast majority of enzymes used in industrial processes are not recovered following completion of their catalytic conversion(s). In some instances, however, enzymes are utilized in an immobilized form such that they may be recovered at the end of the catalytic process. This facilitates reutilization of the enzyme

preparation, which is of obvious economic benefit. The decision to use immobilized or free enzyme in any given situation depends on economic, technical and practical considerations. The more expensive the enzyme preparation, the greater the impetus to utilize it in an immobilized form. However, immobilization must not seriously adversely affect enzyme stability, kinetic or other relevant properties if it is to be viable. Immobilization often has the added advantage of stabilizing enzyme activity, often by several hundred to several thousand fold. This can allow continuous use of the immobilized enzyme in bioprocessing operations for weeks or months. In many instances (e.g. enzymes employed therapeutically, or processes employing bulk quantities of crude enzymes), subsequent recovery of an immobilized enzyme is impractical. Some of the more notable industrial processes that do utilize immobilized enzymes are listed in Table 11.7.

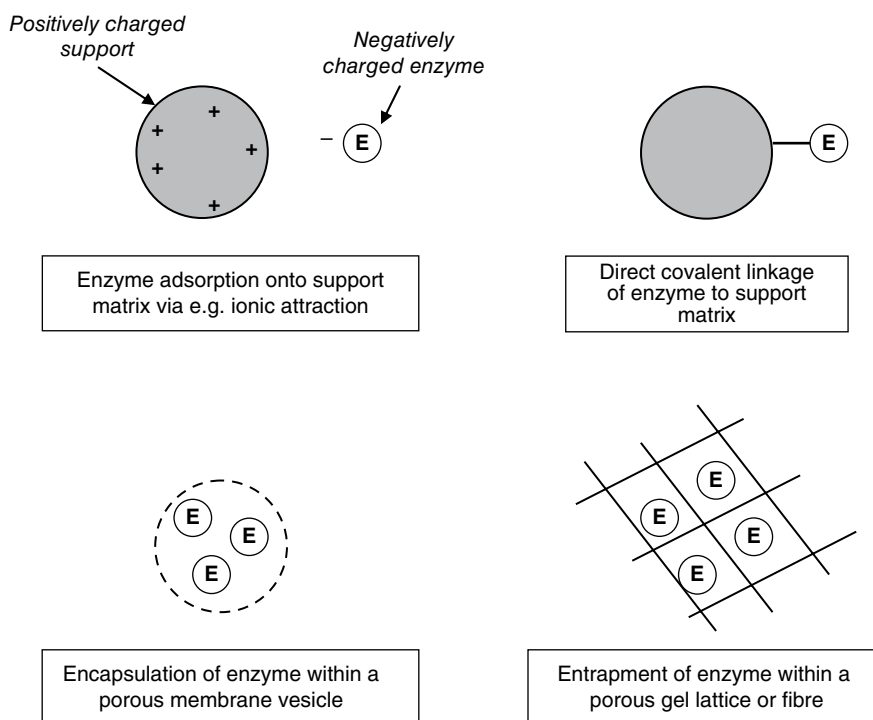
A variety of methods may be used to immobilize any enzyme and the preparation methodologies generally involve either entrapping the enzyme within a confined matrix or binding the enzyme to an insoluble support matrix (Figure 11.3). A specific industrially utilized immobilized enzyme system is outlined in Figure 11.4.

### 11.5.1 Gel/fibre entrapment

Enzymes may be entrapped within the matrix of a polymeric gel. This is achieved by incubating the enzyme together with the gel monomers and then

**Table 11.7** Some enzymes currently used in an immobilized format for industrial purposes, the reactions catalysed and the industrial processes in which they are used.

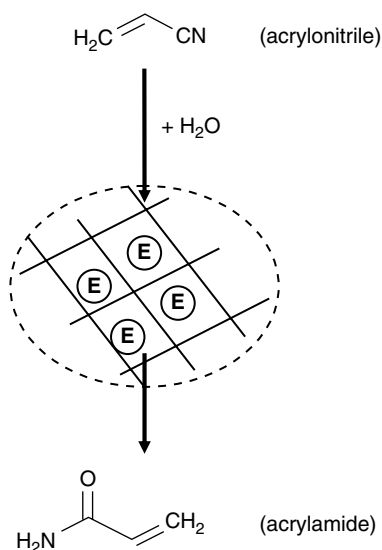
Enzyme	Reaction catalysed	Process
Glucose isomerase	Conversion of glucose to fructose	Production of high-fructose corn syrup (Chapter 12)
Amino acid acylase	Deacetylation of L-acetyl amino acids	Production of optically pure L-amino acids (Chapter 13)
Penicillin acylase	Removal of side chains from naturally produced penicillin, thus yielding 6-amino-penicillanic acids	Production of semi-synthetic penicillins (Chapter 13)
Nitrile hydrolase	Conversion of acrylonitrile to acrylamide	Production of acrylamide (Chapter 11)
Thermolysin	Synthesis of peptide bonds (under certain conditions)	Production of aspartame (Chapter 12)
Lipases	Transesterification reactions (under certain conditions)	Production of biodiesel (Chapter 13)
Lactase	Hydrolysis of lactose thus yielding glucose and galactose	Hydrolysis of lactose

**Figure 11.3** Most common means by which enzymes are immobilized.

promoting gel polymerization. Enzymes entrapped within polyacrylamide or polymethacrylate gels serve as two such examples. In order to function successfully, the gel pore size generated must be small enough to retain the entrapped enzyme but must be large enough to allow free passage of enzyme reactants and products. Enzymes may also

be entrapped within molecular-sized pockets formed during spinning of industrial fibres such as cellulose acetate, in liposomes, in hollow fibres and by using ultrafiltration membranes to physically retain the enzyme molecules.

Encapsulation involves entrapping enzymes within a spherical semi-permeable membrane.



**Figure 11.4** Schematic diagram of the production of acrylamide from acrylonitrile and water using immobilized nitrile hydratase. The enzyme is not immobilized in isolated form. Instead whole *Corynebacterium* cells containing high levels of this enzyme are entrapped in polyacrylamide gel particles. The enzymatic process is used by Nitto (Mitsubishi Rayon) in Japan. It is undertaken at temperatures below 10°C. The same reaction process can be achieved using chemical (copper)-based catalysis at 100°C. The enzyme-based approach is more environmentally friendly, using less energy and avoiding potential heavy metal contamination via process wastewater streams.

Smaller molecules, such as enzyme substrates, cofactors and products, must be able to pass freely through such pores, while enzymes or other macromolecules are retarded. Cellulose nitrate and nylon-based membranes have been extensively used in this regard.

### 11.5.2 Immobilization via adsorption

Enzymes may also be immobilized by promoting their binding to an insoluble matrix. A variety of matrices and methods of attachment have been developed. Perhaps the simplest such method involves physical adsorption of the enzyme to a suitable carrier substance. This may often be achieved

by directly mixing the enzyme and support, under incubation conditions (of ionic strength, pH and sometimes temperature) at which maximal adsorption is observed. These parameters are normally determined empirically. The enzyme is retained on the support matrix via physical interactions such as hydrogen bonding, hydrophobic interactions and van der Waal's forces. Leakage of enzyme from the support is often a problem, as the forces of attraction retaining the enzyme on its surface are relatively weak. Supports, such as aluminium hydroxide, are most often utilized and a variety of enzymes have been immobilized by this procedure.

Enzymes may also be immobilized by promoting ionic interactions with a suitably charged matrix. Ion-exchange resins have found particular favour in this regard. Anion-exchange resins such as DEAE-cellulose or DEAE-Sephadex have been used in the immobilization of negatively charged enzymes, while cation-exchange media such as CM-cellulose may be used to immobilize positively charged enzymes. A variation of this approach involves the initial coating of the support material with a thick layer of ionic polymer displaying high charge density (e.g. polyethylenimine or dextran sulfate). This provides a higher concentration of ionic groups (allowing greater enzyme loading levels and/or stronger enzyme interactions), as well as being more flexibly adaptable to multi-point interactions with individual enzyme molecules. This method of enzyme immobilization is technically undemanding and economically attractive. Enzyme leakage normally does not present a major problem, providing the column is operated under appropriately controlled conditions. Such immobilization systems may also be regenerated by passing through a solution of soluble enzyme.

Perhaps the best-known industrial-scale enzyme used in immobilized form on DEAE-Sephadex is amino acylase, used in the production of synthetic L-amino acids (see Chapter 13). Glucose isomerase may also be immobilized by this method. However, glucose isomerase is an intracellular enzyme and immobilized forms generally consist of dead (sometimes lysed) microbial cells that have been cross-linked by glutaraldehyde and subsequently pelleted. The pelleted cellular preparations may be poured

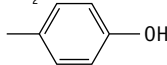
into a reactor column, through which the glucose syrup is passed. The main disadvantage associated with this approach is the relatively limited mechanical stability of the immobilized system. In the region of 1500 tonnes of immobilized glucose isomerase is used to produce in excess of 7 million tonnes of high-fructose corn syrup annually (see Chapter 12).

The most widely used method of enzyme immobilization involves covalent attachment of the enzyme to a suitable insoluble support matrix. Covalent attachment is technically more complex than most other methods of immobilization, and requires a variety of often expensive chemical reagents. Immobilization procedures are also often time-consuming. However, the covalent nature of

the binding renders the immobilized enzyme preparations very stable and leaching of the enzyme from the column is minimal.

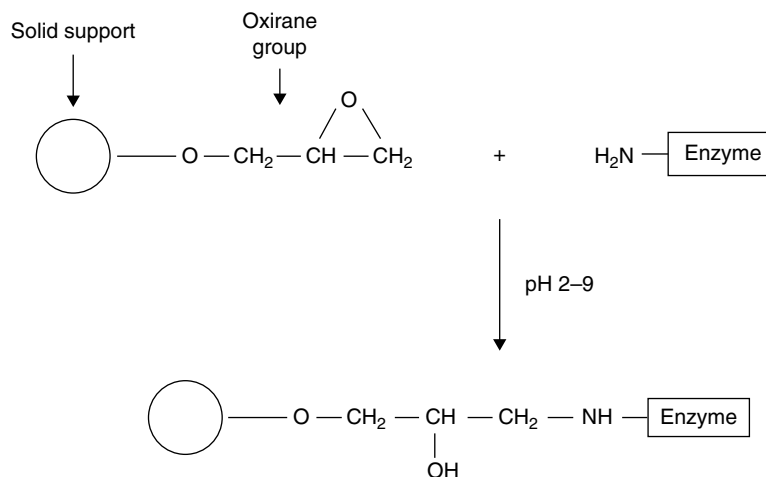
A wide variety of different immobilization procedures have been developed. All promote the formation of a covalent bond between a suitable reactive group on the surface of the insoluble matrix and a suitable group on the surface of the protein. The latter's functional groups obviously must not play an essential role in the enzyme's catalytic mechanism. Hydroxyl groups present in carbohydrate-based matrices such as cellulose, dextrans or agarose often participate in covalent bond formation. Amino, carboxyl and sulfhydryl groups present in various amino acids are also generally involved in covalent bond formation (Table 11.8). A selected example of the chemistry of enzyme immobilization (oxirane-based immobilization) is presented in Figure 11.5.

**Table 11.8** Some amino acid residues normally involved in the formation of covalent linkages in enzyme immobilization.

Amino Acid	Functional group
Lysine	NH <sub>2</sub>
Arginine	NH <sub>2</sub>
Tyrosine	
Aspartate	COOH
Glutamate	COOH
Cysteine	SH
Methionine	SCH <sub>3</sub>

## 11.6 Extremophiles

To date the majority of microbial enzymes used industrially have been sourced from mesophilic organisms (e.g. *Aspergillus*). With a few exceptions industrial processes in which they are used operate at temperatures ranging between 35 and 60°C, and at pH values between 4 and 8. The discovery of extremophiles



**Figure 11.5** Covalent attachment of an enzyme to a solid support containing a reactive oxirane group. The enzyme amino group participating most frequently in the cross-linking reaction is contributed by a surface lysine residue.

**Table 11.9** Various categories of extremophiles and the habitats in which they live.

Extremophile type	Natural habitat	Relevant growth parameter	Example microbe
Hyperthermophiles	Geothermal habitats (e.g. hot springs)	Optimum temperature for growth at or above 80°C	<i>Pyrococcus furiosus</i>
Halophiles	Hypersaline waters	Grow in up to 5 mol/L NaCl	<i>Halobacterium halobium</i>
Psychrophiles	Extremely cold environments (e.g. Antarctic sea water)	Grow at temperatures as low as -15°C	<i>Alteromonas</i> sp.
Alkaliphiles	Alkaline environments	Grow at pH values above 9	<i>Natronobacterium</i> sp.
Acidophiles	Acidic environments	Grow at pH values lower than 4	<i>Thermoplasma acidophilum</i>

provided a potential source of industrial enzymes capable of functioning well outside these relatively narrow operational parameters. Such enzymes could find use in a limited number of existing appropriate applications (e.g. high-temperature starch processing), in applications currently being perfected (e.g. lignocellulosic pretreatment), or facilitate the development of entirely new applications.

Extremophiles represent microorganisms that have successfully colonized ecological niches displaying one or more extreme environmental parameter (temperature, pH, ionic strength, pressure). An overview of extremophile classification is presented in Table 11.9. Some extremophiles have colonized niches characterized by two or more extreme environmental parameters, for example deep ocean barophiles grow at temperatures below 2°C in pressures of up to 120 MPa (1200 bar), while thermoacidophiles grow at elevated temperatures in an acidic environment.

### 11.6.1 Hyperthermophiles

Thermophiles are organisms living at high temperatures. Various different life forms thrive/survive over differing temperature ranges, and the maximum temperature at which life is sustainable remains one of the most fascinating (and unanswered) questions in biology. The upper temperature limit for growth (and often survival) of fish and other aquatic species is approximately 38°C, that of most plants and insect is 50°C, while that of eukaryotic microorganisms (certain protozoa, algae and fungi) is in the region of 60°C. Certain prokaryotic microorganisms (e.g. blue-green algae and photosynthetic bacteria) can

grow at temperatures of 70–73°C. Scientists have long studied thermostable organisms in an attempt to understand how life processes function at elevated temperatures. In addition they have exploited the inherent thermostability of biomolecules produced by such microorganisms by applying them to various industrial processes (e.g. thermostable DNA polymerase is used in recombinant DNA technology, incorporation of thermostable proteases in biologically acting detergents). This field of academic and applied endeavour was revolutionized in the mid 1980s with the discovery of microorganisms capable of growth at or above 100°C. Approximately 20 different such genera are now known (Table 11.10).

All the hyperthermophiles thus far discovered have been isolated from various geothermal habitats. Such habitats are worldwide in distribution, and are associated primarily with tectonically active zones where major crustal movements of the earth occur. In such areas magmatic material is close to the earth's surface, serving as a source of heat for sea water or ground water, thus generating deep-sea geothermal vents, thermal springs or geysers, all potential sources of hyperthermophiles.

Although a few such hyperthermophiles are aerobic, most are strictly anaerobic, which is not surprising given the low solubility of oxygen at high temperatures. Many are heterotrophs, utilizing complex peptide mixtures as a source of energy, carbon and nitrogen. Most are dependent on the reduction of elemental sulfur to H<sub>2</sub>S for significant growth. Carbohydrates are utilized by only a few species. Successful culture of these microorganisms generally requires some specialized fermentation equipment and an element of specialized microbiological training.



**Table 11.10** (Hyper)thermophilic Archaea and their respective maximum growth temperatures.

Order	Genus (maximum growth temperature)
Thermococcales	<i>Pyrococcus</i> (105°C) <i>Thermococcus</i> (97°C)
Sulfolobales	<i>Sulfolobus</i> (87°C) <i>Acidianus</i> (96°C) <i>Desulfurolobus</i> (87°C) <i>Metallosphaera</i> (80°C) <i>Styoiolobus</i> (88°C)
Thermoproteales	<i>Pyrodictium</i> (110°C) <i>Thermodiscus</i> (98°C) <i>Desulfurococcus</i> (90°C) <i>Staphylothermus</i> (98°C) <i>Thermoproteus</i> (92°C) <i>Pyrobaculum</i> (102°C) <i>Thermofilum</i> (100°C)
Thermoplasmatales	<i>Thermoplasma</i> (67°C)
Methanogenic Archaea	<i>Methanothermus</i> (97°C) <i>Methanococcus</i> (91°C) <i>Methanopyrus</i> (110°C)
Sulfate-reducing Archaea	<i>Archaeoglobus</i> (95°C)
Unclassified	<i>Hyperthermus</i> (110°C) <i>ES-1</i> (91°C) <i>ES-4</i> (108°C) <i>GE-5</i> (102°C) <i>GB-D</i> (103°C)

Considering the extreme environments in which hyperthermophiles grow, it is perhaps not surprising that many aspects of their cellular architecture and metabolism are novel. The plasma membrane of several such Archaea has been studied and has proven quite different to mesophilic plasma membranes in terms of composition. The membrane lipids contain almost no fatty acid ester groups for example. Proteins isolated from such hyperthermophiles are inherently thermostable, with some retaining biological activity at temperatures as high as 140°C.

### 11.6.1.1 Enzymes from hyperthermophiles

A range of enzymes displaying remarkable thermal stability have been isolated from various hyperthermophiles (Table 11.11). This has prompted further applied research aimed at identifying high-temperature enzymes suitable for industrial use. Such enzymes

would prove desirable in the case of certain industrial processes, as high temperature operation would:

- reduce viscosity of processing fluid (e.g. in the case of starch hydrolysis);
- discourage microbial growth (important in food applications);
- render many reaction substrates more soluble/susceptible to hydrolysis.

Furthermore, thermostable enzymes are usually more resistant to inactivation by other denaturing influences such as detergents, organic solvents, chaotropic agents and oxidizing agents. In some cases thermostable enzymes have also proven extremely resistant to proteolysis.

However, enzymes from hyperthermophiles should not automatically be assumed to be suited for all industrial applications as:

- many processes are not amenable to high-temperature operation (because of thermolability of reactants/products for example);
- low-temperature operation is often more economic and more environmentally friendly due to lower process energy costs incurred;
- in some instances (e.g. detergent applications) commercial trends favour low-temperature operation;
- there may be no obvious advantage to increasing the operating temperature;
- sufficiently thermostable enzymes may already be in use;
- for certain food applications (e.g. amylases used in breadmaking) the added enzyme is heat inactivated after it has achieved its catalytic effect, thus heat inactivation would prove unrealistic in the case of extremely thermostable enzymes;
- many enzymes derived from (anaerobic) archaeal sources are oxygen sensitive and hence not suited to industrial application.

Most hyperthermophiles are difficult to culture. The majority are strict anaerobes, and grow only to a low final cell density. Many produce end products of metabolism that are toxic and/or corrosive (e.g. H<sub>2</sub>S production in many anaerobic Archaea). As a

**Table 11.11** Temperature optimum and stability of selected enzymes isolated from various hyperthermophiles which may have potential industrial application.

Enzyme	Source	$T_{opt}$ (°C)	Thermostability ( $T_{50}$ )
Protease	<i>Thermobacteroides vulgaris</i>	85°C	NR
Protease	<i>Pyrococcus furiosus</i>	>115°C	33 hours at 98°C
DNA polymerase	<i>Pyrococcus furiosus</i>	>75°C	20 hours at 95°C
$\alpha$ -Amylase	<i>Pyrococcus woesei</i>	100°C	6 hours at 100°C
$\alpha$ -Amylase	<i>Methanocaldococcus jannaschii</i>	120°C	50 hours at 100°C
Amylopullulanase	<i>Thermoproteus tenax</i>	118°C	20 hours at 98°C
Xylanase	<i>Thermotoga</i> sp.	105°C	1.5 hours at 95°C
Cellobiohydrolase	<i>Thermotoga</i> sp.	105°C	1 hour at 108°C
Glucose isomerase	<i>Thermotoga neapolitana</i>	95°C	NR
$\alpha$ -Glucosidase	<i>Pyrococcus furiosus</i>	100°C	48 hours at 98°C
$\alpha$ -Galactosidase	<i>Pyrococcus furiosus</i>	110°C	15 hours at 100°C
$\beta$ -Glucosidase	<i>Pyrococcus furiosus</i>	105°C	85 hours at 100°C or 13 hours at 110°C
$\beta$ -Galactosidase	<i>Pyrococcus woesei</i>	90°C	3.5 hours at 100°C
Pullulanase	<i>Pyrococcus woesei</i>	100°C	4 hours at 100°C
Lactate dehydrogenase	<i>Thermotoga maritima</i>	>90°C	1.5 hours at 90°C

NR, not recorded;  $T_{50}$ , time required to lose 50% of catalytic activity when incubated at the indicated temperature.

consequence the industrial production of moderate to large quantities of hyperthermophile-derived enzymes will depend on recombinant DNA technology. While many such thermozymes have been expressed in *Escherichia coli* and additional recombinant systems, high-level production of catalytically active product can prove difficult. Incompatible codon usage patterns can prevent expression, certainly at high levels, although this can be potentially overcome by codon engineering or by using *E. coli* engineered for rare codon usage. Even then, recombinant product may accumulated in inactive, inclusion body or aggregated form, or may be oxygen sensitive. Thus far the only such enzymes that have found wide industrial application are heat-stable DNA polymerases (see Chapter 13).

### 11.6.2 Thermoacidophiles and their enzymes

Thermoacidophiles are microorganisms that grow at pH values less than 4 and at temperatures above about 60–70°C. They have hence evolved to grow optimally

in near boiling acid. The vast majority are Archaea, although a small number (e.g. various species of *Alicyclobacillus*) are eubacteria (Table 11.12). Members of the genus *Picrophilus* are the most acidophilic microbes characterized to date, several members being capable of growth at pH 0, with optimal growth at pH 0.7. They lose cellular viability at pH values above 4.0.

Thermoacidophiles maintain their intracellular pH at values typical of other cell types (i.e. approaching neutrality); hence their intracellular enzymes, while thermostable, are rarely active at low pH. Their extracellular enzymes, on the other hand, must function at both elevated temperature and low pH. In most instances such enzymes possess a C-terminal transmembrane helical domain that tethers the enzyme to the cell surface. Apart from their obvious academic interest, some such enzymes could be of industrial use (Table 11.13). Thermoacidophilic lignocellulases could have potential application in lignocellulosic bioethanol production, where the initial phase of lignocellulose deconstruction generally relies on acid treatment at elevated temperature (Chapter 12). Thermoacidophilic enzymes that retain appreciable activity at lower temperatures could be of

**Table 11.12** Some thermoacidophiles and the temperature and pH values at which they grow optimally.

Organism	Temp optimum (°C)	pH optimum
<i>Sulfolobus acidocaldarius</i>	80	2.0–3.5
<i>Thermoplasma acidophilum</i>	59	1.0–2.0
<i>Picrophilus oshimae</i>	60	0.7
<i>Picrophilus torridus</i>	60	0.7
<i>Ferroplasma acidiphilum</i>	35	1.7
<i>Desulfurolobus ambivalens</i>	81	2.5
<i>Alicyclobacillus acidiphilus</i>	50	3.0
<i>Sulfobacillus</i> <i>thermosulfidooxidans</i>	48	2.0
<i>Alicyclobacillus acidiphilus</i>	50	3.0

**Table 11.13** Some enzymes derived from thermoacidophiles which may have potential industrial application.

Organism	Enzyme	Optimum temperature (°C)	Optimum pH
<i>Sulfolobus solfataricus</i>	Endoglucanase	80	1.8
<i>Sulfolobus solfataricus</i>	Amidase*	95	7.5
<i>Picrophilus torridus</i>	Glucoamylase	90	2
<i>Picrophilus torridus</i>	Esterase*	80	6.5
<i>Alicyclobacillus acidocaldarius</i>	Endoglucanase	80	4
<i>Alicyclobacillus acidocaldarius</i>	α-Amylase	75	3
<i>Ferroplasma acidiphilum</i>	Esterase	50	1.5

\* Intracellular.

interest as stomach-targeted supplemental digestive enzymes in the animal feed industry (Chapter 13).

### 11.6.3 Enzymes from additional extremophiles

Much of the research on psychrophiles thus far has focused on fish obtained from Antarctic waters which are thus continually exposed to temperatures

of about  $-1.8^{\circ}\text{C}$  (the freezing point of sea water). The freezing of body fluids in these fish is prevented by the presence of 'antifreeze' glycoproteins, which function to inhibit ice crystal growth. Enzymes isolated from these and other psychrophiles (e.g. Antarctic microorganisms) typically display maximum activity at temperatures close to  $0^{\circ}\text{C}$ . While a proportion of these enzymes display little or no residual catalytic activity at temperatures above  $6-10^{\circ}\text{C}$ , many retain some level of activity up to temperatures approaching  $45^{\circ}\text{C}$ .

While no cold-adapted enzyme has proven itself of actual biotechnological use to date, a number of potential application fields have been proposed, including:

- use in biotransformation reactions/processes where heat-labile substrate(s) and/or products(s) are consumed/produced;
- use in low-temperature food processing, thus discouraging microbial growth;
- use in low-temperature industrial processes, thus reducing processing costs by decreasing energy requirements;
- as cold-active detergent enzymes for use in cold washing.

It remains to be seen if any such applications will prove commercially viable.

Barophiles (also called piezophiles) are microorganisms that can grow at elevated pressures. The SI unit of pressure is the pascal (Pa). Standard atmospheric pressure is approximately 100,000 Pa (0.1 MPa). Pressures at the bottom of deep oceans can reach or surpass 100 MPa, and many barophiles can thrive at these pressures. Exposure to pressures exceeding 200 MPa can lead to dissociation of oligomeric proteins, while pressures greater than 400 MPa typically denature proteins. Enzymes derived from barophiles are considerably more resistant to pressure denaturation than enzymes currently used in industry. As such, there may be potential to explore the application of such enzymes in the context of high-pressure food processing (where pressures of several hundred megapascals are routinely used).

Halophiles are microorganisms that require a minimum of 0.2 mol/L NaCl for growth. Extreme

halophiles, which are invariably Archaeobacteria, can grow in solutions containing up to 5 mol/L NaCl (~30% NaCl solution). Various proteins isolated from halophiles have also attracted some interest from the biotechnological community. Both intracellular and extracellular enzymes produced by halobacteria (extremely halophilic Archaea) are remarkably halophilic/halotolerant. Most such enzymes are quite stable, and are more than usually resistant to potential denaturing influences such as elevated temperatures. Stability is a highly desirable characteristic for any industrial enzyme. Perhaps the most likely field of application for halophilic enzymes is in biotransformation reactions carried out in organic solvent-based media (see section 11.7). Under such conditions of low water activity, some solvents tend to strip the essential hydration layer from the proteins, thus often leading to their inactivation. Halobacterial enzymes naturally function under conditions of very low water activity.

## 11.7 Enzymes in organic solvents

Until relatively recently it was assumed that enzymes added to organic solvents (e.g. benzene, acetone, toluene, cyclohexane) would lose activity and likely be denatured. Proteins have evolved to function in aqueous-based media, and one major force promoting protein folding and subsequent stabilization of the biologically active conformation is the 'hydrophobic effect'. This describes the fact that apolar amino acid residues are largely buried in a protein's hydrophobic core, with polar amino acids largely residing on the protein's surface, interacting with the surrounding water molecules (see Chapter 1).

Addition of enzymes to non-polar organic solvents should thus prompt the protein to turn, in essence, inside out. In practice, when many enzymes are added (usually in lyophilized format) to various organic solvents, they retain catalytic activity even though there is little or no water in the system. This may be partly explained by the fact that such solvents have a much lower dielectric constant than that of water. Lowering the dielectric constant

strengthens electrostatic forces within the protein, thus stabilizing it. An apolar solvent environment also promotes increased intramolecular hydrogen bonding.

The fact that enzymes can retain activity when placed in organic solvents has attracted biotechnological interest. Some potential advantages to industry of using enzymes under such conditions include:

- increased solubility of non-polar reactants/products;
- suppression of water-dependent side reactions;
- increased enzyme thermostability;
- altered enzyme substrate specificity;
- shifting of thermodynamic equilibria to favour synthesis rather than hydrolysis (e.g. formation of peptide bonds by proteases);
- enzyme reusability, due to ease of recovery by filtration or centrifugation;
- use of solvents with low boiling points facilitates easier product recovery by solvent evaporation;
- most organic solvents discourage/prevent microbial growth.

One of the main advantages is that lipophilic substrates, sparingly soluble in water, are generally freely soluble in organic solvents. This renders more practical the application of enzymes to the biotransformation of apolar substances. Enzymes also exhibit greater thermostability when present in organic as opposed to aqueous-based media (Table 11.14). Although the molecular basis of increased thermostability of enzymes has not been fully elucidated, it is probably due to the enhancement of intramolecular (stabilizing) electrostatic interactions, as already described. As enzymes are insoluble in most organic solvents, they usually form suspensions rather than true solutions when added to such solvents. This allows recovery (and hence reuse) of the enzyme by filtration or centrifugation.

When placed in organic solvents enzymes usually display altered substrate specificity. Specificity can be relaxed and kinetic parameters such as  $K_{\text{cat}}$  and  $K_{\text{m}}$  are generally altered. In some cases reaction equilibria can be reversed (e.g. proteases such as subtilisin can catalyse peptide bond synthesis rather than hydrolysis). Many of these effects are explained, in part at least, by the fact that binding of an enzyme to its

**Table 11.14** Comparison of the thermal stability of selected enzymes in aqueous versus non-aqueous media.

Enzyme	Solvent	Half-life at indicated temperature
Lipase (porcine)	Aqueous (buffer)	<1 min at 100°C
	Organic (tributyrin)	12 hours at 100°C
Lysozyme	Aqueous (buffer)	8 min at 100°C
	Organic (cyclohexane)	140 hours at 100°C
Chymotrypsin	Aqueous (buffer)	<1 min at 100°C
	Organic (octane)	270 min at 100°C
Ribonuclease	Aqueous (buffer)	<10 min at 90°C
	Organic (nonane)	>6 hours at 110°C

substrate(s) is influenced by the solvent. The binding energy of an enzyme to its substrate is determined by the difference between the energy of the enzyme–substrate complex and that of the enzyme and substrate free in solution, each separately interacting with solvent molecules. The natural stereospecificity exhibited by enzymes is also usually altered by the use of organic solvents. Subtilisin for example can synthesize peptides from D-amino acids as well as L-amino acids in organic media.

One impediment to the more widespread industrial application of enzymes in organic solvents is that most enzymes are less active when placed in such solvents. In some cases the reduction in activity can be relatively modest. However, in many instances, a  $10^4$  to  $10^7$  fold reduction in activity is observed. This can render uneconomic the use of an enzyme in some industrial processes. The observed reduction in enzyme activity in organic solvents may be due to a number of factors. The fact that the enzyme is in suspension rather than true solution may impose diffusional limitations on substrate accessibility. The enzyme's conformation may be altered sufficiently in the organic environment to reduce catalytic efficiency. Furthermore, pH has a profound effect on enzyme activity in water, but the concept of pH in the case of organic solvents has no meaning. However, the influence of the latter point can be mitigated by freeze-drying the enzyme in a buffer adjusted to its optimal pH for activity prior to its addition to the organic solvent media. Such 'pH memory' is likely due to the fact that ionizable groups on the protein are retained in their optimal ionization state for activity when placed in the organic solvent.

Despite such difficulties, more and more research and development work is underway aimed at using enzymes in organic solvents for applied purposes, particularly with regard to the use of enzymes in organic synthesis.

## 11.8 Industrial enzymes: the future

The range and market value of industrial enzymes will likely grow substantially over the coming years. Genomics and proteomics continue to uncover new enzymes from various sources which may prove industrially useful. The continued discovery of microorganisms living in extreme environmental conditions (extremophiles) provides a particularly rich source of novel, potentially useful enzymes. Recombinant methods now ensure that most enzymes can be produced economically and at the scale required. Furthermore, protein engineering continues to allow scientists to tailor enzymes to optimize their industrial utility. Both academic and industrial research groups continue to investigate and develop new enzyme-mediated industrial processes. For example, more and more enzyme-based processes for the production of pharmaceuticals are being developed (often involving enzyme use in organic solvents, as discussed in section 11.7). Additionally, the successful development of enzyme-mediated biofuel production processes such as bioethanol production from lignocellulosic material (Chapter 12) and lipase-mediated biodiesel production (Chapter 13) will likely increase substantially the range and market value of industrial enzymes.

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# Chapter 12

## Industrial enzymes: proteases and carbohydrases

### 12.1 Proteolytic enzymes

Proteolytic enzymes (proteases) catalyse the cleavage of peptide bonds in protein-based substrates. The degradation of proteins, ultimately to amino acid level, can serve an obvious nutritive/catabolic role. However, proteolysis is at the heart of many additional biological processes. For example, proteases:

- regulate the targeting and localization of many proteins;
- regulate the biological activity of many proteins (e.g. proteolytic activation of proenzymes);
- modulate protein–protein interactions;
- play a role in some forms of cell signal transduction.

Proteases are a diverse family of enzymes, both structurally and functionally. They can range in size from small single-chain polypeptides of about 20kDa to enormous multi-subunit complexes of up to several thousand kilodaltons (e.g. the proteasome, see section 12.1.1). Some proteases (e.g. proteinase K) display extremely broad substrate specificity, while others (e.g. angiotensin-converting enzyme) display

extreme specificity, hydrolysing only a single peptide bond in a single protein substrate.

Quantitatively, proteolytic enzymes constitute the single most important group of industrial enzymes currently in use. They have enjoyed a long history of use in the food and detergent industries and they have more recently been employed in leather processing and as therapeutic agents. An overview of their industrial uses (excluding medical uses, which are discussed in Chapter 6) is provided in Table 12.1. Proteolytic

**Table 12.1** Overview of the various industrial uses of proteolytic enzymes.

Industry	Use
Beverage	Solubilization of grain proteins, stabilization of beer
Detergent	Catalytic degradation of protein-based stains on clothing
Bread/confectionery	Modification of gluten elasticity
Cheese production	Coagulation of casein, hence forming curds; ripening cheese
Leather processing	Dehair hides and bate (soften) leather
Meat	Tenderize meat

**Table 12.2** Peptide bonds hydrolysed by various types of exopeptidases.

Exopeptidase	Peptide bond cleaved
Aminopeptidase	$\text{NH}_2-\overset{\downarrow}{\text{O}}-\text{O}-\text{O}-(-\text{O}-)_n-\text{O}-\text{O}-\text{O}-\text{COOH}$
Depeptidyl peptidase	$\text{NH}_2-\text{O}-\text{O}-\overset{\downarrow}{\text{O}}-\text{O}-(-\text{O}-)_n-\text{O}-\text{O}-\text{O}-\text{COOH}$
Tripeptidyl peptidase	$\text{NH}_2-\text{O}-\text{O}-\text{O}-\overset{\downarrow}{\text{O}}-\text{O}-(-\text{O}-)_n-\text{O}-\text{O}-\text{O}-\text{COOH}$
Carboxypeptidase	$\text{NH}_2-\text{O}-\text{O}-\text{O}-\text{O}-(-\text{O}-)_n-\text{O}-\text{O}-\overset{\downarrow}{\text{O}}-\text{COOH}$
Peptidyl dipeptidase	$\text{NH}_2-\text{O}-\text{O}-\text{O}-\text{O}-(-\text{O}-)_n-\text{O}-\overset{\downarrow}{\text{O}}-\text{O}-\text{COOH}$

enzymes used industrially are obtained from a wide range of source material (see Chapter 2), and the most recent innovations in this regard relate to their recombinant production and engineering (see Chapter 11).

### 12.1.1 Classification of proteases

Proteases may be classified using various criteria, but are most often grouped on the basis of either the positioning of the peptide bond hydrolysed or the molecular mechanism by which such hydrolysis is achieved. Based on the relative position of the susceptible bond within the protein substrate, proteases may be described as exopeptidases or endopeptidases. Exopeptidases may be further classified into aminopeptidases, dipeptidyl peptidases, tripeptidyl peptidases, carboxypeptidases and peptidyl dipeptidases (Table 12.2). Endopeptidases cleave peptide bonds that are found internally in the protein, usually some distance from the carboxyl or amino terminus. Most such enzymes (both endopeptidases and exopeptidases) also display some level of selectivity towards the peptide bond they hydrolyse, for example chymotrypsin hydrolyses peptide bonds adjacent to aromatic amino acid residues.

On the basis of mechanism of action, proteolytic enzymes may be divided into six categories; serine, cysteine, aspartic, glutamic and threonine proteases, as well as metalloproteases (Table 12.3).

**Table 12.3** The major classes of proteolytic enzymes (classified on the basis of their mechanism of catalytic conversion). Some better-known examples of each class are also presented.

Protease class	Examples
Serine proteases	Trypsin, chymotrypsin, elastase, subtilisins, proteinase K
Aspartic proteases	Pepsin, rennin (chymosin), microbial aspartic proteases
Cysteine proteases	Papain, ficin, bromelain
Metalloproteases	Collagenase, elastase, thermolysin
Glutamic proteases	Several fungal proteases
Threonine proteases	The proteasome

1. *Serine proteases* are characterized by the presence of an essential serine residue at their active site. They are the most common class of protease, being produced by Archaea, bacteria, Eukarya and viruses. Although exceptions exist, most serine proteases display a molecular mass of between 18 and 35 kDa and are maximally active somewhere between pH 7 and 11. They may be divided into a number of subfamilies based on structural similarities. From an applied perspective, the bacterial subtilisins are the subgroup of serine proteases of greatest industrial significance. Subtilisins, particularly those produced by selected bacilli, have found widespread application as detergent additives.



2. *Aspartic proteases*, as the name suggests, are a group of acidic proteases that contain an essential aspartic acid residue at the catalytic site. Most aspartic proteases display maximum activity at pH 3–4 and have isoelectric points between 3 and 4.5. They generally exhibit molecular masses of 30–45 kDa. Pepsin and rennin represent two animal stomach-derived aspartic proteases. Microbial aspartic proteases are produced mainly by *Aspergillus*, *Penicillium*, *Rhizopus* and *Mucor* spp., and the best-known application of aspartic proteases is in cheese manufacture.
3. *Cysteine proteases* are widely distributed in nature and are characterized by the presence of a cysteine and a histidine residue at the active site, which forms a catalytic dyad essential for biological activity. These proteases, the best known of which is papain, are generally active under reducing conditions and tend to display optimum activity at neutral pH values. Several members find industrial application, mainly in the food industry.
4. *Threonine proteases*, as the name suggests, contain an essential threonine residue at the active site. The threonine participates directly in a nucleophilic attack on the target peptide bonds of the protein substrate. The best-known threonine proteases are the proteasomes. Proteasomes are protease complexes found in eukaryotes, some bacteria and Archaea. They function to proteolytically degrade damaged or excess proteins in the cell, and thus play a major role in normal cellular protein turnover. The proteasome also plays an important role in several additional headline cellular processes, including the cell cycle and apoptosis, as well as response to cellular stress.
5. *Glutamate proteases* were initially classified as a family of aspartic proteases. However, they are now known to contain a unique catalytic dyad consisting of a glutamic acid residue and a glutamine residue, which together hydrolyse the peptide bond. These proteases are predominantly, if not exclusively, associated with filamentous fungi.
6. *Metalloproteases*, as the name suggests, are a family of proteases characterized by their requirement for (divalent) metal ions to sustain

biological activity. Removal of these ions, for example by incubation of the enzyme with chelating agents, abolishes their catalytic activity. Most metalloproteases display maximum activity at neutral to alkaline pH values. The single best-known member of this family is microbial thermolysin, a very heat-stable neutral protease.

Proteases of different classes can be further grouped into families on the basis of sequence comparison at the amino acid level, and families can be grouped into clans based on similarities in their three-dimensional structures. The availability of genome sequences and bioinformatic tools to interrogate sequence information, along with the full three-dimensional structural elucidation of increasing numbers of proteases, has greatly contributed to protease classification. There is also increasing interest in determining the entire protease capability or complement of entire organisms (i.e. the degradome) and degradome-related information is available from databanks such as MEROPS ([merops.sanger.ac.uk](http://merops.sanger.ac.uk)) and the degradome database ([degradome.uniovi.es](http://degradome.uniovi.es)).

### 12.1.2 Detergent proteases

The bulk of proteolytic enzymes produced commercially are incorporated into detergents. Enzymes were first introduced into detergent preparations at the turn of the last century. Few such products were successful as the enzymes chosen, generally from animal sources, were invariably inactivated by other detergent components or by the ensuing washing process.

Most clothing becomes soiled by substances such as dyes, biological molecules, soil and miscellaneous particulate matter. Biological 'dirt' includes protein, lipid and carbohydrate-based stains. Such dirt components may be derived directly from humans or animals (e.g. shed skin or blood) or may be derived from other sources such as foodstuffs or grass. Detergent formulations typically contain a range of ingredients capable of efficiently removing both biological and non-biological dirt. Typical ingredients are listed in Table 12.4.

**Table 12.4** Principal ingredients of detergent preparations.

Detergent component	Function
Soap and surfactants	Removal of dirt particles, especially hydrophobic molecules, from fabrics
Sodium perborate	Removal of dyes and stains from fabrics
Sodium tripolyphosphate	Used to soften water
Enzymes	Removal of biological dirt, which is mainly protein
Sodium carbonate and silicate	Maintenance of an alkaline pH
Polycarboxylates	Helps disperse dirt particles in water
Silicones	Controls foaming
Perfume	Imparts an appropriate scent to fabrics

Soaps and surfactants remove the majority of dirt particles from fabrics. Perborates exhibit a bleaching action and thus help remove dyes and stains, such as those from tea, coffee and wine. Proteolytic enzymes function to degrade protein dirt such as blood, egg and gravy. Proteins are often denatured and aggregated by the washing process. This renders them even more difficult to remove from clothing fibres. The addition of proteolytic enzymes active under the washing conditions employed greatly facilitates the degradation and subsequent removal of such stubborn stains. Phosphates, such as sodium tripolyphosphate, function to soften the water thus ensuring maximal detergent efficiency. Hard water contains appreciable quantities of calcium ions. Calcium and other divalent ions such as  $Mg^{2+}$  or  $Fe^{2+}$  will react with soap, forming a precipitate. Precipitate formation in detergent solutions would obviously be undesirable. Calcium-containing water may be softened by a number of means. One popular method involves the addition of sequestering agents such as sodium polyphosphates. The sequestering agent complexes or sequesters the divalent cations in solution and thus prevents precipitate formation. One consequence of employing such sequestering agents is that divalent cation-dependent proteases may not be used. The presence of sodium carbonate in the detergent formulation ensures the maintenance of an alkaline environment, required for maximal cleaning efficiency.

The exact formulation of detergents sold in various world regions can vary somewhat. This largely reflects different washing practices characteristic of these regions. In Europe, for example, automatic washing machines are generally used with washing cycles of about 1 hour and at temperature settings of 30–60°C. In the USA, washing cycles are much shorter (usually 10–15 minutes), and in all world regions the trend is towards lower washing temperatures.

Proteolytic enzymes incorporated into detergent formulations must exhibit satisfactory catalytic activities in the presence of other detergent components, and under standard washing conditions. Such enzymes must therefore be stable at alkaline pH, often at relatively high temperatures and in the presence of sequestering agents, bleach and surfactants. Of the various classes of proteolytic enzymes (Table 12.3), only the serine proteases are potentially suited to application in the detergent industry. Aspartic proteases will be totally inactive at alkaline pH values. Metalloproteases will be inactivated by sequestering agents in the detergent formulation, while cysteine proteases will be inactivated by the oxidizing environment (due to the bleach) and high pH values.

Screening of serine proteases from various (particularly bacterial) sources quickly identified members of the subtilisin subfamily as being the most appropriate for detergent application. These generally:

- display maximum activity at pH values typical of detergent-containing wash water;
- are modestly/highly stable in the presence of other detergent ingredients;
- generally display broad substrate specificity (i.e. cleave peptide bonds linking many different amino acid residues).

Currently, the vast majority of detergent proteases are subtilisins isolated from *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus alcalophilus* or *Bacillus amyloliquefaciens* (Table 12.5). These (extracellular) proteases may be produced in large quantities by fermentation technology, using the native producer organism. However, many are now produced by recombinant means in

**Table 12.5** The major commercialized subtilisin proteases, their sources, properties and manufacturers.

Subtilisin source	Active pH range	Active temperature range (°C)	Trade name (and producer)
<i>Bacillus licheniformis</i>	7–10.5	50–65	Alcalase (Novozymes) Maxatase (Danisco)
<i>Bacillus lentus</i>	9–12	45–70	Savinase (Novozymes) Esperase (Novozymes) Purafect (Danisco)
<i>Bacillus alcalophilus</i>	9–12	45–60	Maxapem (DSM)
<i>Bacillus amyloliquefaciens</i>	9–12	45–60	Maxacal (Danisco)

order to achieve even higher expression levels and to facilitate protein engineering.

One of the first proteolytic enzymes successfully used in detergent products was that of subtilisin Carlsberg. Subtilisin Carlsberg is a single-chain serine protease produced by *B. licheniformis*. It consists of 274 amino acids and has a molecular mass of 27.5 kDa. It is a single-domain polypeptide whose active site is formed by Ser<sup>221</sup>Asp<sup>32</sup>His<sup>64</sup>. The enzyme exhibits typical Michaelis–Menten kinetics and broad product specificity. The protein is maximally active at alkaline pH values of 8–10 and is stable at temperatures in excess of 50°C. The subtilisin gene has been cloned and expressed in various production systems. Its relatively straightforward structure and kinetic mechanism, along with its almost unparalleled industrial importance, has rendered it subject to extensive investigation.

Subtilisin BPN', produced by *B. amyloliquefaciens*, also found early application in the detergent industry. The enzyme consists of 275 amino acids and its three-dimensional structure is quite similar to that of the Carlsberg enzyme, although their kinetic properties do vary. Both enzymes function effectively when used in liquid detergent formulations (the resulting wash water pH will typically fall between 7.5 and 9.5). Powdered detergent formulations generally produce wash water of even higher pH (usually 9–11). Subtilisin sourced from *B. lentus* is often included in such formulations, as this enzyme exhibits an even more alkaline pH vs. activity profile than that of subtilisin BPN' or Carlsberg. The *B. lentus* subtilisin is slightly smaller (269 amino acids) than subtilisin Carlsberg and

BPN', and it displays in the region of 60% sequence homology with each of the latter proteases.

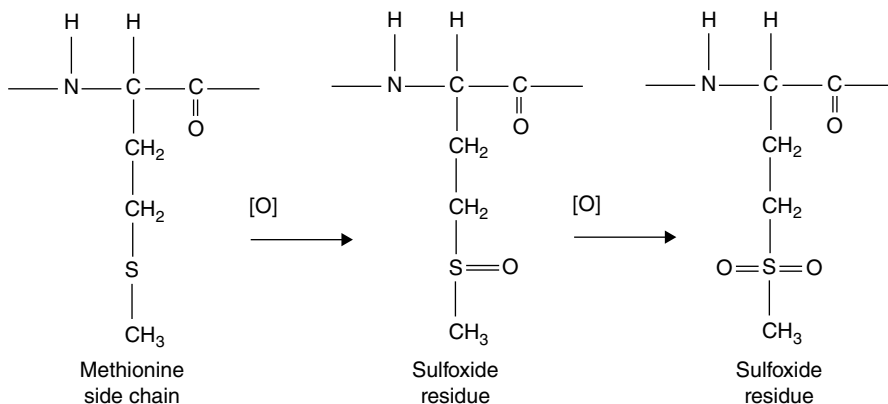
Although subtilisins display biochemical characteristics that make them suitable for detergent application, various modified forms have been generated by protein engineering. Although earlier engineering studies aimed to increase the thermal stability of the enzymes, the general trend towards decreased washing temperatures renders this goal less important. Most commercial attention in this area has focused on developing detergent proteases resistant to oxidation.

The bleach constituent of all modern detergents invariably promotes the oxidation of sensitive surface amino acid residues such as methionine and cysteine. The subtilisins commonly included in detergent preparations (Table 12.5) all contain a particularly susceptible surface methionine located adjacent to the active site Ser<sup>211</sup>. Oxidation of the methionine residue (Figure 12.1) reduces the enzyme's catalytic activity, usually quite significantly. Site-directed mutagenesis allows scientists to replace the methionine with a non-oxidizable amino acid residue, and this approach has resulted in the development of second-generation oxidation-resistant engineered subtilisins. Such commercialized products include Maxapem (DSM), Durazyme (Novozymes) and Purafect OXP (Danisco). Although methionine replacement with some amino acids caused an unacceptable decrease in activity, replacement with serine or alanine promoted considerably lower levels of activity loss.

In addition to removing protein-based stains, detergent proteases can also show lytic activity

towards microorganisms, viruses and prions. Such potential effects may become more practically important given the trend toward lower washing temperatures and milder detergent formulations containing reduced levels of bleaching agents for

example. Detergent proteases have also been included in dishwasher tablets and show promise for cleaning applications in various other industries, such as the dairy processing industry (Box 12.1.).



**Figure 12.1** Oxidation of a methionine residue, forming a sulfoxide and finally a sulfone.

### Box 12.1 Enzymes for cleaning-in-place in the dairy industry

Dairy processing facilities are characterized by numerous stainless steel vessels, pipework and heat exchangers (heated stainless steel plates) through which large volumes of milk are pumped during processing operations such as pasteurization. Effective and regular cleaning of such process equipment is essential to the production of safe high-quality dairy products. As most such equipment is not readily disassembled, it is usually cleaned in place (CIP). CIP typically involves circulating 0.5–1.5% NaOH solutions at elevated temperatures (70–80°C) throughout the system for periods of 1 hour, followed by extensive rinsing with water in order to remove all traces of alkali. Such procedures have a number of negative consequences from an industrial and environmental standpoint:

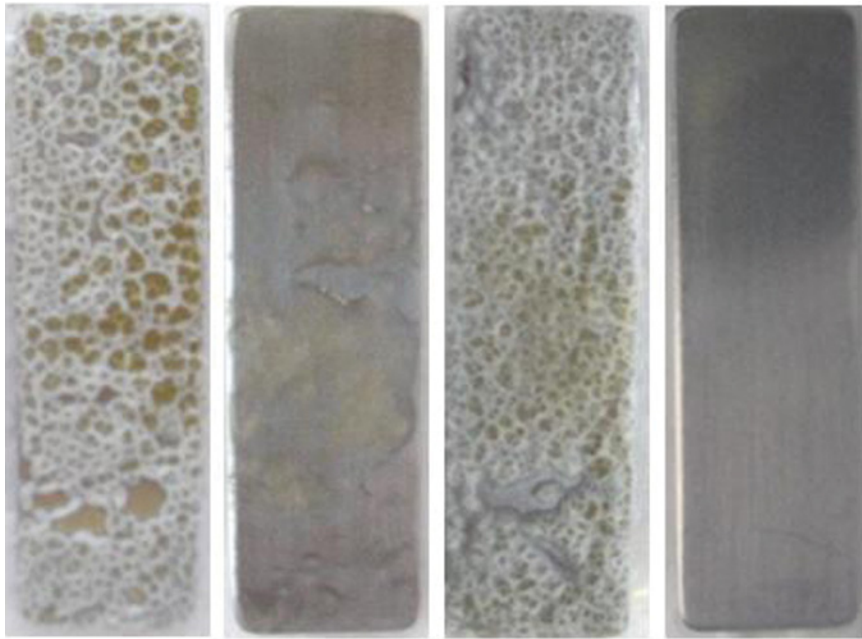
- high energy usage (CIP typically accounts for 30% of total energy use in dairy processing);
- high rinse water usage levels;

- health and safety risks associated with using hot alkali;
- potential for equipment corrosion;
- potential for environmental damage if the high pH waste/rinse water is not appropriately treated before disposal.

Protein represents the major constituent of milk fouling deposits formed on the insides of pipes/vessels and particularly on heated surfaces (heat exchangers) during processing operations. Proteolytic enzymes thus represent a potential alternative to NaOH-based CIP operations and the use of enzymes would negate, entirely or in part, the associated disadvantages linked to NaOH. Several studies suggest that the use of enzymes would be technically and economically viable. For example, one study (Boyce, A. *et al. Biofouling*, 2010, **26**, 837–850) found that cleaning performance at 40°C of several already commercialized detergent proteases was comparable to that of 1%

NaOH at 60°C, as assessed at both laboratory scale and at initial bioprocess scale. Moreover, the associated cost comparisons indicated the enzyme cost to be no greater than for NaOH. The photographs below illustrate this cleaning concept using stainless steel panels experi-

mentally fouled with milk (from left to right: stainless steel panel experimentally fouled with milk; fouled panel cleaned with NaOH; fouled panel cleaned with buffer alone; fouled panel cleaned with commercially available protease in buffer).

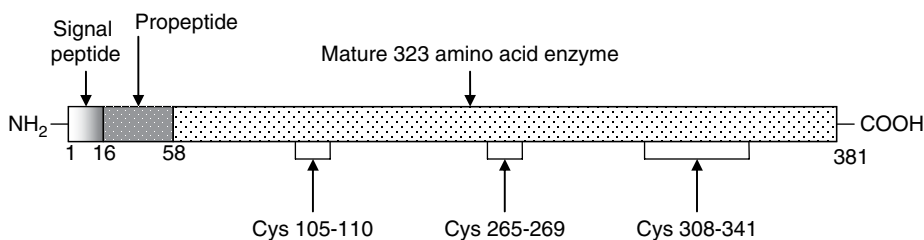


### 12.1.3 *Proteases used in cheese manufacture*

Rennin, also termed chymosin, represents another proteolytic enzyme subject to considerable industrial demand. This protease finds application in the cheese manufacturing process. The initial step in cheesemaking involves the enzymatic coagulation of milk. Rennin catalyses limited proteolytic cleavage of milk  $\kappa$ -casein. This destabilizes casein micelles and promotes their precipitation, thus forming curds (three casein subunit types are found in milk,  $\alpha$ ,  $\beta$  and  $\kappa$ ). These aggregate spontaneously, forming spherical particles called micelles (this is considered further in Chapter 14). The remaining liquid or whey is removed and the curd is further processed, yielding cheese or other dairy products.

Rennin is obtained from the fourth stomach of suckling calves. Extraction often involves prolonged treatment of dried strips of calf stomach with a salt solution containing boric acid. The enzymatic extract obtained is termed rennet and it contains a variety of proteolytic and other enzymatic activities. Rennin accounts for no more than 2–3% of such preparations.

Rennin (chymosin) is an aspartic proteinase and is synthesized as pre-prorennin. The hydrophobic leader sequence, which directs the newly synthesized molecule through the endoplasmic reticulum, is cleaved yielding prorennin, the inactive rennin zymogen. Prorennin undergoes autocatalytic activation under the acidic conditions associated with the stomach thus yielding the active enzyme. Rennin has a molecular mass



**Figure 12.2** Schematic diagram of bovine pre-prorennin from which mature biologically active rennin is derived. Cysteine residues participating in 3 intrachain disulfide linkages are indicated. Refer to text for further details.

of 35 kDa and consists of 323 amino acids (Figure 12.2) and is optimally active at pH 4.0 and a temperature of 30°C.

Calf rennin catalyses the proteolytic cleavage of a single peptide bond, the Phe<sup>105</sup>—Met<sup>106</sup> bond in the  $\kappa$ -casein molecule, thus inducing protein precipitation and curd formation. The extreme specificity of rennin towards its casein substrate renders this enzyme ideally suited to cheesemaking operations. Further proteolytic cleavage of casein would result in the production of inferior-quality unpalatable cheeses.

The rate of slaughter of young calves reflects market demand for veal. Fluctuation in this regard has an obvious effect on the availability and price of rennet. Thus many alternative sources have been screened in an effort to identify a suitable replacement protease. Pepsin preparations, obtained from a variety of slaughterhouse animals, have had limited success in this regard and are sometimes used in combination with rennet preparations. Of all the microbial enzymes thus far screened, few induce fully satisfactory curd formation. Several members of the *Mucor* family of thermophilic fungi have been found to produce acceptable alternative activities to calf rennin. The *Mucor* enzymes may be produced economically and in satisfactory quantities by fermentation technology. Like rennin, these enzymes catalyse limited cleavage of the casein molecule and function adequately under the conditions utilized in the cheesemaking process. However, most such *Mucor* enzymes are thermostable and high temperatures are required to bring about their subsequent inactivation.

An alternative approach involves the expression of rennin cDNA in recombinant microbial species. The chymosin cDNA has been expressed in a variety of such systems, including *Escherichia coli*, *Aspergillus nidulans*, *Saccharomyces cerevisiae* and *Trichoderma reesei*. Recombinant rennin produced as a heterologous protein product in *E. coli* K-12 was the first food ingredient produced by recombinant DNA technology approved for human use (in the USA by the FDA in 1990).

The recombinant rennin forms inclusion bodies in *E. coli* and recovery of the inclusion bodies, followed by solubilization and subsequent renaturation, yields active enzyme. The recombinant product displays biological properties identical to those of native rennin. Alternative recombinant systems capable of synthesizing large quantities of rennin produced as an extracellular protein would have obvious production advantages. A number of such commercialized products have come on the market (Table 12.6) and one such product (Maxiren) is considered in Box 12.2.

Recombinant chymosins display a number of potential advantages over products extracted directly from the stomach of calves:

- product manufacture is independent of the rate of slaughter of calves;
- high expression levels facilitate economic production;
- the product is no longer sourced from an animal and hence is kosher and vegetarian approved;
- the recombinant product is highly pure and is devoid of pepsin.

**Table 12.6** Some proteolytic preparations (recombinant and non-recombinant) used to promote curd formation in cheesemaking.

Product brand name	Product description
Delvoren or Caglio Camoscio	Animal rennet extracted directly from the fourth stomach of young calves. Clotting activity due to a mixture of chymosin and some pepsin
Fromase	Microbial product derived from the fermentation of the fungus <i>Rhizomucor miehei</i> . The clotting activity is due to a fungal acid protease
Maxiren	Recombinant rennin produced in <i>Kluyveromyces lactis</i>
Chy-max	Recombinant rennin produced in <i>Aspergillus niger</i>
Chymostar	Recombinant rennin produced in <i>Trichoderma reesei</i>

The final point is particularly significant, as traditional rennins typically contain 10–20% pepsin as a contaminant. Pepsin has broad proteolytic activity and proteolytic activity contributes to flavour development in cheese. A number of peptides are known to impart a characteristic flavour to food-stuffs. So-called bitter peptides have been isolated from a number of cheese types. Most bitter peptides exhibit highly hydrophobic amino acid content, and their presence in cheese is generally considered undesirable.

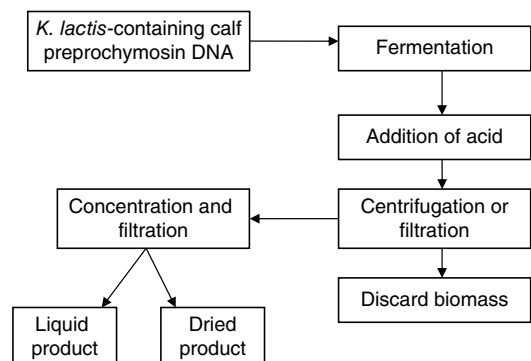
### 12.1.4 Proteases and meat tenderization

Tenderness is rated by most consumers as the most important attribute of meat. It is primarily connective tissue collagen that renders meat tough and hence necessitates its cooking prior to consumption. Individual collagen molecules in young animals are cross-linked to a low degree and cooking readily promotes their solubilization into gelatin, so meat from young animals is generally very tender. As animals get older, significant

### Box 12.2 Product case study: Maxiren

The company DSM market a recombinant chymosin (trade name Maxiren) produced in an engineered strain of *Kluyveromyces lactis*. This organism was chosen as the production strain as it has a long and safe history of industrial usage. It is non-pathogenic, non-toxic and has GRAS status. It was cultured in the 1960s and 1970s and used directly as a health food and protein supplement. It has also been used for many years in the (non-recombinant) production of lactase.

The chymosin production strain houses the calf preprochymosin gene, spliced to an appropriate yeast leader sequence so that the recombinant product is efficiently excreted from the cell. After fermentation is complete acid is added, promoting autolysis of the inactive chymosin precursor to yield active chymosin. The cell biomass is then removed by centrifugation or filtration, leaving behind the chymosin-rich extracellular media. As *K. lactis* excretes very little protein, the extracellular chymosin is very substantially pure. Concentration and formulation is followed by direct marketing of the liquid product, although a substantial proportion of the product is sold in a dried format (as powder, granules or tablets). The recombinant chymosin is biologically and chemically identical to its natural counterpart, and is kosher and halal approved. The production scheme for Maxiren is summarized below.



additional quantities of collagen are deposited in their connective tissue (to help support their greater bulk), and individual collagen molecules become progressively more cross-linked. The cooking of meat from older animals thus promotes only partial collagen solubilization and hence the cooked meat remains tough.

The tenderness of meat may be maximized by storing fresh carcasses in a cold room for several days after slaughter. This process is termed conditioning (also ageing or ripening). During storage, proteolytic and other hydrolytic enzymes are released as the physiological integrity of some muscle cells (i.e. the meat) breaks down.

Artificial tenderization (particularly of meat from older animals) may be achieved using papain, a plant-derived cysteine protease. Papain tenderizes the meat by degrading myofibrillar proteins (contractile elements of muscle fibre) and connective tissue proteins. Its tenderization action occurs during the cooking process. Papain displays little activity against native intact collagen. However, the enzyme has an unusually high optimum temperature (50°C) and is quite thermostable. At temperatures in excess of 50°C, the native collagen structure is loosened, facilitating attack by near maximally active papain. Collagen breakdown occurs during cooking when the meat temperature is between 55 and 65°C. The papain is likely fully inactivated when meat temperature reaches 80–90°C.

Commercially available powdered papain preparations may be rubbed or dusted onto the meat before cooking, although this will mainly promote a surface action. For larger meat cuts, the enzyme must be injected into the joint by commercially available equipment. An alternative approach entails injecting a papain solution into the animal approximately 30 minutes before slaughter. This facilitates even body distribution of the enzyme. Injection of an active protease into an animal's bloodstream will promote activation of the serum complement system, leading to shock and death. Papain injected into live animals is first chemically oxidized. Oxidation of the essential catalytic-site cysteine residue inactivates the enzyme. As long as

the animal is alive the papain remains oxidized and hence inactive. After slaughter, cellular glycolysis continues for several hours. This quickly consumes muscle oxygen, resulting in the generation of a reducing environment. This in turn reduces the papain, restoring its catalytic activity. Ficin and bromelain, additional plant proteases, have also been used to tenderize meat. However, papain remains the enzyme of choice, mainly on economic grounds.

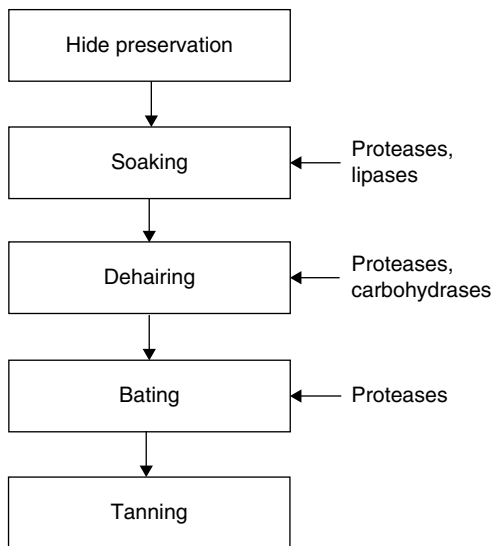
### **12.1.5 Proteases and leather production**

The production of leather from animal hides is a multistep process (Figure 12.3), with enzymes being utilized in several of the steps. Animal skin (Figure 12.4) is composed largely of the following components: water (64%), protein (mainly collagen, 33%), lipid (2%) and minerals (0.5%). The leather production process essentially involves removing the lipid, water and some of the surface protein (e.g. hair), partial disruption of the collagen, and its subsequent cross-linking during tanning.

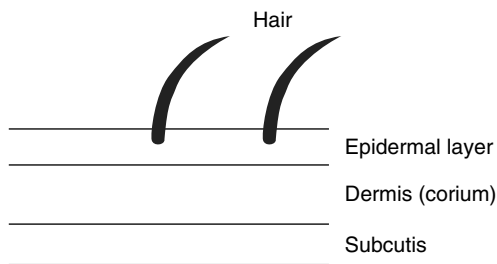
A range of proteases are employed during leather manufacture, including animal pancreatic proteases and microbial proteases (acidic, neutral and alkaline bacterial and fungal proteases). Application of plant-derived proteases (papain and bromelain) have been recorded, as has the use of carbohydrases and lipases, although these latter activities have not gained widespread application in the industry. The enzymes help degrade and hence remove unwanted skin components, but a secondary effect is the consequent reduction in chemical treatments required. This has environmental benefits as less chemical waste is generated.

Raw fresh animal hide is very susceptible to microbial attack. The first step in leather manufacture is thus generally hide preservation (by air drying or dehydrating and curing by packing with dry salt). This allows the hides to be stored/shipped safely. Leather manufacture begins with rehydration of the hides in the so-called soaking process. The





**Figure 12.3** An overview of the leather production process, emphasizing steps which are at least partially dependent on enzymatic activity. Refer to text for specific details.



**Figure 12.4** The structure of skin. The epidermal layer, which constitutes approximately 1% of the skin, houses hair/hair roots, sebaceous (oil/wax-producing) glands and sweat glands. The dermis (which constitutes approximately 84% of skin) largely consists of collagen/connective tissue while the subcutis (constituting approximately 15% of the skin) houses blood vessels, nerves, muscle, connective tissue and fat.

hide is immersed in water containing a detergent, preservatives and enzymes for several hours. In addition to rehydrating the hide, this process also helps remove non-fibrous proteins (globulins and albumins) from the hide. Pancreatic proteases have traditionally found most application in this regard, as they efficiently degrade globular proteins while

leaving the collagen intact (finished leather is almost pure collagen). Neutral and alkaline bacterial proteases now also find application in the soaking process. Pancreatin (which contains lipase activity in addition to proteolytic activity) is also sometimes used and research has shown that additional lipase activity can help clean the hide by initiating the degradation of its lipid components.

Dehairing/dewooling was traditionally achieved by rubbing a paste containing a mixture of lime and sodium sulfide onto the outer hide surface. The combination of alkaline conditions and sulfide quickly solubilizes the hair root proteins, discharging the hair from the hide surface. Subsequent alkaline treatment swells the hide. In addition to removing surface hair/wool, these processes also strip away most of the epidermal layer, exposing the corium (Figure 12.4) for further processing. Nowadays, the dehairing process includes the use of proteolytic enzymes (alkaline microbial proteases), which allows significant reductions in the quantities of (polluting) chemicals required. A synergistic effect between proteases and carbohydrases in promoting hair removal has been reported. This may be as a result of carbohydrase-mediated degradation of proteoglycans in the basal membrane area surrounding the hair root.

After dehairing, the hide is usually immersed in acid to reduce its pH to neutrality. The enzyme-mediated bating (softening) process then begins. The purpose of bating is to remove non-collagen proteins from the hide. These proteins include various glycoproteins, proteoglycans, some keratin and elastin. Their removal not only purifies the collagen network remaining, but also loosens it (yielding softer leather). Pancreatic proteases (mainly trypsin) in combination with microbial (*Bacillus* and *Aspergillus* derived) proteases find most application in the bating process, as they show least ability to degrade collagen while degrading the 'contaminant' proteins. Higher concentrations of enzymes are used if highly bated (i.e. very soft/pliable) leather is required. The bated leather is then ready for chemical treatments in the subsequent tanning process, which cross-links the collagen fibres and yields finished leather.

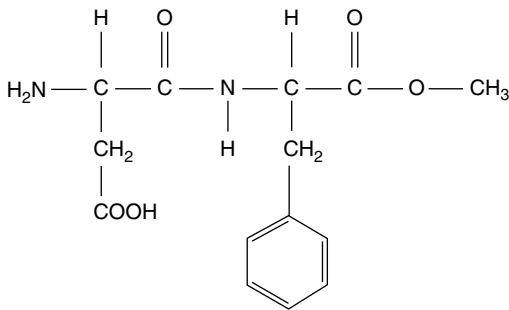


Figure 12.5 The structure of aspartame.

### 12.1.6 Synthesis of aspartame

Aspartame is a dipeptide consisting of L-aspartic acid linked via a peptide bond to the methyl ester of phenylalanine (Figure 12.5). It is approximately 200 times as sweet as table sugar (sucrose), and finds extensive use as a low-calorie sweetener. Its sweet taste depends on the L-configuration of the two amino acids, and it may be synthesized chemically or enzymatically. Chemical synthesis is costly due to the need to preserve amino acid stereospecificity. Enzymatic synthesis, which automatically preserves stereospecificity, has found favour. The neutral metalloprotease thermolysin (sourced from *Bacillus thermoproteolyticus*) is used in immobilized form. Under certain kinetically controlled conditions, the protease synthesizes peptide bonds rather than hydrolysing them.

### 12.1.7 Protease enzymes used in the brewing and baking industries

The cooling of beer after brewing often promotes haze formation. The haze is composed largely of protein, carbohydrate and polyphenolic compounds. Haze formation can be arrested by addition of proteolytic enzymes to the beer. Although various microbial enzymes have been assessed, plant-derived proteases such as papain and bromelain are most commonly used for such purposes.

Fungal proteases also enjoy limited application in the baking industry. Such enzymes, generally sourced from *Aspergillus*, are used in order to modify the protein components of flour and thus

alter the texture of the dough. Gluten represents a major protein fraction of flour. It is a complex between two protein types, gliadin and glutenin. When flour is wetted during dough preparation, gluten binds a portion of the water and expands to form a lattice-like structure. This promotes resistance to dough stretching. The addition of low levels of a neutral protease results in limited degradation of the gluten lattice, thereby reducing the dough's resistance to stretching. This better facilitates retention within the dough of CO<sub>2</sub> produced by yeast fermentation. In turn this influences pore structure of leavened bread and allows the dough to rise uniformly during baking.

### 12.1.8 Enzymatic conversion of protein waste

The food production and processing industries generate large quantities of waste protein. Such waste includes dead animals, and inedible portions of animals such as heads, feet, guts and feathers. Such substances are at best of marginal economic value and are often regarded simply as generating a waste disposal problem. Some waste material may be effectively recycled as food by what are termed rendering facilities. These facilities process or render dead animals and animal offal, usually by cooking at high pressure. This yields a valuable source of protein which can potentially be incorporated into livestock feed. The process also sterilizes the rendered material, thus preventing potential transmission of disease from microbial pathogens. However, the process may not inactivate all potential pathogens such as prions, and the advent of bovine spongiform encephalopathy (mad cow disease) has limited the application this technology to some extent, certainly in the context of rendering cattle-derived waste back into cattle feed.

The rendering process requires dedicated well-equipped facilities. High energy inputs are also required in the cooking process. An alternative method of converting such biological waste into a nutrient source involves the use of degradative enzymes. Such a biological process would require less sophisticated processing facilities and should function with lower energy costs. Enzymatic conversion of

waste is also more flexible than traditional rendering processes, and may expand the range of convertible waste products. Enzymatic digestion of poultry feathers represents one such example. Worldwide, several hundred million chickens are sacrificed each week. Typically, each bird has up to 125 g of feathers. Thus the weekly worldwide production of feather waste would be in the range of several thousand metric tons. Feathers are poorly digested by animals, mainly due to the weak ability of the latter to hydrolyse the highly ordered structure of the  $\alpha$ -keratins.

Individual  $\alpha$ -keratin polypeptides are densely packed and strongly stabilized by high levels of hydrogen bonds as well as hydrophobic interactions and disulfide linkages, rendering their enzymatic degradation challenging. However, a range of microorganisms producing keratinolytic enzymes have been identified (Table 12.7). In general, keratinases are monomeric extracellular enzymes of molecular mass 20–50 kDa. Most are neutral to alkaline proteases, displaying pH optima in the 7–9

range. Most are endo-acting, and most display broad substrate activity, usually degrading soluble proteins more effectively than keratins.

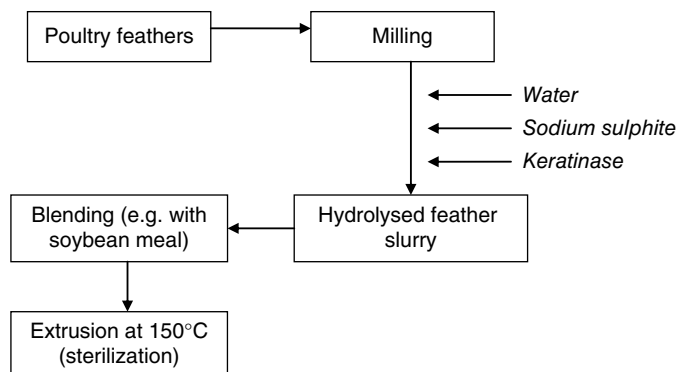
Microbial keratinolysis in nature seems to rely not only on the production of keratinases but also on:

- the breakage (reduction) of disulfide bonds by a variety of processes, including disulfide reductase enzymes or chemical reduction via the concurrent production of reducing agents;
- mechanical pressure on the substrate, via mycelial growth/penetration in the case of fungi.

Although not widely adopted in industry, as yet at least, various enzyme-based processes achieving the hydrolysis of poultry feather have been developed. The processes are variants of each other and rely on both chemical and enzymatic attack (Figure 12.6). Essentially, it entails blending a slurry of ground feathers with sodium sulfite (which chemically reduces the disulfide linkages) and keratinase. The

**Table 12.7** Some microorganisms that produce keratinases and selected biochemical properties of the keratinases they produce.

Producer microbe	Protease class	Mass (kDa)	Optimum temperature (°C)	Optimum pH
<i>Bacillus licheniformis</i> (MSK 103)	Serine protease	26	60–70	9–10
<i>Bacillus subtilis</i> (KD-N2)	Serine protease	30	55	8.5
<i>Streptomyces</i> (S7)	Serine protease	44	45	11
<i>Aspergillus oryzae</i>	Metalloprotease	60	50	8
<i>Trichoderma atroviride</i> (F6)	Serine protease	21	50–60	8–9



**Figure 12.6** Conversion of poultry feathers from waste to a source of amino acids.

enzyme activities hydrolyse the ground feather protein, yielding a peptide-rich product. The process is facilitated by elevated temperature in an alkaline environment; thus keratinases produced by some strains of *B. licheniformis* would likely be most suited to this approach.

The feather digest may be formulated with another protein source such as soybean meal in order to upgrade the overall nutritional value of the product ( $\alpha$ -keratins contain low levels of the essential amino acids methionine and phenylalanine). The resultant mixture can then be sterilized by heat in order to prevent potential transmission of pathogens. Feather digest products are used in the animal feed industry and are fed to both poultry and pigs. It has also been included in the diets of fish and domestic pets. An alternative approach could entail the direct incorporation of keratinases into animal feed directly supplemented with feather meal, and at least one such keratinase (trade name Versazyme) has been evaluated in feed trials for this application. The use of enzymes in animal feed is considered in Chapter 13.

Animal hair and wool consist almost entirely of  $\alpha$ -keratins. Thousands of tons of such material are deposited in landfill sites annually. Such waste products also represent potential substrates for enzymatic digestion. A process similar in design to that utilized in feather digestion could be used to convert such substrates into a potential nutrient source.

The fish processing industry also generates large quantities of waste byproducts that are proteinaceous in nature. Such products include not only inedible portions of fish, but also undersized or damaged whole fish. Such waste products may be enzymatically converted into protein hydrolysates of high nutritional value and fish hydrolysates are frequently incorporated into the diets of animals such as mink.

Partially degraded protein preparations also find use as additives in human food. Protein scraps can be recovered from the mechanical fleshing of beef, chicken, turkey or pig bones. Partial proteolytic hydrolysis of such scraps can yield a product containing a high meat extract flavour, which can be added to products such as soups, sauces and prepared meals. Partially hydrolysed vegetable

protein (e.g. soybean protein) also finds application as a food/flavour additive.

## 12.2 Carbohydrases

Polysaccharide-degrading enzymes represent one of the most significant groups of industrially important bulk enzymes. Such enzymes include amylases, cellulases and pectinases.

### 12.2.1 Amylases

Enzymes that participate in the hydrolytic degradation of starch are referred to as amylolytic enzymes or amylases and such enzymes have found widespread industrial application (Table 12.8). Specific enzymes classified within this group include  $\alpha$ -amylase,  $\beta$ -amylase, glucoamylase (also known as amyloglucosidase), pullulanase and isoamylase. Enzymatic degradation of starch yields glucose, maltose and other low-molecular-mass sugars. Furthermore, enzymatically mediated isomerization of glucose yields high-fructose syrups.

Abundant supplies of starch may be obtained from seeds and tubers, such as corn, wheat, rice, tapioca and potato. The widespread availability of starch from such inexpensive sources, coupled with

**Table 12.8** Major industrial applications of amylolytic enzymes.

Industry/process	Amylolytic enzymes employed
Production of glucose/maltose syrups	$\alpha$ -amylases, $\beta$ -amylase, debranching enzymes
Brewing/alcohol production	$\alpha$ -amylase, $\beta$ -amylase, amyloglucosidase
Animal feed additive	$\alpha$ -amylase
Baking industry	$\alpha$ -amylase, $\beta$ -amylase, amyloglucosidase, debranching enzymes
Laundry detergent additive	$\alpha$ -amylase
Production of dextrans	$\alpha$ -amylase
Fruit juice processing	$\alpha$ -amylase, amyloglucosidase
Textile disizing	$\alpha$ -amylase

large-scale production of amylolytic enzymes, facilitates production of syrups containing glucose, fructose or maltose, which are of considerable importance in the food and confectionery industry and are also a starting point for the production of first-generation bioethanol fuel.

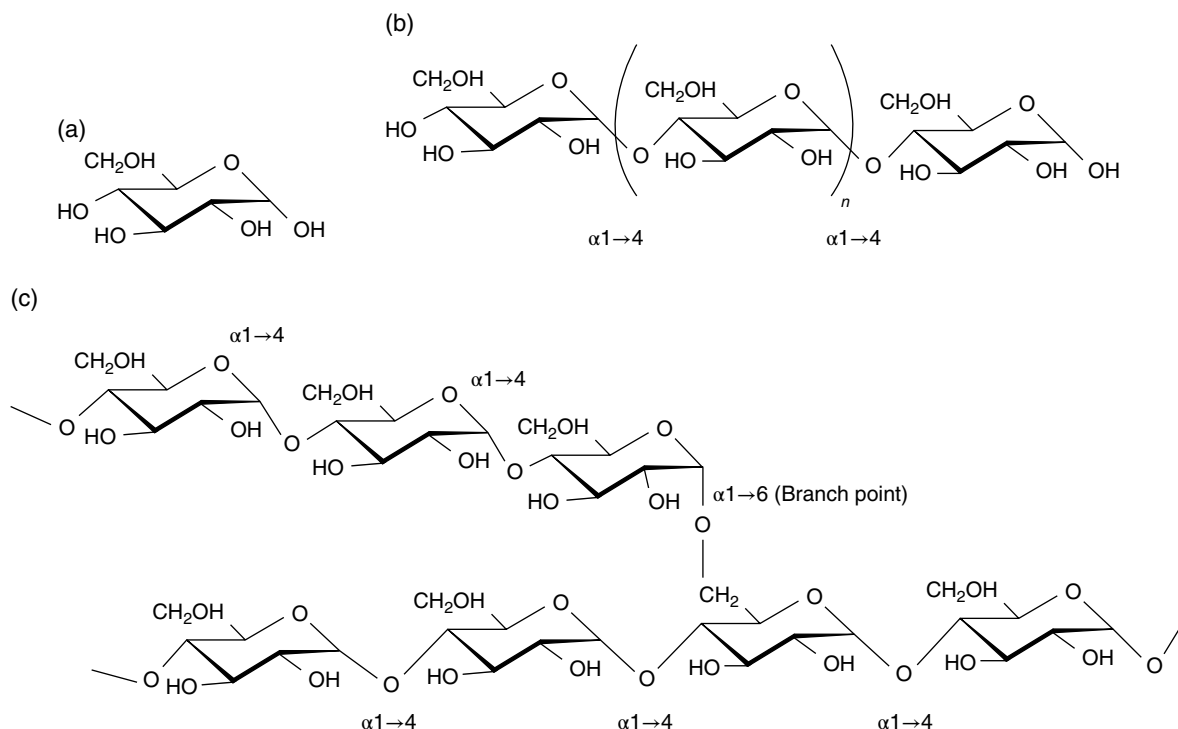
### 12.2.1.1 The starch substrate

Starch represents the most abundant form of polysaccharide stored in plants and next to cellulose is the most abundant polysaccharide on earth. As previously mentioned, it is especially abundant in seeds such as corn and in a variety of tubers. It is stored in granular form in the plant cell.

The starch polymer consists exclusively of glucose units. Two forms exist, namely  $\alpha$ -amylose and amylopectin (Figure 12.7).  $\alpha$ -Amylose is a long

linear polymer, in which successive D-glucose molecules are linked by an  $\alpha 1 \rightarrow 4$  glycosidic bond. Individual  $\alpha$ -amylose chains may vary in length and hence in molecular mass. The larger chains have molecular masses in the region of 500 kDa.

Amylopectin, on the other hand, is a highly branched molecule. Successive glucose residues are linked via  $\alpha 1 \rightarrow 4$  glycosidic linkages along the linear portion of the molecule, with branch points consisting of  $\alpha 1 \rightarrow 6$  glycosidic linkages. Such branch points generally occur every 25–30 glucose residues. Starch isolated from most plants consists of 70–80% amylopectin. In some cases, such as waxy rice, the starch granule consists exclusively of amylopectin (Table 12.9). Well over half of all starch utilized by humans is used in the manufacture of human food and animal feed. However, modified starches also find application in the textile, pharmaceutical, cosmetic, laundry, fuel and soap industries.



**Figure 12.7** Structures of (a)  $\alpha$ -D-glucose (b) segment of amylose chain (c) section of amylopectin. Glycosidic bonds between successive glucose residues link carbon atom no. 1 of one glucose residue to carbon atom no. 4 of the adjacent glucose residue. The bonds are in the  $\alpha$  configuration and hence are termed  $\alpha 1 \rightarrow 4$  glycosidic bonds. At branch points found in amylopectin (c), carbon atom no. 6 of the glucose residue in the main chain is linked to carbon no. 1 of the first glucose residue in the branch. This bond is thus termed an  $\alpha 1 \rightarrow 6$  glycosidic bond.

**Table 12.9** The relative percentage content of amylose and amylopectin in the starch derived from various plant sources.

Source	Amylose content (wt%)	Amylopectin content (wt%)
Corn		
Normal	28	72
Waxy	0	100
High amylose	65–85	15–35
Sorghum	28	72
Tapioca	16	84
Arrowroot	21	79
Sago	26	74
Potato	20	80
Wheat	30	70
Rice		
Normal	20–30	70–80
Waxy	0	100

Starch may be hydrolysed by chemical or enzymatic means. Chemical hydrolysis was used formerly and involves heating in the presence of acid. However, this method has been very largely superseded by the use of specific enzymes. Enzymatic hydrolysis generates fewer byproducts and produces higher yields of end product compared with the chemical method.

### 12.2.1.2 $\alpha$ -Amylase

The initial step in starch hydrolysis entails disruption of the starch granule. Solubilization of the granules, the process of 'gelatinization', facilitates subsequent catalytic degradation. Gelatinization is normally achieved by heating the starch slurry to temperatures in excess of 100°C for several minutes.  $\alpha$ -Amylase may be added immediately prior to the heating step, in order to render more efficient the process of granule disruption. Once the granules have been disrupted, additional  $\alpha$ -amylase is added in order to liquefy the starch. This process reduces the viscosity of the starch solution.

$\alpha$ -Amylase activity is widely distributed in nature. The enzyme may be isolated from microbial

sources and from animal and plant tissues.  $\alpha$ -Amylase is an endo-acting enzyme, catalysing the random hydrolysis of internal  $\alpha 1 \rightarrow 4$  glycosidic linkages present in the starch substrate. These enzymes are incapable of hydrolysing  $\alpha 1 \rightarrow 6$  glycosidic linkages present at branch points of amylopectin chains. One exception to this is the  $\alpha$ -amylase produced by *Thermoactinomyces vulgaris*, which can hydrolyse both  $\alpha 1 \rightarrow 6$  and  $\alpha 1 \rightarrow 4$  glycosidic linkages. These enzymes can also generally catalyse the cleavage of internal  $\alpha 1 \rightarrow 4$  glycosidic bonds in glycogen and a variety of additional oligosaccharides.

Structurally,  $\alpha$ -amylases are invariably single-chain polypeptides folded into three domains, A, B and C. The N-terminal A domain is the catalytic domain, which consists of eight parallel stretches of  $\beta$  strands arranged in a barrel, encircled by eight stretches of  $\alpha$ -helix, i.e. a  $(\beta/\alpha)_8$  barrel, also known as a TIM barrel. The B domain varies in structural detail between various amylases and plays a role in substrate binding. The C domain is found towards the C terminus of the molecule. Almost all  $\alpha$ -amylases house one or more characteristic calcium ions, which are generally located at the interface between domains A and B. Although usually essential to activity, the calcium is not located at the active site and thus does not participate directly in catalytic transformation. These ions appear to play a structural stabilizing role. Some amylase enzymes contain additional domains, D and E. While the function of domain D remains unknown, domain E represents a starch-binding domain. Although present in only some 10% of amylases, there is significant interest in this domain as it facilitates enzyme binding to raw (i.e. unsolubilized) starch, with subsequent granule degradation. Mammalian  $\alpha$ -amylases also contain disulfide linkages, although bacterial amylases are generally devoid of such bonds.

Two of the  $\alpha$ -amylases traditionally applied at an industrial level are derived from *B. amyloliquefaciens* and *B. licheniformis*. *Bacillus* amylases exhibit a pH optimum in or around neutrality, and are stabilized by the presence of calcium ions.  $\alpha$ -Amylase produced by *B. licheniformis* is particularly suited to industrial applications due to its thermal stability. This enzyme

consists of 483 amino acids and has a molecular mass of 55.2kDa. Its pH optimum is 6.0 and its temperature optimum is 90°C. Most other  $\alpha$ -amylases, including those produced by *B. amyloliquefaciens*, are rapidly inactivated at temperatures in excess of 60°C.

The advent of recombinant DNA technology has facilitated the cloning and expression of genes coding for various  $\alpha$ -amylases in a variety of recombinant organisms, and the majority of new  $\alpha$ -amylases now coming on the market are produced in this way. One such example (Termamyl® SC) is considered in

Box 12.3.  $\alpha$ -Amylase activities are also produced by a variety of fungal species. Fungal  $\alpha$ -amylases most commonly used industrially are produced by species of *Aspergillus*, most notably *A. oryzae* (Table 12.10).

### 12.2.1.3 Glucoamylase

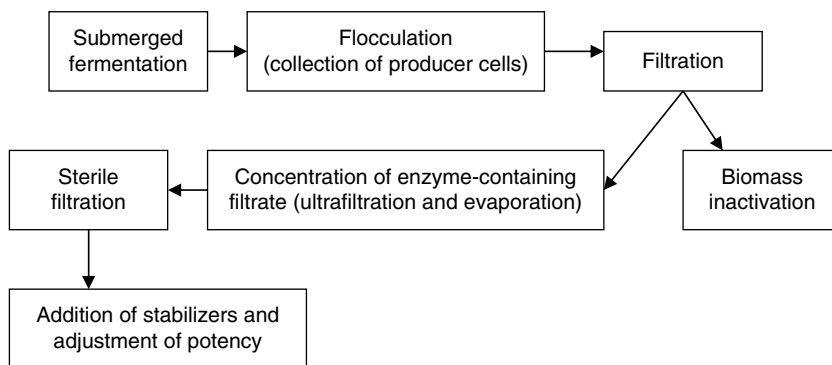
Glucoamylase, also known as amyloglucosidase, catalyses the sequential hydrolysis of  $\alpha 1 \rightarrow 4$  glycosidic bonds from the non-reducing end of the starch molecule. The enzyme also catalyses the hydrolysis

#### Box 12.3 Product case study: Termamyl® SC

Termamyl® SC is the trade name given to a recombinant  $\alpha$ -amylase preparation commercialized by Novozymes. It is applied in the starch processing industry in order to liquefy (degrade) the starch substrate, as outlined in the main text. The production organism is a well-characterized *Bacillus licheniformis* strain that is sporulation deficient and alkaline protease negative. It carries a modified gene coding for  $\alpha$ -amylase originally derived from a specific *Bacillus stearothermophilus* strain. Engineering of the *B. stearothermophilus* gene was undertaken by site-directed mutagenesis, and involved deleting two amino acids (Ile<sup>181</sup> and Gly<sup>182</sup>), as well as substituting Asn<sup>193</sup> with a phenylalanine. These changes increased the stability of the enzyme at low Ca<sup>2+</sup> concentrations and at low pH, with some consequent processing advantages.

Product manufacture is via submerged fermentation, and is overviewed schematically below.

Each production batch is initiated using a lyophilized stock culture of the production microorganism. The fermentation medium contains starch and/or starch hydrolysate as the major carbon source, as well as potato protein, corn steep liquor and soy as nitrogen sources, and various minerals (phosphates and sulfates). After fermentation the enzyme is recovered from the spent culture broth by a pH adjustment step and the addition of flocculation agents, followed by a filtration step. The enzyme-containing extracellular media is then concentrated by a combination of ultrafiltration and evaporation. This is followed by a final filtration step designed to ensure that no production microorganisms are present in the final product. The enzyme concentrate is pH adjusted and diluted as necessary to achieve final product activity and pH specifications, with sodium chloride and sucrose also being added to a final concentration of 14% and 30%, respectively.

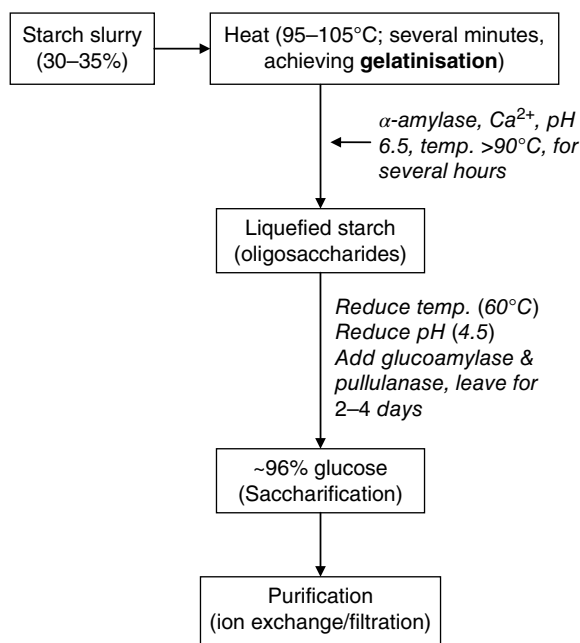


**Table 12.10** Sources and characteristics of some industrially significant amylolytic enzymes.

Enzyme	Endo- or exo-acting	Glycosidic bond cleaved	Source	pH optimum	Temperature optimum (°C)
$\alpha$ -Amylase	Endo	$\alpha 1 \rightarrow 4$	<i>Bacillus subtilis</i>	6.0	65
			<i>Bacillus licheniformis</i>	6.0	90
			<i>Bacillus amyloliquefaciens</i>	5.5	60
			<i>Aspergillus oryzae</i>	4.5	60
Amyloglucosidase (glucoamylase)	Exo	$\alpha 1 \rightarrow 4$	<i>Aspergillus niger</i>	4.5	60
$\beta$ -Amylase	Exo	$\alpha 1 \rightarrow 4$	<i>Bacillus</i> sp.	5.0	60
			<i>Clostridium</i> sp.	5.5	80
Pullulanase	Endo	$\alpha 1 \rightarrow 6$	<i>Klebsiella aerogenes</i>	5.0	60
Isoamylase	Endo	$\alpha 1 \rightarrow 6$	<i>Pseudomonas</i> sp.	4.0	55

of  $\alpha 1 \rightarrow 6$  glycosidic bonds present at branch points in amylopectin, although at a much slower rate. The enzyme is produced extracellularly, in particular by a variety of fungal species, most notably members of *Aspergillus* and *Rhizopus*. The fungal enzymes are invariably glycosylated, exhibiting varying degrees of O- and/or N-linked sugar side chains. Indeed one glucoamylase isolated from a specific strain of *S. cerevisiae* is noteworthy in that its glyco-component accounts for 80% of its total mass. Glucoamylases find industrial application in starch processing, after the liquefaction of the starch by bacterial  $\alpha$ -amylase in order to produce glucose syrup. This process is described as saccharification of starch. The overall scheme involved in the production of glucose syrups from starch is summarized in Figure 12.8.

The enzyme produced by *Aspergillus niger* is the most widely used industrially, and these fungal enzymes are traditionally manufactured at process-scale by fermentation using a production medium of 20% corn and 2.5% corn steep liquor at 30–35°C. More recently, recombinant production of such enzymes has come to the fore (Box 12.4.). Amyloglucosidases from most traditional sources are relatively thermolabile enzymes, typically being unstable at temperatures in excess of 60°C. Thus the temperature of liquefied starch must be adjusted downwards before addition of the saccharifying enzyme. pH adjustment to more acidic values is also required to ensure optimal enzyme activity.



**Figure 12.8** Controlled hydrolysis of starch, yielding up to a 96% glucose syrup. The various steps and conditions used are illustrated diagrammatically. Pullulanase may be added in addition to glucoamylase during the saccharification process. This enzyme hydrolyses  $\alpha 1 \rightarrow 6$  glycosidic bonds. The glucose syrup produced by this method is often used directly by the food industry, as a substrate for first-generation bioethanol production via fermentation, and for the production of crystalline glucose used for various pharmaceutical purposes. A further application entails conversion of some of the glucose to fructose by the enzyme glucose isomerase, thus producing high-fructose syrup, which again is used in the food industry.



**Box 12.4 Product case study: Distillase®/Diazyme®**

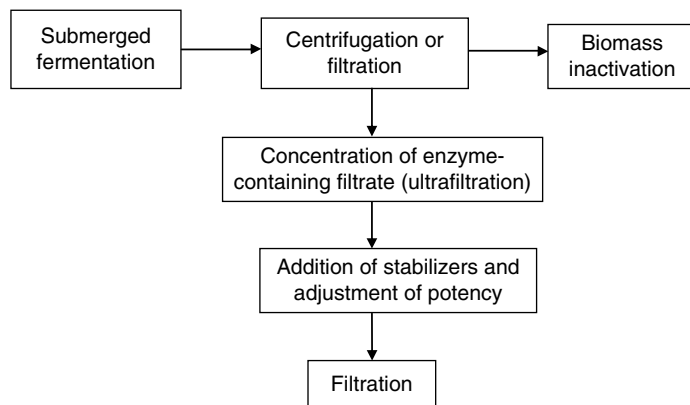
Distillase®, also marketed as Diazyme®, is a recombinant glucoamylase preparation commercialized by DuPont. The enzyme, which catalyses the hydrolysis of terminal 1 → 4 α-D-glucose linkages from the non-reducing end of starch molecules, is used in the starch processing industry for the ultimate production of glucose, fructose, ethanol or organic acids such as lactic acid.

The production organism is a well-characterized strain of *Trichoderma reesei*, which is genetically modified by inactivation of several cellulase genes. It is originally derived from a *T. reesei* strain (RL-P37), a cellulase overproducer, itself derived via classical mutagenesis techniques from a wild-type *T. reesei* (strain QM6a) which is available in several public culture collections such as ATCC (American Type Culture Collection).

Inactivation of the producer strain's cellulases prevents unnecessary cellulase production and

also facilitates the use of a powerful cellulase (cellobiohydrolase; see section 12.2.2.3) promoter to drive high-level expression of the inserted glucoamylase gene, which is derived from a different *T. reesei* strain.

Product manufacture is via submerged fermentation, and is overviewed schematically below. Each production batch is initiated using a lyophilized stock culture of the production microorganism. After fermentation the extracellular enzyme is recovered from the spent culture broth using a centrifugation or filtration step. The enzyme solution is then concentrated by an ultrafiltration step with subsequent addition of glucose and potassium sorbate as stabilizers and sodium benzoate as a preservative, followed by pH adjustment to 4.0–4.5. Finally the product is filtered to ensure total removal of the producer microorganism.



Over the past several years a number of glucoamylases have been identified and produced by recombinant and/or traditional means, from a variety of aerobic and anaerobic microbial species. Most are maximally active at acidic pH and a few display an optimum activity temperature above 60°C (Table 12.11).

A glucoamylase displaying a higher optimum activity temperature, particularly in combination

with optimal activity at more neutral pH, would likely be of industrial interest. Attempts to identify and produce new glucoamylases of industrial interest entail:

- bioinformatic interrogation of the genomes and/or direct screening of natural thermo(acido)philes;
- protein engineering of mesophile-derived glucoamylases in order to improve their thermal stability/optimum activity.

**Table 12.11** Optimum temperature and pH for glucoamylases isolated from the indicated sources.

Source	Optimum temperature (°C)	Optimum pH
<i>Aspergillus awamori</i>	60	4.5
<i>Aspergillus oryzae</i>	60	4.5
<i>Aspergillus terreus</i>	60	4.5
<i>Cephalosporium charticola</i>	60	5.5
<i>Corticium rolfsii</i>	50	4.5
<i>Clostridium thermosaccharolyticum</i>	70	5.0
<i>Humicola lanuginosa</i>	65	4.9
<i>Lactobacillus amylovorus</i>	45	6.0
<i>Mucor rouxianus</i>	55	4.6
<i>Paecilomyces variotii</i>	55	5.0
<i>Penicillium oxalicum</i>	60	5.0
<i>Picrophilus torridus</i>	90	2.0
<i>Rhizopus delemar</i>	40	4.5
<i>Thermoplasma acidophilum</i>	90	2.0

#### 12.2.1.4 $\beta$ -Amylase

In contrast to  $\alpha$ -amylase,  $\beta$ -amylases are exo-acting enzymes catalysing the sequential hydrolysis of alternate  $\alpha 1 \rightarrow 4$  glycosidic linkages present in starch, from its non-reducing end. This reaction produces maltose units with inversion to the  $\beta$  form (Figure 12.9). As is the case for  $\alpha$ -amylases,  $\beta$ -amylases are incapable of hydrolysing  $\alpha 1 \rightarrow 6$  linkages. Conversion of amylopectin to maltose by  $\beta$ -amylase is therefore limited by branch points. The amylose molecule, on the other hand, being devoid of such branch points, may be fully degraded by  $\beta$ -amylases. Overall, hydrolysis of most starches by  $\beta$ -amylase yields a product mix of maltose and larger oligosaccharides, often termed  $\beta$ -limit dextrans.

$\beta$ -Amylase is produced by many higher plants. Molecular masses vary from about 60 kDa (cereal  $\beta$ -amylase) to 150 kDa (sweet potato  $\beta$ -amylase).

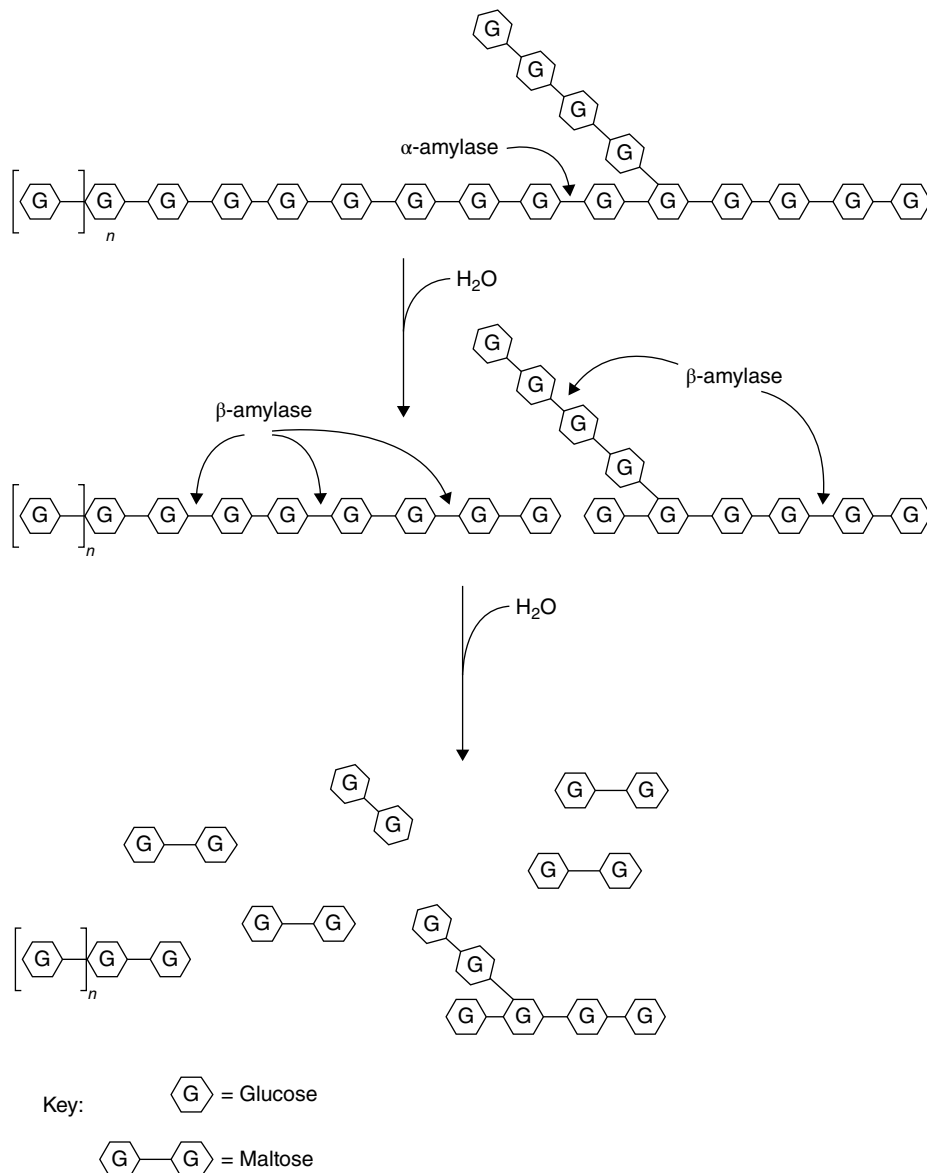
Plant-derived  $\beta$ -amylases do not require metal ions for activity, are generally maximally active at pH values slightly below neutrality, and are inactivated when incubated at temperatures above 60°C for any length of time. Plant  $\beta$ -amylases used commercially are obtained from barley or, in some world regions, soybeans. Hydrolysis of starch using  $\alpha$ -amylase and these  $\beta$ -amylases yields a syrup typically consisting of 2–8% glucose, 40–60% maltose, 10–25% maltotriose and lower levels of  $\beta$ -limit dextrans.

$\beta$ -Amylase is also produced by a range of microorganisms, most notably *Bacillus*, as well as various species of *Pseudomonas* and *Streptomyces*. Those from *Bacillus* have gained greatest commercial use and again are optimally active in the 55–60°C range. A more thermostable  $\beta$ -amylase would be of industrial interest and at least one such enzyme has been reported. *Clostridium thermosulfurogenes*  $\beta$ -amylase retains considerable activity up to temperatures of 85°C.

#### 12.2.1.5 $\alpha 1 \rightarrow 6$ Glucosidases

$\alpha 1 \rightarrow 6$  Glucosidases represent a group of amylolytic enzymes that are capable of hydrolysing the  $\alpha 1 \rightarrow 6$  linkages present at branch points in amylopectin. Amyloglucosidase is one such  $\alpha 1 \rightarrow 6$  glucosidase. Such debranching enzymes play a central role in the complete degradation of starch as neither  $\alpha$ - nor  $\beta$ -amylases possess the catalytic ability to hydrolyse the  $\alpha 1 \rightarrow 6$  bonds of amylopectin. Several additional  $\alpha 1 \rightarrow 6$  glycosidases hydrolyse branch-point linkages much more efficiently and rapidly than do amyloglucosidase. The most important such enzymes are pullulanase and isoamylase. These enzymes are often utilized to aid the saccharification process outlined in Figure 12.8.

Although both pullulanase and isoamylase cleave the  $\alpha 1 \rightarrow 6$  linkages of amylopectin, they may be differentiated by their ability to degrade the polysaccharide pullulan. Pullulanase degrades pullulan, whereas isoamylase does not. Pullulan is a linear polysaccharide consisting of up to 1500 glucose molecules. The basic recurring structure consists of three glucose residues linked via two  $\alpha 1 \rightarrow 4$  glycosidic linkages. Each such maltotriosyl unit is

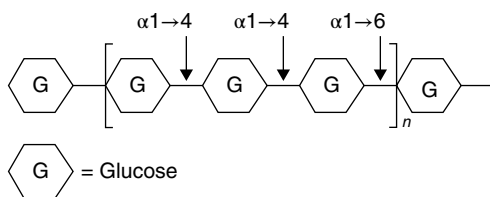


**Figure 12.9** Hydrolysis of starch (in this example amylopectin) by  $\alpha$ -amylase and  $\beta$ -amylase.  $\alpha$ -Amylase catalyses the random hydrolysis of internal  $\alpha$ 1  $\rightarrow$  4 glycosidic linkages. It is incapable of cleaving  $\alpha$ 1  $\rightarrow$  6 linkages.  $\beta$ -Amylase catalyses the sequential removal of maltose units from the non-reducing end of the starch molecule. It too fails to hydrolyse  $\alpha$ 1  $\rightarrow$  6 glycosidic linkages found at branch points.

linked to the next via an  $\alpha$ 1  $\rightarrow$  6 bond, as shown in Figure 12.10.

Pullulanase was first discovered in a species of *Aerobacter* in the early 1960s. It is produced by a variety of bacteria including some bacilli and species

of streptococci. The pullulanases used commercially have been largely sourced from *Klebsiella aerogenes* and *Bacillus subtilis*. More recently, recombinant pullulanases have become available, as exemplified by Novozymes recombinant pullulanase preparation



**Figure 12.10** Structure of pullulan. Refer to text for specific details.

produced by *B. subtilis* expressing the gene encoding a pullulanase from *Bacillus acidopullulyticus* (Box 12.5).

Isoamylase, produced by a number of microbial species, was initially isolated from yeast. This enzyme is also synthesized by a variety of bacteria, including some bacilli. Extracellular isoamylase produced in large quantities by a particular mutant

### Box 12.5 Product case study: Novozym® 26062

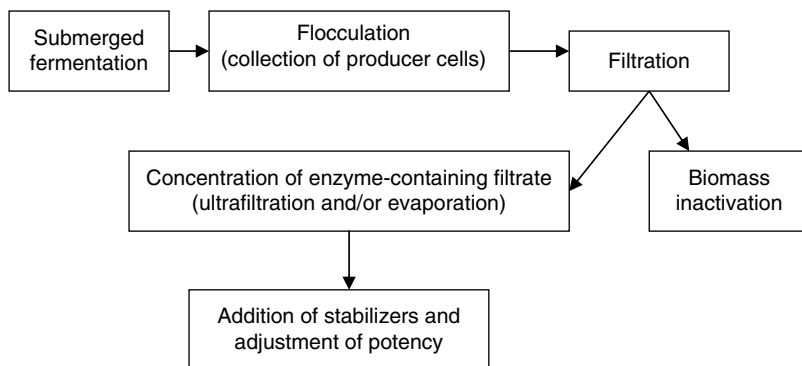
Novozyym® 26062 is a recombinant pullulanase preparation developed by Novozymes. The enzyme, which catalyses the hydrolysis of 1 → 6 α-glucosidic linkages in amylopectin, is used in the starch processing industry as a debranching enzyme after liquefaction. The production organism is a well-characterized strain of *Bacillus subtilis* (A164) which, in addition to housing a recipient pullulanase gene, is genetically modified by the deletion of five genes:

- *spollAC* gene, which encodes a sporulating factor;
- *nprE* gene, which encodes an extracellular neutral metalloprotease (reduces protease production);
- *aprE* gene, which encodes the *B. subtilis* extracellular serine protease subtilisin;
- *amyE* gene, which encodes extracellular α-amylase;

- *srfC* gene, which encodes the peptide antibiotic surfactin.

The production organism is thus engineered to express little or no extracellular amylase or protease activity, no surfactin antibiotic and to be unable to sporulate. These modifications result in more efficient pullulanase production to higher yield and/or enhance the microbe's GRAS status.

The gene-deleted strain also houses a gene coding for pullulanase derived from *B. acidopullulyticus*, under the control of a powerful promoter system that facilitates high-level product expression. Batch manufacture of product is outlined schematically below. Final product formulation entails the addition of sucrose and glucose (to a final concentration of 40%), as well as the preservatives sodium benzoate and potassium sorbate (to final concentrations of 0.1% and 0.3%, respectively).



strain of *Pseudomonas amyloclavata* enjoys wide-spread industrial application.

More recently, a number of pullulanases exhibiting novel activities have been isolated from several thermophilic organisms. Such producer microorganisms include a variety of *Clostridium* and a number of strains of *Thermoanaerobium* and *Thermus*. Some of these enzymes have been produced as heterologous protein products in recombinant systems such as *E. coli* and *B. subtilis*. Most exhibit excellent thermal stability and remain active for prolonged periods at temperatures in excess of 90°C. Perhaps the single most striking attribute of many such novel pullulanases, sometimes termed amylopullulanases, is their ability to hydrolyse  $\alpha 1 \rightarrow 6$  linkages in some carbohydrates such as pullulan and  $\alpha 1 \rightarrow 4$  linkages in others such as starch. Conventional pullulanase fails to hydrolyse the  $\alpha 1 \rightarrow 4$  glycosidic linkages of either pullulan or amylose. Purified pullulanase from *Thermoanaerobium brockii*, for example, hydrolyses only  $\alpha 1 \rightarrow 6$  glycosidic bonds in pullulanase but exhibits an almost exclusive preference for  $\alpha 1 \rightarrow 4$  bonds in starch.

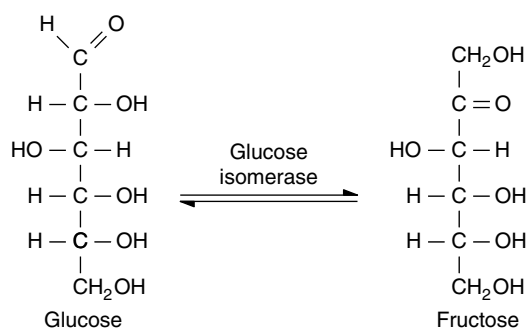
### 12.2.1.6 Glucose isomerase

The enzymatic hydrolysis of large quantities of relatively inexpensive, readily available starch facilitates the economic production of large quantities of glucose syrup. Although glucose syrups may be used directly for food application, many are first converted into high-fructose syrups. The conversion of glucose into fructose is desirable in so far as fructose is substantially sweeter than glucose (Table 12.12) and is therefore more attractive industrially when utilized as a sweetener in confectionery, ice cream and soft drinks.

Glucose may be converted into fructose by chemical or enzymatic isomerization (Figure 12.11). Chemical conversion relies on the use of alkali at high temperatures. Low yields and undesirable side reactions (e.g. production of non-metabolizable sugars such as psicose as well as colour and some off-flavour sugar derivatives) limit the applicability of this particular method. The enzyme glucose isomerase catalyses the required isomerization

**Table 12.12** The relative sweetness of a number of commonly occurring sugars of industrial importance. For comparative purposes, sucrose has been assigned a relative sweetness of 100%.

Sugar	Relative sweetness (%)
Sucrose	100
Glucose	70
Fructose	130
Maltose	40



**Figure 12.11** The isomerization of D-glucose, an aldohexose, forming D-fructose, a ketohexose.

reaction at ambient temperatures and at near neutral pH values, yielding a syrup containing about 42% fructose. Enzymatic isomerization of glucose to fructose was first undertaken on an industrial scale in the late 1960s in the USA. Recently developed process refinements now allow production of syrups of even higher fructose content.

Glucose isomerase obtained from a wide variety of microbial species have found industrial application in the production of high-fructose syrups. Bacterial species from which this enzyme may be obtained include a variety of *Aerobacter*, *Bacillus* and organisms such as *Streptomyces albus*, *Lactobacillus brevis* and *Actinoplanes missouriensis*. The main sources of commercially available glucose isomerase are *A. missouriensis*, *Bacillus coagulans* and various species of *Streptomyces*.

Glucose isomerases from these and many other (mainly microbial) sources have been purified and characterized. From such studies, the following generalizations can be made. Glucose isomerases are usually capable of isomerizing several sugars: the most frequent isomerizations catalysed are the interconversion of D-glucose and D-fructose and the interconversion of D-xylose and D-xylulose. However, the enzyme from various sources also exhibits the ability to isomerize D-ribose, D-allose, L-arabinose and L-rhamnose. Glucose isomerases generally require the presence of a divalent cation (e.g.  $Mg^{2+}$ ,  $Co^{2+}$  or  $Mn^{2+}$ ) for maximum activity. Molecular masses reported range from 50 to 190 kDa and active glucose isomerases tend to be homodimers or homotetramers, with individual subunits being held together by non-covalent interactions. Optimum temperatures of microbial glucose isomerases tend to range from 60 to 80°C, while optimum pH values range between 6 and 9.

Glucose isomerase, as produced by most microorganisms, is an intracellular enzyme. Isolation of the enzyme thus requires disruption of the producer cells. Its intracellular location renders the production of purified glucose isomerase more technically and economically demanding than production and isolation of extracellular enzymes.

Many of the initial studies designed to investigate the industrial potential of glucose isomerization used soluble enzymes. However, most industrial-scale isomerization systems now utilize an immobilized form of the enzyme. On an economic level this is quite significant, as it facilitates reuse of the enzyme (see Chapter 11).

As with most other enzymes of commercial interest, a range of glucose isomerases have been produced by recombinant means in both homologous and heterologous systems. Protein engineering studies have been undertaken, with site-directed mutagenesis studies aiming to increase the enzyme's thermal stability, lower its pH optimum or alter its substrate specificity. Several studies aimed at increasing the enzyme's thermal stability concentrated on altering surface amino acid residues in order to strengthen non-covalent interactions between individual enzyme subunits (thereby holding them together at higher

temperatures). Results obtained were mixed, with the introduction of disulfide linkages and additional salt bridges having no effect, although alterations of surface hydrophobic amino acid residues did enhance thermostability in at least one case.

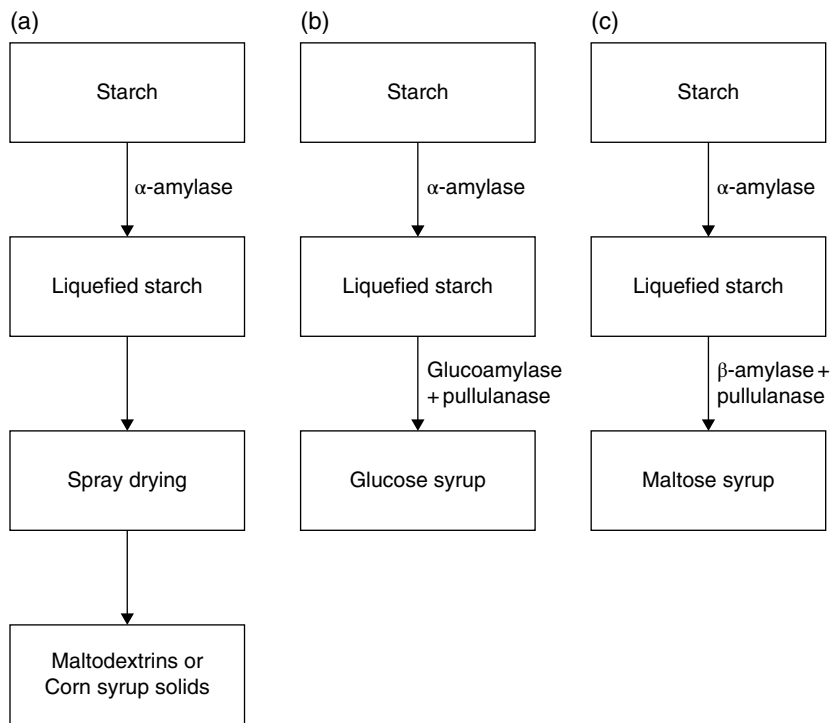
#### 12.2.1.7 Industrial importance of starch conversion

Starch may be enzymatically hydrolysed using varying combinations of amylolytic enzymes in order to generate specific end products. The major end products produced commercially are maltodextrins/corn syrup solids, maltose syrup, glucose syrup or high-fructose corn syrup (Figure 12.12), as well as cyclodextrins (discussed in Chapter 13).

Maltodextrins are produced by partial enzymatic hydrolysis of starch using  $\alpha$ -amylase (Figure 12.12). The product is often defined as a non-sweet nutritive saccharide mix, largely containing glucose units linked primarily by  $\alpha 1 \rightarrow 4$  glycosidic linkages. Although the exact product composition can vary, it generally consists of glucose (~1%), maltose (3–5%), maltotriose (5–10%), maltotetraose (6%), with the remainder (75% or more) being saccharides of higher molecular mass.

When the desired degree of starch hydrolysis is attained by  $\alpha$ -amylase, the pH value of the slurry is dropped from 6.5 to 3.0 and the slurry is then heated to boiling for about 5 minutes in order to totally inactivate the  $\alpha$ -amylase. Filtration and passage through a carbon column removes any particulates and colour, and the resultant purified maltodextrin mix is evaporated and subsequently spray-dried to yield a powdered product.

Maltodextrins display a number of useful functional properties, including low hygroscopicity, a bland non-sweet flavour and the ability to retard ice crystal growth in ice creams and related frozen products. They are used as an ingredient in soft sweets, where they contribute to viscosity and chewiness. They are also added to many harder sweets, helping to maintain their moisture levels and extending their shelf-life. Higher-molecular-mass maltodextrin preparations (i.e. where 95% of the product consists of pentasaccharides and higher



**Figure 12.12** Hydrolytic degradation of starch, yielding industrially important end products. In (a) the use of  $\alpha$ -amylase alone facilitates the production of starch fragments, sold as maltodextrins or corn syrup solids. In (b) a combination of  $\alpha$ -amylase, glucoamylase and pullulanase yields a glucose-rich syrup, whereas in (c) a combination of  $\alpha$ -amylase,  $\beta$ -amylase and pullulanase yields a maltose syrup. Process parameters such as adjustments of pH and temperature, and addition of stabilizers have been omitted for clarity.

saccharides) can be used as a fat replacement in some low-fat foods. It appears that such maltodextrins can provide a fat-like texture while containing less than half the calories of fat on a weight basis. In pharmaceutical manufacturing, maltodextrins are used as binding agents in tablet manufacture and as coating agents for some tablets/capsules. Corn syrup solids are similar to maltodextrins, except they are more extensively hydrolysed. In general, they have similar applications to those of maltodextrin products.

Glucose syrups are used to produce crystalline glucose, as a raw material for the production of various organic acids, ethanol and other chemicals, and as a raw material for the production of high-fructose corn syrup. High-fructose syrups are as sweet as sucrose (or sweeter depending on the exact fructose content). On the basis of its sweetening power, high-fructose corn syrup can be 10–20% cheaper

than sucrose. As a result, it has largely replaced sucrose as a food ingredient in many world regions, particularly in the USA. High-fructose corn syrups find application as a sweetening ingredient in cakes, confectionery and soft drinks, canned foods, jams, jelly and ketchup. Maltose syrups are characterized by low viscosity and hygroscopicity, good heat stability and mild sweetness. They are used as ingredients in various foods, confectionery, soft drinks and in ice cream where they help control ice crystal formation. Maltose syrups are also sometimes used medically in the intravenous feeding of diabetics.

Starch-degrading enzymes are also utilized in the production of alcoholic beverages and in breadmaking. The production of alcoholic beverages by brewing relies on the ability of yeast to ferment carbohydrates present in the malted barley and other added sugars. Wild-type yeast traditionally

used in brewing do not possess the enzymatic ability to degrade starch, as they utilize only glucose or other simple monosaccharides/disaccharides as substrates for growth. Germination of the grain is thus promoted prior to the fermentation step. The germinating seeds produce endogenous enzymes capable of hydrolysing not only the stored starch but also cellulose and other structural polysaccharide components of the seed. Germination is subsequently arrested by controlled heat, in order to prevent further seed growth. The seed now contains enzymes such as  $\alpha$ - and  $\beta$ -amylases that are capable of hydrolysing stored starch, thus producing glucose and other sugars which the yeast cells are capable of fermenting. This process is called malting. This traditional process, by which the seeds are induced to produce amylolytic enzymes, may now be supplemented or replaced by the addition of exogenous amylolytic enzymes obtained from microbial sources.

The level of  $\beta$ -amylase present in cereals remains relatively constant but the content of  $\alpha$ -amylase is low prior to germination. Milled flour therefore often contains low concentrations of  $\alpha$ -amylase. Supplementation of such flour with fungal  $\alpha$ -amylase results in more effective degradation of flour starch, hence rendering the dough easier to work and allowing yeast fermentation to proceed. This in turn promotes leavening, which increases loaf volume and enhances bread texture.

#### 12.2.1.8 $\alpha$ -Amylase: detergent applications

Carbohydrate-based food stains constitute one of the most common causes of soiled clothing. Foodstuffs such as potatoes, oatmeal, gravy, spaghetti, chocolate, puddings and baby food contain a high proportion of starch/modified starch. Satisfactory removal of starch-based stains can be achieved at higher washing temperatures, but the addition of  $\alpha$ -amylase to detergent preparations allows starch removal under less aggressive washing conditions.

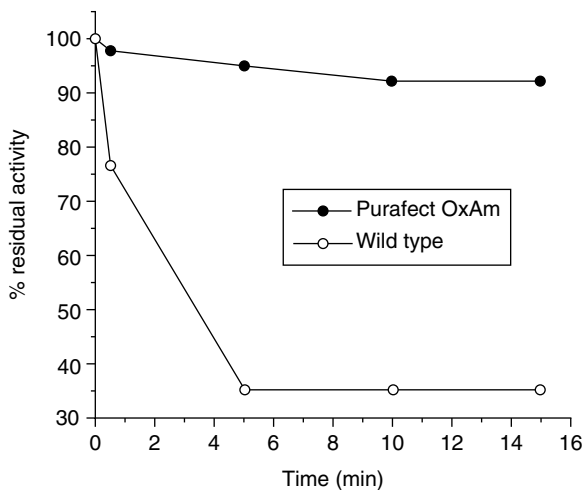
Amylases were first introduced into detergents in the early 1970s, although this practice only gained widespread favour in the 1980s. By the mid-1990s, well over 90% of laundry detergents and a growing

percentage of dishwashing detergents contained  $\alpha$ -amylase. Most traditional detergent amylases are produced by fermentation of *B. subtilis*, *B. amyloliquifaciens* or *B. licheniformis*. The  $\alpha$ -amylase from *B. licheniformis* has gained most widespread use, because of its thermal stability, resistance to proteolytic degradation and good wash performance. It is available commercially under trade names such as Maxamyl (Danisco) and Thermamyl (Novozymes). These (and other) traditional detergent amylase preparations were initially developed for use in starch processing as opposed to being developed specifically for detergent use. These  $\alpha$ -amylases display acceptable storage stability in detergents devoid of bleaching agents. However, most such amylase preparations lose significant activity in bleach-containing detergents (typically greater than 50% loss of activity is witnessed after storage in such detergents for 6 months).

The three-dimensional structure of several microbial  $\alpha$ -amylases has been elucidated and several of these enzymes have been subject to protein engineering studies. As in the case of detergent proteases, replacement of oxidation-sensitive amino acid residues in  $\alpha$ -amylases should (and does) render them more bleach stable. In the case of *B. licheniformis*  $\alpha$ -amylase, site-directed mutagenesis indicated that replacement of Met<sup>197</sup> with a non-oxidizable amino acid results in the production of an oxidation-resistant product (Figure 12.13). Two such engineered products, Purafect OxAm (Genencor Int.) and Duramyl (Novozymes), were introduced to the market in the mid-1990s.

Additional protein engineering experiments sought to develop a more thermostable  $\alpha$ -amylase with improved stability in the presence of low calcium ion concentrations. Calcium ions serve as cofactors for most bacterial  $\alpha$ -amylases, enhancing their thermal stability and increasing resistance to proteolytic degradation. Site-directed mutagenesis was used to introduce additional negatively charged amino acid residues adjacent to the enzyme's calcium-binding site. The rationale was to generate an enzyme with increased affinity for calcium ions. Several such modified enzymes did show increased thermostability, even at low calcium concentrations. These may prove useful in the starch





**Figure 12.13** Stability of wild-type *Bacillus licheniformis*  $\alpha$ -amylase, and its engineered form Purafect OxAm in the presence of peracetic acid, the active component of a common detergent bleach. Reproduced with permission from *Enzymes in Detergency* (eds J.H. van Ee, O. Misset and E.J. Baas), 1997, p. 223, Marcel Dekker Inc., New York.

processing industry and as detergent amylases. Detergent sequestering agents ensure that free calcium ion concentrations in the washing liquor are very low and these agents could even potentially strip weakly bound calcium ions from the enzyme surface, thereby destabilizing it.

### 12.2.1.9 $\alpha$ -Amylase: applications in textile desizing

Modern methods of textile weaving places considerable mechanical stress on the fabric threads. To prevent breakage, the fibre strands are generally coated with a substance known as 'size'. The size serves to strengthen the fibre prior to weaving. Essential attributes of sizing materials include good adhesion to the textile threads, ease of removal after weaving (it is necessary to remove the size after weaving as it would subsequently prevent proper dyeing/bleaching of the finished product) and inexpensiveness. A range of natural and synthetic substances have been used in textile sizing (Table 12.13). Starch remains a popular sizing agent due to its low cost and ready availability. In Europe potato starch is normally

**Table 12.13** Various substances used in textile sizing. Refer to text for details.

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Starch
Methylcellulose
Gelatin
Carboxymethylcellulose
Guar gum
Polyvinyl alcohol
Carbo bean meal
Methacrylate

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used whereas cornstarch and rice starch find wider application in the USA and Far East, respectively.

Subsequent removal of the starch ('desizing') may be achieved by steam heating in the presence of NaOH, or by oxidants. However, such treatments can damage the textile and will generate a process effluent that must be treated before disposal. Desizing using  $\alpha$ -amylase has thus become popular. Generally, thermostable bacterial (e.g. *B. licheniformis*)  $\alpha$ -amylases are mainly used. Depending on the enzyme concentration and environmental parameters chosen, the desizing process may last from several minutes to several hours.

Like textiles, paper is also often sized using starch. Sizing of paper protects it from mechanical damage during manufacture, and also enhances the stiffness and feel of the finished product (in this case desizing is not subsequently carried out). Natural unprocessed starch slurries are too viscous to be used in paper sizing, so  $\alpha$ -amylase is used to partially degrade the starch in order to yield a product of appropriate viscosity for the task at hand.

### 12.2.2 Lignocellulose-degrading enzymes

The most abundant carbohydrate reserves on the planet are present in plant biomass. Biomass has been described as everything that grows. Plant biomass consists largely of three polymeric substances: cellulose (40–55%, depending on the exact source, e.g. hardwood, softwood or grasses), hemicellulose (25–50%) and lignin (10–40%). Perpetual renewal of plant biomass via the process of photosynthesis ensures an inexhaustible

supply of such material. It has been estimated that approximately 100 billion tonnes of cellulose is synthesized annually by higher plants. In practice, given that the world population stands at 7 billion, this means that almost 40 kg of cellulose is synthesized per person per day. Enzymes capable of degrading cellulose therefore attract obvious industrial interest.

### 12.2.2.1 The cellulose substrate

Cellulose is a linear unbranched homopolysaccharide consisting of glucose subunits linked via  $\beta 1 \rightarrow 4$  glycosidic linkages. Individual cellulose molecules vary widely with regard to polymer length, with some molecules containing as few as 100 glucose residues, whereas others may contain as many as 20,000 units. The majority of cellulose molecules consist of between 8000 and 12,000 glucose molecules. Each glucose molecule present in cellulose is rotated at an angle of  $180^\circ$  with respect to its immediately adjacent residue. The actual repeating structural subunit is therefore cellobiose (Figure 12.14).

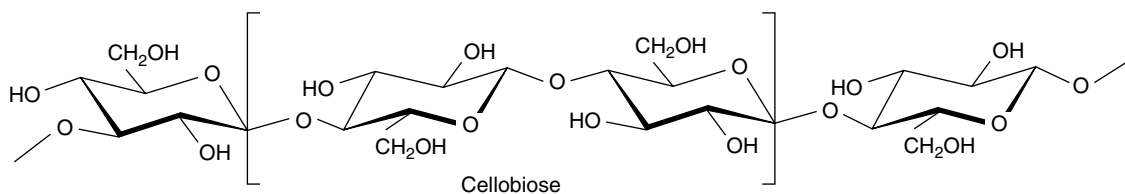
The cellulose molecule is chemically very stable, with an estimated half-life of 5–8 million years for  $\beta$ -glucosidic bond cleavage at  $25^\circ\text{C}$ . The underlying molecular structure confers on cellulose properties which are very different to those of amylose, which also consists exclusively of glucose residues linked by  $1 \rightarrow 4$  glycosidic linkages. Because of the  $\beta$  form of the linkage and the spatial arrangement of alternate glucose molecules, cellulose adopts an extended conformational structure. Furthermore, individual cellulose molecules are usually arranged in bundles or fibrils consisting of several parallel cellulose molecules that are held in place by an

extensive network of intermolecular hydrogen bonds. Glucose residues within the cellulose molecule also engage in intramolecular hydrogen bonding, which contributes to the overall rigidity of the molecule. Within cellulose fibrils, there are extended areas exhibiting a completely ordered structure (crystalline areas) that are water insoluble and very resistant to chemical or enzymatic attack, and smaller areas that are less well ordered (amorphous regions).

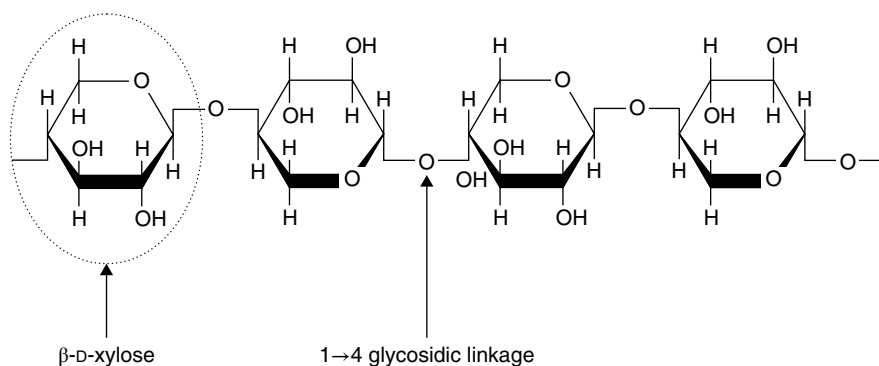
Vertebrates are devoid of endogenous enzymatic activities capable of hydrolysing the cellulolytic  $\beta 1 \rightarrow 4$  linkages, and therefore they are incapable of digesting cellulose and utilizing it as a nutrient source. Some microorganisms, in particular various fungi, do synthesize cellulase enzymes. Ruminant animals, by virtue of the fact that their digestive system contains cellulolytic microorganisms, can indirectly gain nutritional benefit from ingested cellulose.

### 12.2.2.2 Hemicellulose and lignin

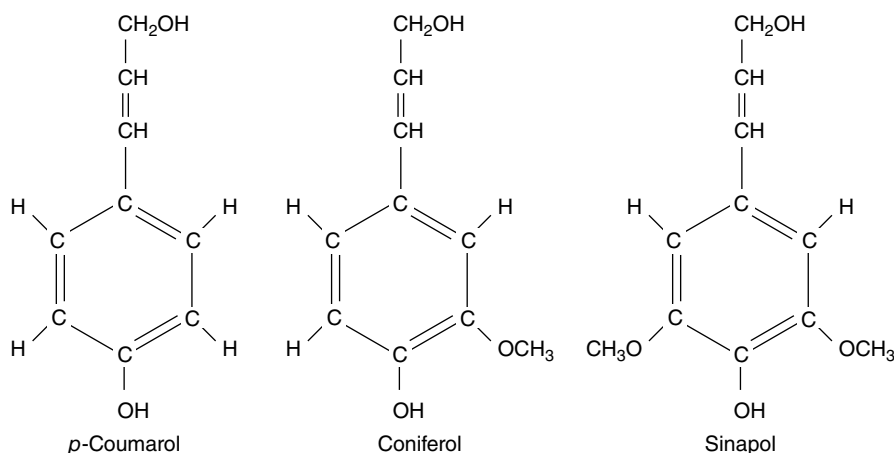
While cellulose is the principal constituent of the plant cell wall, it is rarely found in pure form as it is in intimate association with other polymeric substances termed hemicelluloses and lignin. Hemicelluloses are generally lower-molecular-mass polysaccharides. They consist predominantly of *D*-xylose, *D*-mannose, *D*-glucose, *D*-galactose, *L*-arabinose and 4-*O*-methyl-*D*-glucuronic acid. The most abundant hemicellulose types present in the cell walls include glucans, mannans and xylans. Xylan, the single most abundant hemicellulose, is a polymer consisting of  $\beta 1 \rightarrow 4$  linked *D*-xylosyl backbone (Figure 12.15) to which additional sugars (mainly *L*-arabinose, *D*-glucuronic acid and 4-*O*-methyl-*D*-glucuronic acid) are attached.



**Figure 12.14** Structure of a portion of the cellulose backbone. Individual glucose molecules are linked via  $\beta 1 \rightarrow 4$  glycosidic bonds. Successive glucose residues are rotated at an angle of  $180^\circ$  relative to the preceding glucose residue.



**Figure 12.15** Structure of a portion of the xylan backbone, consisting of repeating molecules of the pentose  $\beta$ -D-xylose linked via 1  $\rightarrow$  4 glycosidic linkages.



**Figure 12.16** Structure of the alcohol molecules found in lignin.

Lignin is a complex aromatic polymer found in higher plants, predominantly located within the plant cell wall, interspersed with hemicellulose. This mixture forms a cement-like matrix in which the ordered cellulose fibrils are embedded. The presence of hemicellulose and lignin serves to increase the overall structural strength of the cell wall. The woody portion of tree trunks consists of over 20% lignin. In such woody tissues, lignin is also present in the intercellular spaces, the middle lamella, where it serves as an adhesive, holding adjacent cells together.

Unlike cellulose or hemicellulose, lignin is not a polysaccharide. It is a polymeric molecule composed mainly of three alcohol subunits: coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (Figure 12.16). Seemingly random cross-linking of

such alcohols yields highly dispersed lignin molecules. Angiosperm lignins generally contain equal quantities of coniferyl alcohol and sinapyl alcohol monomers. Gymnosperm lignins, on the other hand, consist largely of coniferyl units alone whereas grass lignins contain all three alcohols.

### 12.2.2.3 Cellulases

Enzymes capable of hydrolysing cellulose are termed cellulases. Serious research on cellulases dates back to the Second World War, when the US Army noted a rapid deterioration of cellulose-based materials (e.g. clothes, tents) while fighting in the South Pacific. Investigations revealed cellulase-producing fungi to be the major culprit.

Agricultural and household waste contains appreciable quantities of cellulose. Wood and wood pulp consist of over 40% cellulose, straw and bagasse contain 30–50% cellulose, while newspapers can contain up to 80% cellulose. Cotton is almost pure cellulose. The complete hydrolysis of cellulose yields glucose. Any process that could efficiently and economically achieve conversion of cellulolytic material to glucose would be of immense industrial significance.

Cellulose is not degraded by a single enzyme but by a combination of enzymes which function in a concerted manner. Degradation of cellulolytic material occurs slowly in nature for a number of reasons.

- Very few microbial species actually produce complete cellulase systems capable of total and systematic degradation of cellulose to glucose molecules.
- The ordered, tightly packed crystalline structure of individual cellulose molecules present in cellulose fibres renders enzymatic attack very difficult. Amorphous non-structured areas of cellulose, on the other hand, are degraded more rapidly.
- The close natural association of cellulose with hemicellulose, lignin and sometimes pectin further reduces the accessibility of cellulases to their substrates.

Cellulolytic enzymes are synthesized by a number of microorganisms, most notably fungi (Table 12.14). Some bacterial species also exhibit cellulose-degrading ability.

Fungal cellulases have received most attention. Some fungal species, most notably *Trichoderma* species such as *T. viride*, *T. reesei* and *T. koningii*, as well as *Penicillium funiculosum*, produce cellulases capable of degrading, at least in part, crystalline regions of native cellulose.

Most cellulolytic enzymes produced by fungi may be classified as one of three major types: (i) endocellulases (endo- $\beta$ 1  $\rightarrow$  4-D-glucanases, often simply called cellulases); (ii) cellobiohydrolases; and (iii)  $\beta$ -glucosidases. Any one fungal species capable of degrading cellulose may produce multiple forms

**Table 12.14** Some well-characterized microbial sources of cellulolytic enzymes.

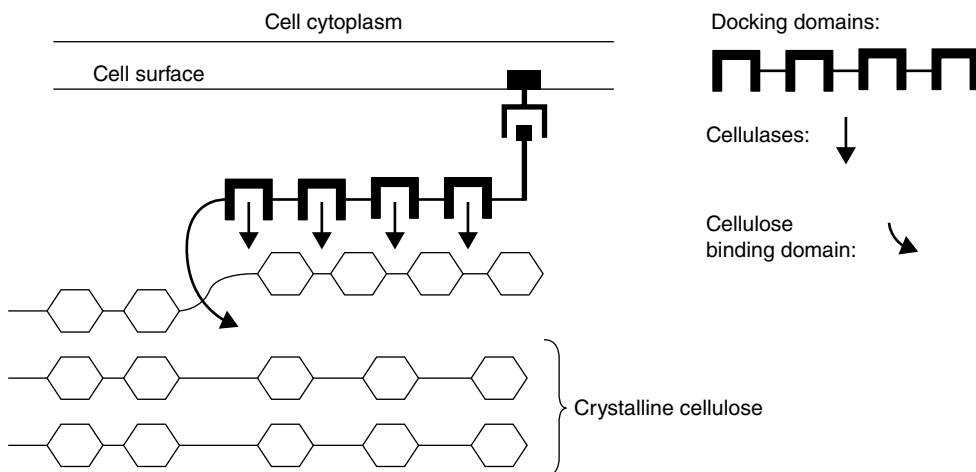
Fungal sources	Bacterial sources
<i>Trichoderma viride</i>	<i>Bacillus</i> spp.
<i>Trichoderma reesei</i>	<i>Cellulomonas</i> spp.
<i>Trichoderma koningii</i>	<i>Thermomonospora</i> spp.
<i>Penicillium pinophilum</i>	<i>Clostridium thermocellum</i>
<i>Penicillium funiculosum</i>	<i>Acetivibrio cellulolyticus</i>
<i>Sporotrichum pulverulentum</i>	<i>Bacteroides cellulosolvens</i>
<i>Talaromyces emersonii</i>	<i>Bacteroides succinogenes</i>
<i>Sporotrichum thermophile</i>	<i>Ruminococcus albus</i>
<i>Humicola insolens</i>	<i>Ruminococcus flavefaciens</i>

of each of these three enzymatic activities. Some such multiple forms are genetically distinct whereas others may result from partial proteolysis or differential glycosylation of a single protein. All these differing cellulolytic activities act synergistically to solubilize the cellulose substrate.

The endocellulases (endoglucanases) catalyse the random internal hydrolytic cleavage of the cellulose molecule. As many as six endocellulase activities may be associated with some fungi. Endocellulases appear to hydrolyse cellulose chains primarily within amorphous regions and display low hydrolytic activity towards crystalline cellulose.

Cellobiohydrolases generally catalyse the sequential removal of cellobiose units from the non-reducing end of the cellulose molecule. The  $\beta$ -glucosidases, on the other hand, hydrolyse short-chain oligosaccharides derived from cellulose in addition to cellobiose, yielding glucose monomers. Many cellobiohydrolases exhibit product inhibition, as their activity is decreased in the presence of increasing concentrations of cellobiose.  $\beta$ -Glucosidase activity thus prevents such product inhibition.

A wide range of both aerobic and anaerobic bacteria produce cellulase enzymes. Cellulolytic bacteria mainly produce endocellulases, with only a few species being capable of producing cellobiohydrolase.  $\beta$ -Glucosidase is produced by some, but it is often cell-bound.



**Figure 12.17** Generalized proposed structure of a bacterial cellulosome. A cellulose-binding domain attached to the docking platform binds a cellulose strand in the cellulose microfibril, thereby disturbing the latter's crystalline structure. This in turn makes this cellulose stretch accessible to the various cellulolytic activities of the cellulosome. Refer to the text for further details.

### *The cellulosome*

Many anaerobic microorganisms, both bacterial and fungal, produce a high-molecular-mass enzyme complex termed a cellulosome. Cellulosomes, which are particularly prevalent in clostridia and rumen microorganisms, normally consist of several endoglucanases as well as some exoglucanases. These cellulases are bound to a large non-catalytic protein (the 'scaffolding subunit') via short sequences ('docking domains'). This highly ordered structure probably allows more efficient degradation of the cellulose substrate. Some cellulosomes contain hemicellulase as well as cellulase activities. Cellulosomes may be anchored on the surface of the producer microbe and these microbes may therefore be required to dock on the cellulose surface in order to commence substrate hydrolysis (Figure 12.17).

### *Cellulase structure*

Limited proteolysis of many endocellulases and some exocellulases produce two protein fragments, one capable of binding to cellulose (originally termed the cellulose-binding domain or CBD, but now renamed the carbohydrate-binding module or CBM) and a catalytic domain. Not all cellulases contain CBMs, and the size and three-dimensional shape of those that do can vary significantly. By

targeting the cellulase to the substrate, the CBMs help achieve more efficient cellulose hydrolysis. CBMs are linked to the enzyme catalytic domain by a short linker sequence. Depending on the cellulase studied, this linker sequence can vary in length from 6 to 59 amino acid residues. Linker sequences are frequently rich in proline and hydroxylated amino acids, and are often highly glycosylated. Catalytic domains, based on comparative amino acid sequences, display considerable variability from cellulase to cellulase. They are invariably much larger than the cellulose-binding domains, typically accounting for 70% or more of the cellulase mass.

This summary outlining cellulose hydrolysis is likely incomplete. The enzymatic degradation of this polymer is complex and controversy still exists regarding the number of enzymes required in this process, the exact role that each enzyme plays in the overall degradative pathway, the mechanism of action of some of these enzymes, particularly pertaining to those hydrolysing crystalline cellulose, and the role of accessory proteins in cellulose degradation.

#### 12.2.2.4 Accessory proteins

In addition to cellulases, which directly attack the cellulose substrate enzymatically, a number of

accessory proteins/systems exist that appear to facilitate or complement enzyme-based cellulose degradation. This is an area of ongoing active research and a comprehensive understanding of these accessory elements has yet to be developed. This area of research was triggered in particular by the discovery of expansins, a family of non-catalytic plant proteins associated with the plant cell wall. Expansins are typically 250–300 amino acids long and consist of two domains (1 and 2). Domain 1 displays distant homology to the catalytic domain of many fungal endoglucanases, although it appears to be devoid of actual catalytic activity. Domain 2 displays homology to group 2 grass pollen allergens, which are of unknown function. Expansins play a role in plant cell growth and fruit ripening or other processes where cell wall loosening occurs. The plant cell wall consists of a scaffold of cellulose microfibrils to which hemicellulose (e.g. xylans) are bound, forming a tough but flexible load-bearing network. Expansins may function by disrupting non-covalent cellulose–hemicellulose interaction, allowing transient slippage (relative movement) of the cellulosic and hemicellulosic cell wall elements.

Expansin-like proteins have been identified (or inferred via bioinformatic analysis) in some microorganisms including plant pathogens, for whom the production of a cell wall-disrupting agent would have obvious benefit. Additional proteins (swollenin) that share some homology with expansins have been identified in some cellulolytic bacteria and fungi, where they appear to aid cellulose hydrolysis.

An additional accessory system used by brown rot fungi involves the secretion of peroxidases, which facilitate the generation of peroxide and hydroxyl radicals. These partially oxidize the cellulose.

#### 12.2.2.5 Cellulase engineering

Much effort continues to be invested in engineering cellulase enzymes in order to improve some application-relevant characteristic such as thermostability, optimum pH, or the ability to better degrade crystalline cellulose in particular. Approaches using both directed evolution and site-directed mutagenesis continue to be pursued, although both have

associated limitations. For example, a site-directed mutagenesis approach to enhancing activity against crystalline cellulose is hampered by an as yet incomplete understanding of the mechanism of crystalline cellulose hydrolysis. Additional impediments to developing engineered cellulolytic systems include the fact that complete cellulose hydrolysis requires a cocktail of such activities (and that for optimum hydrolysis, these would likely be required to be present in differing ratios) and that altered properties associated with engineered enzymes may be observed only when soluble as opposed to crystalline cellulose is used (or vice versa).

#### 12.2.2.6 Industrial applications of cellulose hydrolysis

The potential industrial applications of the cellulases are enormous. Glucose produced from the cellulose substrate can be used directly in animal/human food. Alternatively, the glucose product could be used as a substrate for subsequent fermentations or other processes which could yield valuable end products such as ethanol, methanol, butanol, methane, amino acids, organic acids and single-cell protein. Cellulolytic enzymes could also be used directly to increase the digestibility of food with high-fibre content, and to enhance food flavour, texture or other qualities.

The main industrial processes to which cellulases have been commercially applied include:

- detergent additive;
- stonewashing of denim;
- maximizing extraction of juice from fruits (in conjunction with pectinases);
- clarification of fruit juices (in conjunction with pectinases);
- removal of external coat from soybean during production of fermented soybean foods;
- deinking in the paper recycling industry;
- improving rehydration of dried soups/vegetables;
- as an additive in animal feed (see Chapter 13).

Cellulases are currently the third largest group of industrial enzymes (by sales value). However, if they can be applied at routine process-scale level for

widespread biofuel (bioethanol) production, they will quickly become the single most lucrative category of industrial enzyme.

Commercial cellulase preparations have traditionally been sourced from fungi such as *Trichoderma longibrachiatum*, *T. reesei* and *Humicola insolens*. Commercial bacterial preparations are generally sourced from bacilli. More recent commercial products are produced via recombinant means (Box 12.6).

#### Detergent applications

Cellulases were first added to commercial detergent preparations in Japan in the late 1980s. This practice was subsequently adopted in Europe and the USA in the 1990s. Not all cellulases display physicochemical properties conducive to detergent use, although several have been identified which are active at alkaline pH and are stable in the presence of typical

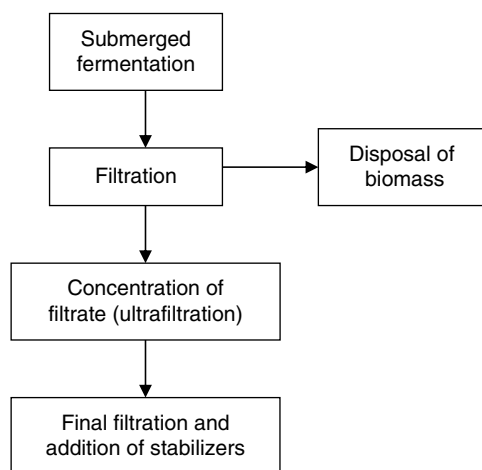
detergent components. Prominent cellulase preparations used for detergent application are presented in Table 12.15.

A screening programme undertaken by the KAO Company in Japan identified a detergent-compatible cellulase from an alkalophilic *Bacillus* (*Bacillus* KSM-635). Chromatographic purification using a combination of anion exchange and gel filtration steps yielded two peaks of cellulase (endoglucanase) activity. The endoglucanases displayed molecular masses of 130 and 103 kDa, were maximally active at 40°C and at pH 9.5, and were stable when incubated at pH values ranging from 6 to 11. These activities were very resistant to incubation with various surfactants and chelating agents and were found to be inhibited by Hg<sup>2+</sup> and Cu<sup>2+</sup>. They were activated by Co<sup>2+</sup> and required either Co<sup>2+</sup>, Ca<sup>2+</sup>, Mg<sup>2+</sup> or Mn<sup>2+</sup> for thermal stability. Novozymes' cellulase preparation Celluzyme is sourced from the

#### Box 12.6 Product case study: CeluStar CL

CeluStar CL is a recombinant cellulase preparation developed by Dyadic International. The enzyme is an endocellulase (endoglucanase), the coding sequence for which was derived from a *Myceliophthora thermophila* strain. Multiple copies of the gene have been incorporated into the chromosome of a *M. thermophila* recipient strain, which serves as the producer microorganism. The transferred genes are under the control of a powerful cellobiohydrolase promoter and the recombinant strain produces approximately 20-fold elevated levels of cellulase activity as a result. In addition to the recombinant enzyme, the product contains lower levels of other cellulases and xylanase native to the producer strain. Batch manufacture of product is outlined schematically below. Cultures used to initiate production of individual product batches are taken directly from a bank of frozen stock culture stored at -80°C in glycerol as a cryopreservant. The final product is formulated and marketed in liquid form and is used as a processing aid in the food industry. Potential applications could include:

- enhancement of juice extraction from citrus and other fruits;
- enhancement of starch recovery from grain;
- enhancement of essential oil extraction from plant material;
- removal of fibre from edible oil press cakes;
- treatment of distiller's mash in brewing;
- tenderization of fruits and vegetables prior to cooking.



**Table 12.15** Sources, characteristics and industrial producers of some detergent cellulases.

Name	Company	Source	pH range	Temperature (°C)
KAC	KAO Corp.	Alkalophilic <i>Bacillus</i> sp.	7–10	40 (optimum)
Celluzyme	Novozymes	<i>Humicola insolens</i>	7–9	50 (max.)
Carezyme	Novozymes	Recombinant <i>H. insolens</i> endoglucanase	10	50 (max.)
Ecostone	AB enzymes	<i>Melanocarpus albomyces</i>	6–8	20–50

thermophilic fungal strain *Humicola insolens* DSM 1800. The preparation contains a mix of endoglucanases, cellobiohydrolase and  $\beta$ -glucosidase activities. Novozymes' Carezyme product is a recombinant endoglucanase isolated from *H. insolens*, but whose gene is expressed in an engineered strain of *A. oryzae*.

Cellulases added to detergents promote a number of effects, including stain removal, colour revival, depilling and fabric softening. They achieve these effects by promoting a very limited hydrolysis of cellulose fibres in cellulose-based fabrics (cotton/cotton blends, rayon and flax). It appears that a proportion of 'dirt' molecules in a stain on cotton fabrics may be trapped within the amorphous regions of cellulose microfibrils. This makes their effective removal difficult. Cellulase activity in detergent preparations is believed to partially cleave cellulose molecules in the amorphous regions of fibres, thus allowing the dirt to be removed more easily while not promoting extensive degradation of the cotton fibre.

The effects of colour revival, depilling and fabric softening are based on the same mechanism of action. Repeated wearing/washing of garments results in mechanical damage to the cotton fibres. As a result, microfibrils protrude from the surface of the originally smooth cotton fibre. This results in a reduction in colour brightness that is independent of bleaching of the colour. This appears to be due to the now uneven fibre surface

dispersing incident light, thereby promoting a dulling effect. Treatment with cellulases enzymatically removes the damaged microfibrils, thus restoring a smooth surface to the cellulose fibre (it appears that mechanical damage sustained at the anchor point of the microfibril renders the area more susceptible to cellulase activity).

Ongoing mechanical damage can result in the microfibrils gathering into little balls called pills. Again cellulases can promote a depilling effect by enzymatic hydrolysis of the pills' anchor point on the fabric surface. Removal of microfibrils also appears to improve the texture and grip of the fabric, by preventing interlocking of individual fibres. This effect is perceived as fabric softening.

#### *Stonewashing and biopolishing*

Cellulases also find application in the textile industry, promoting stonewashing of denim and the biopolishing of cotton fabrics. Stonewashing is the process whereby denim is treated to give it a worn/rugged look. Traditionally, this was undertaken by washing the denim in the presence of pumice stones, which achieved their effect through mechanical abrasion. Cellulases can achieve a similar effect enzymatically and have largely replaced the use of pumice stones. This prevents mechanical damage to the washing machines and the absence of the stones allows more denim to be washed in each cycle. When denim is dyed (particularly with indigo), most of the dye molecules are retained on the surface of the denim yarn. Treatment with cellulases promotes a very partial hydrolysis of the cellulose fibres, thus releasing some of the dye, giving the fabric a worn/faded look.

The biopolishing process involves treating new cotton fabrics with cellulases. The aim is to remove any loose or protruding microfibrils that may be present on the surface of the cotton fibres. By ensuring that the fibres are as smooth as possible, the process of pill formation/colour dulling is delayed. As a result, the clothing looks newer for longer.

#### *Enzymatic deinking*

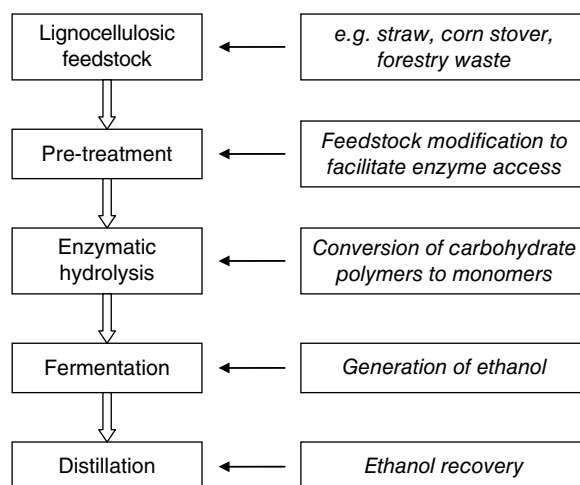
A growing concern relating to the environmental impact of human activity has given impetus to



recycle as much waste as possible. Wastepaper recovery and reuse has become a prominent example of such recycling. Successful reuse of paper requires effective removal of inks from the original paper stock. Deinking can be achieved with relative ease in the case of some paper grades (e.g. newspapers printed using oil-based inks). However, in most cases, deinking can be more problematic. Deinking involves dislodging the ink particles from the paper fibre surface, followed by separation of the dislodged ink from the fibre by washing. This can be achieved chemically but such processes generate waste effluents that must be disposed of carefully. Enzyme-mediated deinking is more environmentally friendly and appears to be economically viable. Lipases may be used to degrade and hence dislodge vegetable oil-based inks. However, other synthetic inks are less susceptible to enzymatic degradation. In such cases cellulases (or sometimes a mixture of cellulases and hemicellulases) are used. These promote deinking by achieving very limited hydrolysis of the cellulose/hemicellulose fibres, thereby releasing ink molecules trapped in the fibre surface.

#### *Second-generation bioethanol production*

Decreasing reserves of fossil fuel and the urgent need to combat climate change has given new impetus to the development of biofuels, of which bioethanol remains at the forefront. So-called first-generation bioethanol is generated from sugar (largely starch)-based substrate. The process typically entails the use of amylolytic enzymes to degrade starch into glucose (as described in Figure 12.8), followed by fermentation of the glucose to ethanol. However, such an approach could only supply a few percent of the global energy demand sustainably (i.e. without unacceptable reductions in land availability for food production). A more sustainable approach would be the use of lignocellulosic feedstock as a source of sugars for fermentation (second-generation bioethanol). Current global demand for oil stands at approximately 30 billion barrels per year. The  $10^{11}$  or so tons of cellulose generated annually via photosynthesis correspond in energy terms to some 640 billion barrels of crude oil. Moreover, lignocellulosic substrates such as straw, corn stover, as well as forest and sawmill



**Figure 12.18** Enzyme-based production of second-generation bioethanol from lignocellulosic substrates. Refer to text for details.

residues are generated in enormous quantities annually as mostly unwanted agricultural processing byproducts. Ethanol can be produced from lignocellulosic material by a number of means but current technologies comprise the same main components, namely pretreatment, hydrolysis, fermentation and distillation (Figure 12.18).

Process-scale development of bioethanol production from lignocellulose remains at an early stage of development. Several large pilot-scale/modest process-scale facilities have been constructed in various world regions, and are currently being used to assess and perfect second-generation bioethanol manufacturing processes. These are sometimes termed biorefineries (the concept of biorefineries pertains to the processing of raw biomass in order to yield some useful product, in this case bioethanol).

Processing of the lignocellulosic feedstock typically entails an initial washing and chopping or even milling of the raw material. The graded raw material is then pretreated with the aim of:

- modifying the lignocellulosic structure (e.g. by solubilizing the hemicellulose and/or the lignin fraction) in order to facilitate enzyme access to the cellulose;
- at least partial decrystallization of the cellulose;
- partial cellulose depolymerization.

Different pretreatment methods have been developed, and include chemical, biological and physical approaches. One of the most commonly applied methods is based on the application of dilute acid. Typically, this entails incubation of the lignocellulose with 0.1–5.0% sulfuric acid at elevated temperatures (generally 140–220°C). Such ‘dilute acid pretreatment’ solubilizes/degrades the hemicellulose fraction, facilitating subsequent cellulase access to the cellulose fraction. The enzymatic hydrolysis phase involves the addition of a cocktail of cellulases, in some cases with supplemental hemicellulases, which promote conversion of carbohydrate polymers to monomeric sugar level. This step is generally undertaken at 45–55°C and at pH 4.5–5.5, conditions conducive to maximal or near-maximal enzyme activity. This is followed by a fermentation phase, which uses yeast (and potentially bacteria or fungi) capable of fermenting the glucose and other monomeric sugars generated into ethanol. Finally, the ethanol can be recovered by distillation.

Most of the major global enzyme producers are actively engaged in developing enzyme products for use in second-generation bioethanol production. Genencor/DuPont, for example, is developing their so-called Accellerase product (currently a blend of cellulases,  $\beta$ -glucosidases and hemicellulases) while Novozymes are developing their Cellic CTec product, which again is based on a cocktail of lignocellulolytic enzymes.

### 12.2.2.7 Xylanases and their applications

As described earlier, hemicellulose constitutes a major fraction of lignocellulosic material, and xylans represent the single most abundant hemicellulose fraction. The complete degradation of xylans requires the cooperation of several enzymes. Endo- $\beta$ -1,4-xylanases (often just called xylanases) catalyse the hydrolysis of the backbone xylan glycosidic linkages, yielding xylooligosaccharides. These in turn can be further degraded to xylose by  $\beta$ -xylosidase. Also required are several side-chain splitting enzymes, such as arabinofuranosidase and acetylxylan esterase. Various microbial species, particularly various fungi, are known to produce various xylan-degrading activities. Among the most notable xylanase producers are species of *Aspergillus*, *Trichoderma*,

*Penicillium* and *Fusarium*. Xylanases traditionally manufactured at industrial level have been produced mainly by strains of *Aspergillus* and *Trichoderma*, although recombinant DNA technology facilitates the production of xylanase genes from many sources in any appropriate producer strain (Box 12.7).

Xylanases find increasing industrial application. Xylanase preparations are used in the baking industry to alter the properties of dough (Box 12.7). Wheat flour contains up to 4% arabinoxylans (highly branched xylans characteristic in particular of the outer cell walls and endosperm of cereals such as wheat, but also barley, oat and rye). Some of these arabinoxylan molecules are ‘soluble’ while the

#### Box 12.7 Product case study: Novozym® 899

Novozym® 899 is an endo-1,4- $\beta$ -xylanase produced by a selected strain of *Fusarium venenatum* expressing the endoxylanase gene derived from a strain of *Thermomyces lanuginosa*. It is used in the breadmaking industry as outlined in the main text. The production strain is derived from a publically available strain (*F. venenatum* ATCC 20334), which has been used as a mycoprotein source for human consumption for several decades. A main element of the xylanase expression plasmid is the *T. lanuginosa* xylanase gene fused to the *F. oxysporum* trypsin gene promoter and terminator. The production strain is also engineered in order to delete a trichodiene synthetase gene, preventing it from producing a variety of secondary metabolites in the trichothecene family (which are potent inhibitors of eukaryotic protein synthesis).

Each production batch is initiated using a lyophilized stock culture of the production microorganism. Fermentation is undertaken in submerged feed-batch mode. After fermentation the enzyme is recovered from the spent culture broth, concentrated and filtered in order to ensure that no producing microorganisms are present in the final formulation. Stabilizers added include dextrin, sorbitol and wheat solids, and the final product is dried and granulated.

majority are coupled to wheat proteins (the insoluble fraction) and this is believed to reduce the elasticity of the gluten (and hence the dough). Xylanases added to the flour can improve the handling and stability of the dough by acting on the insoluble arabinoxylan fraction in particular. Moreover, the added xylanase activity is subsequently thermally destroyed by the baking process. Additional existing and potential xylanase applications include (i) in the paper and pulp industry, (ii) to aid lignocellulosic degradation for the purposes of bioethanol generation, and (iii) in the animal feed industry (see Chapter 13).

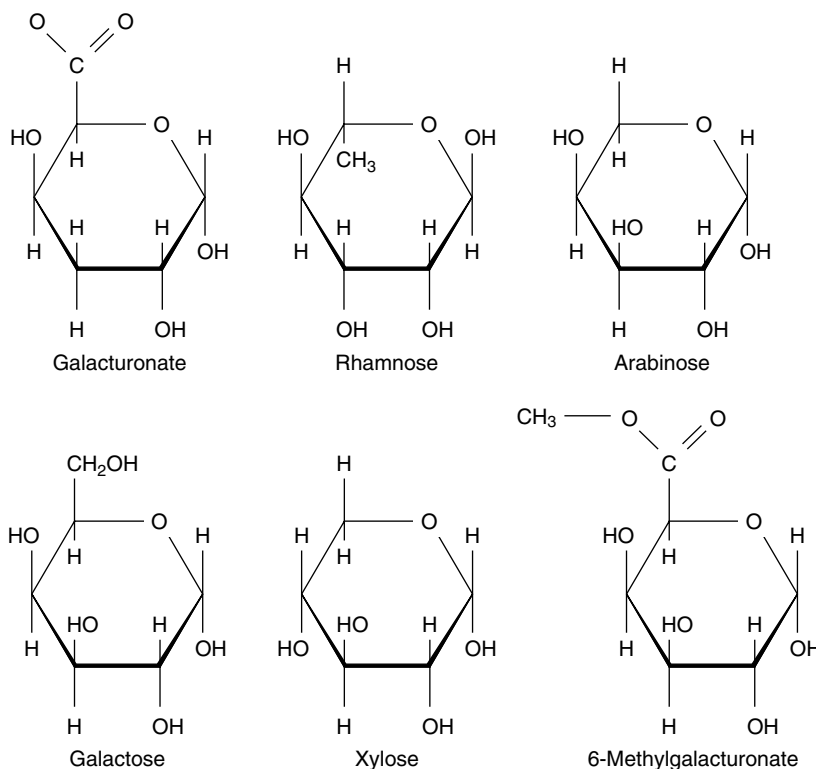
### 12.2.3 Pectin and pectic enzymes

Pectin is yet another structural carbohydrate found in higher plants. It is located primarily in the cell wall and in the middle lamella, where it serves to bind adjacent

cells. Various enzymatic activities capable of degrading pectin may be isolated from both plant and microbial sources. Degradation of pectin plays an important role in the growth of plant cells and the ripening of fruit. Microbial pectic enzymes, mainly produced by fungi, are utilized in many large-scale industrial processes. Advances in recombinant DNA technology has facilitated the detection, cloning and sequencing of genes coding for many such enzymes, and enabled their expression in heterologous systems.

#### 12.2.3.1 The pectic substrate

Pectic substances are a relatively diverse group of polysaccharides that vary in both their composition and molecular mass. Galacturonic acid is the major molecule present, constituting up to 60–80% of some pectic preparations. Other sugars often present in pectic preparations include rhamnose, arabinose, galactose and xylose (Figure 12.19).



**Figure 12.19** Structural formulae of the more common monomers found in pectin. In native pectin, 75% or more of the galacturonic acid units are esterified with methanol, thus forming methylgalacturonides.

The pectin molecule, present in intact immature plant tissue, is often referred to as protopectin. Protopectin is insoluble, which seems to be due to its polymer size and its association with calcium and other divalent cations. All other pectic substances that are soluble are derived from protopectin by hydrolysis. Pectins may thus be described as polysaccharides composed mainly of galacturonic acid, at least 75% of which is esterified with methanol. Enzymatic de-esterification of such galacturonide yields a polymeric substance often termed pectic acid. Pectic substances are often classified as galacturonans, rhamnogalacturonans, arabinans, galactans and arabinogalactans.

Rhamnogalacturonans represent the major constituent of pectic substances. The polysaccharide backbone of rhamnogalacturonans consists mainly of  $\alpha$ -D-galacturonate units linked via  $\alpha 1 \rightarrow 4$  bonds. Molecules of L-rhamnose are interdispersed in the backbone, occurring on average every 25–30 galacturonate units. The rhamnose units are linked via  $\beta 1 \rightarrow 2$  and  $\beta 1 \rightarrow 4$  bonds to the D-galacturonate residues. Side chains of variable length, often consisting of galacturonans, galactans, arabinans or arabinogalactans, branch off from the main chain (Figure 12.20).

### 12.2.3.2 Pectic enzymes

There exists two broad classes of pectic enzymes. The first group are termed pectin esterases, also known as pectin methylesterases. The second class are depolymerases. Pectin methylesterases remove methoxy groups from methylated galacturonides. Pectin esterase activity is present in all higher plants and is particularly abundant in citrus fruits and vegetables. Esterase activities are also found with a variety of microorganisms, most notably fungi.

The depolymerases catalyse the cleavage of glycosidic bonds via hydrolysis (hydrolases) or via  $\beta$ -elimination (lyases). In many instances, pectin esterase must firstly remove methoxyl groups from the galacturonide before depolymerase activity can commence.

Polygalacturonases catalyse the hydrolysis of  $\alpha 1 \rightarrow 4$  linkages between  $\beta$ -galacturonic acid residues. A number of distinct polygalacturonase

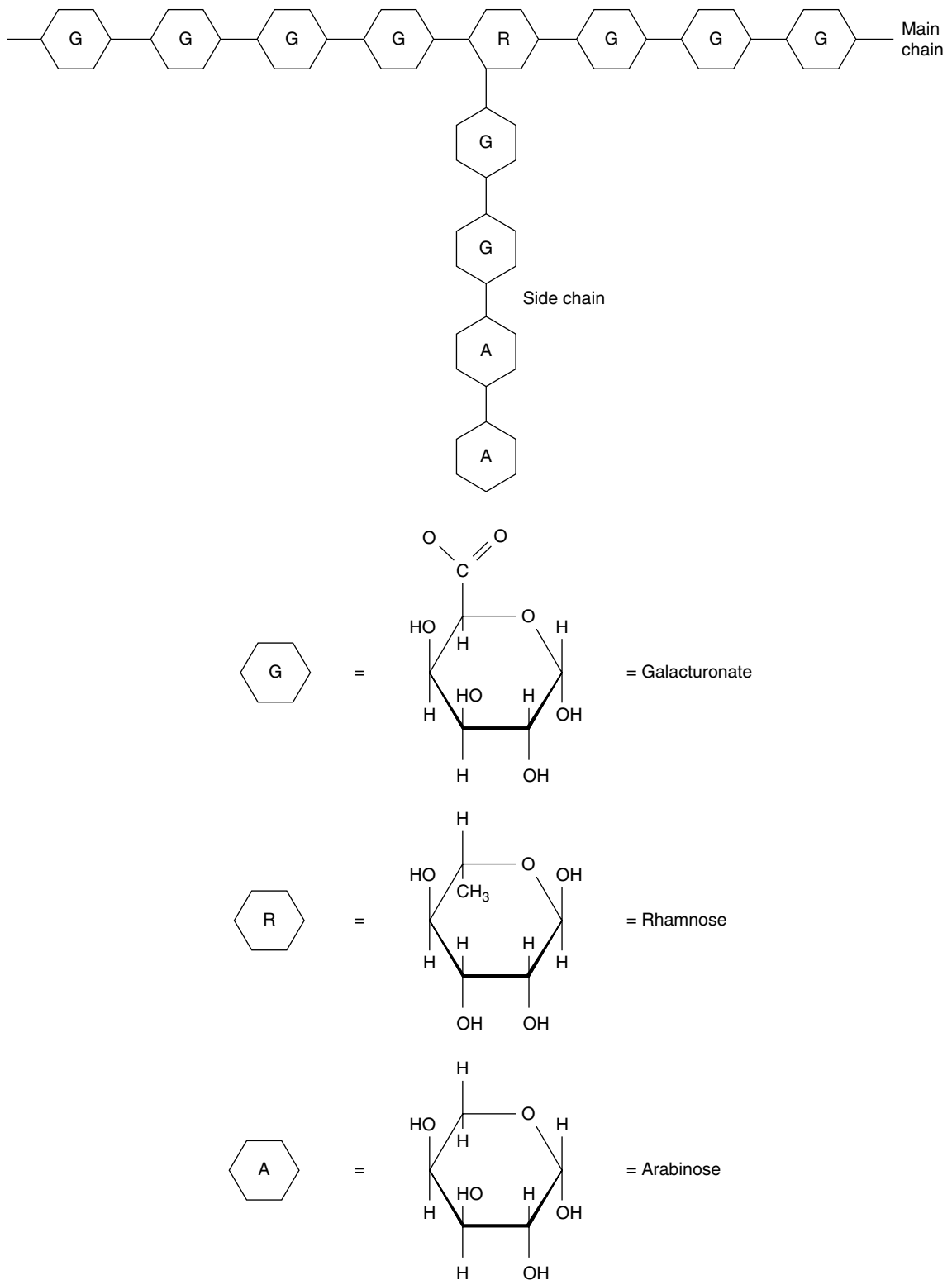
activities have been recognized, both in higher plants and in microbes. Endopolygalacturonases catalyse the hydrolysis of internal  $\alpha 1 \rightarrow 4$  glycosidic linkages in stretches of polygalacturonic acid. As cleavage sites are chosen more or less at random, a number of lower-molecular-mass oligosaccharides are produced. Cleavage by endopolygalacturonases requires the prior removal of methoxy groups by pectin methylesterase. Exopolygalacturonases catalyse the sequential removal of galacturonic acid residues from the non-reducing end of polygalacturonate.

Lyases are a group of pectin-degrading depolymerases produced almost exclusively by microorganisms. These enzymes catalyse the cleavage of  $\alpha 1 \rightarrow 4$  glycosidic bonds which link galacturonic acid residues by  $\beta$ -elimination. Endopectate lyases catalyse the cleavage of internal glycosidic bonds in regions of polygalacturonic acids devoid of methoxy groups. Such enzymes have been isolated from a number of microbial plant pathogens. They have a high pH optimum and require the presence of calcium ions to maintain activity.

Exopectate lyases are found mainly in bacteria. Most such enzymes cleave the penultimate glycosidic bond of galacturonans, thus releasing dimeric molecules composed of galacturonic acid. Some such lyases, however, split the terminal galacturonin glycosidic bond, yielding single galacturonic acid moieties. As in the case of endopectate lyases, most exopectate lyases preferentially attack polygalacturonic acid which is essentially free of methyl ester groups. Pectin lyases display a preference for highly esterified polygalacturonic acid sequences (galacturonins) as substrates. These enzymes are produced mostly by fungi and usually catalyse the random internal cleavage of glycosidic bonds, thus producing esterified oligogalacturonates.

### 12.2.3.3 Industrial significance of pectin and pectin-degrading enzymes

Pectin enjoys widespread industrial application as solutions of pectin are viscous, and readily gel when heated in the presence of sugar under acidic conditions. This renders pectin useful as a gelling agent, emulsifier and thickener in the production



**Figure 12.20** Structure of a segment of rhamnogalacturonans. Regions containing a high density of side chain are termed 'hairy' regions. Such hairy regions are normally separated by extended sequences devoid of side chains, the smooth regions.

of a number of foods. Pectin used for such purposes has been classified as high-methoxy or low-methoxy pectin. As the name suggests, the majority of galacturonic acid residues present are methoxylated in high-methoxy pectin. In low-methoxy pectin, many such methoxy groups have been removed. While gelation of high-methoxy pectin depends on the presence of significant quantities of sugar, low-methoxy pectin may be induced to gel by the addition of certain metal ions, even in the absence of sugar. Use of the latter pectin substrate thus makes possible the manufacture of jams and jellies of low sugar content. Commercial pectin is normally produced by extraction from the rind of citrus fruits or from sugar beet pulp, both of which serve as particularly rich sources of this substance.

Pectic enzymes are also used in a number of industrial processes. Such enzymes have found particular favour within the fruit juice extraction and clarification industries. Fruit juices are normally manufactured by the mechanical pressing of the relevant fruits. In many instances, the physical characteristics of the fruit hinder maximal juice extraction. The addition of commercial preparations of pectic enzymes generally facilitates a greatly enhanced juice yield.

Enzymatic degradation of pectin by fungal pectinase preparations is routinely used to maximize juice yields from grapes and apples. Juice extraction from most soft fruits, such as raspberries, strawberries and blackberries, is also increased by supplementation with exogenous pectinolytic enzymes.

Commercial pectinase preparations are routinely employed to clarify 'sparkling' fruit juice preparations, such as apple juice and pear juice. When freshly pressed, most such juices contain relatively high levels of soluble pectins that contribute greatly to the characteristic viscosity and haziness associated with such products. Haze formation often reflects a decreased solubility of one or more of the components present in the juice, with which pectins associate. Partial degradation of the pectins is achieved by the addition of commercial pectinase preparations, which results in a significant drop in product viscosity. Furthermore, partial degradation

of the pectin destabilizes the haze particles, resulting in their coagulation and precipitation from solution. Subsequent removal of the precipitate is easily achieved by centrifugation or filtration, yielding the sparkling clear juice. The decrease in solution viscosity observed on treatment with pectic enzymes permits the production of concentrated juice extracts.

Pectic enzyme preparations are also used in the maceration of fruits and vegetables. Maceration usually entails the conversion of fruit or vegetable tissue into a suspension of individual intact cells. This may be achieved by selectively hydrolysing the pectin present in the middle lamella, which binds plant cells. Maceration is normally achieved by treatment with pectic enzyme preparations exhibiting high levels of polygalacturonase activity. This enzyme has also been directly associated with the process of fruit softening during ripening. Enzymatic maceration may be used in the production of fruit nectars, 'pulpy' drinks, and in the preparation of some baby foods. Pectic enzymes are also used to aid extraction of various citrus oils and pigments from orange and lemon peel.

Most commonly available pectinase preparations are obtained from fungal sources such as various species of *Aspergillus* or *Penicillium*. A source of pectin (such as apple pomace, citrus peel or dried sugar beet pulp) is normally included in the fermentation medium used to culture pectinase-producing fungi. This enhances not only pectinase production, but also promotes increased secretion of these enzymes from the mycelium. The enzymes produced are usually concentrated and partially purified by techniques such as precipitation. Stabilizers, preservatives and other additives are then incorporated into the final enzymatic preparation, which may be marketed in liquid or powdered form. Commercial pectinase preparations usually contain a variety of pectinolytic activities. Most also contain appreciable quantities of additional enzymes such as cellulases and hemicellulases. Recombinant DNA technology also facilitates the production of specific single pectinase enzymes, as illustrated in Box 12.8.

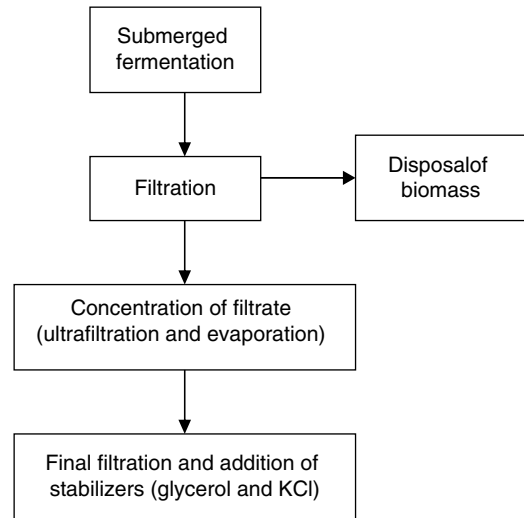
**Box 12.8 Product case study: Rheozyme™**

Rheozyme™ is a Novozymes recombinant pectin esterase preparation produced by an *Aspergillus oryzae* strain expressing a pectin esterase gene derived from a strain of *A. aculeatus*. The production strain is an engineered amylase-negative derivative of an original *A. oryzae* strain obtained from the Institute of Fermentation in Osaka, Japan. The expression plasmid used houses the *A. aculeatus* pectinase esterase gene under the control of the *A. oryzae* TAKA amylase gene promoter and an *A. niger* glucoamylase gene terminator sequence.

Product manufacture (see schematic) is via submerged feed-batch fermentation on a starch/starch hydrolysate-based medium. Additional fermentation components include yeast extract, vegetable protein, supplemental nitrogen such as urea, as well as minerals, trace metals and antifoaming agents. Each batch manufacturing process is initiated using a lyophilized stock culture of the producing microorganism.

The enzyme was developed for use in the food industry to modify (increase) the viscosity of fruit and vegetable-based products. The enzymatic de-esterification of highly methoxylated pectin yields low-methoxylated molecules which, in the presence of calcium ions, form a strong gel.

Therefore the enzyme can be used in processing jams, jellies or ketchup in order to reduce or eliminate the necessity of adding thickening agents in order to achieve appropriate viscosity/gel structure. The enzyme could also be potentially infused into whole fruits or vegetables in order to firm them up.



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# Chapter 13

## Additional industrial enzymes

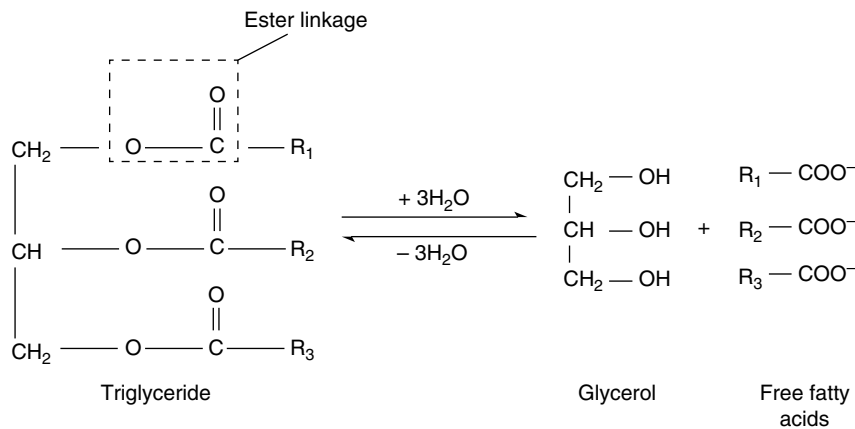
### 13.1 Lipases

Lipases are enzymes that, in aqueous media, catalyse the hydrolysis of lipids, yielding glycerol and fatty acids. The reaction is generally reversible (Figure 13.1), with the reverse (synthetic) reaction being favoured when undertaken in organic solvent-based media. Lipases exhibit little or no activity against soluble substrates in aqueous solutions. Instead they become activated only at the water-substrate (i.e. lipid) interface, a process termed interfacial activation. Lipases have been purified from a wide variety of mammalian, plant, fungal, yeast and bacterial sources. The three-dimensional structure of several lipases has been elucidated. These enzymes generally exhibit molecular masses ranging from 20 to 60 kDa, and despite relatively low sequence identity they all share similar aspects of three-dimensional architecture. For example, they display a characteristic  $\alpha/\beta$  hydrolase fold, with the enzyme's core composed of a  $\beta$  sheet consisting of up to eight stretches of  $\beta$  conformation, connected by  $\alpha$ -helical segments.

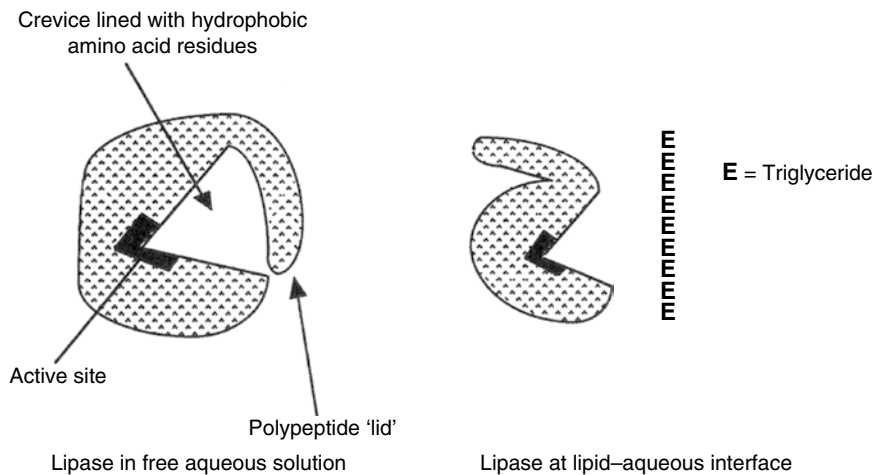
Lipases are often termed serine hydrolases as they possess a catalytic triad somewhat similar to that of

serine proteases (see Chapter 12). The lipase triad is generally composed of a serine and histidine residue along with an aspartic or glutamic acid residue. Active site topography is somewhat unusual in that it is surrounded by a large hydrophobic region. This entire area is in turn shielded/covered by a lid-like polypeptide loop composed of polar/charged (i.e. hydrophilic) amino acid residues. The presence of such a 'lid' likely explains why the enzyme displays no activity against soluble substrates in water. When the enzyme makes contact with the lipid interface, a conformational change is induced that makes the active site freely accessible to substrate molecules (Figure 13.2), hence explaining the aforementioned phenomenon of interfacial activation.

Industrial demand for lipases has grown steadily over the last two decades. The single most extensive application of lipases is their inclusion in detergent preparations. Lipases also find application in the food industry, in organic synthesis, in the paper/pulp processing industry and for the purposes of biodiesel production. The most common sources of commercial lipase preparations are various species of *Candida*, *Pseudomonas* and *Rhizopus*, although some commercial preparations are sourced from



**Figure 13.1** Reaction catalysed by lipases.



**Figure 13.2** Binding of lipase to the lipid-aqueous interface promotes a conformational change in the enzyme which makes the active site available to the substrate. Refer to the text for details.

*Bacillus subtilis*, *Aspergillus niger*, *Mucor*, *Humicola* and *Penicillium* species, as well as porcine pancreas. Some commercial lipases are produced by direct extraction of the enzyme from the native producer. However, greater emphasis is now placed on recombinant production, which overcomes barriers such as low natural enzyme expression levels and facilitates protein engineering.

### 13.1.1 Detergent applications

The first recorded application of a lipase in the laundry industry dates back to 1913, when Rhom

added pancreatic extracts to detergent preparations. However, surfactants and additional detergent components inactivated the pancreatic enzymes and it was not until the 1970s that researchers began to identify lipases suited to detergent application. Today over 1 million kg of detergent lipases are added to in excess of 13 billion tonnes of detergents annually (Table 13.1).

Lipid-based stains (Table 13.2) have long been recognized as the most difficult type of stain to remove from soiled clothing. In the past these stains were best removed by washing at high temperatures. The trend towards milder washing conditions (15–40°C) thus renders effective lipid removal

**Table 13.1** Major detergent lipase preparations now commercially available, their sources and manufacturers.

Trade name	Manufacturer	Lipase gene sourced from	Lipase gene expressed in
Lipolase	Novozymes	<i>Humicola lanuginosa</i>	<i>Aspergillus oryzae</i>
Lipomax	DSM	<i>Pseudomonas alcaligenes</i>	<i>Pseudomonas alcaligenes</i>
Lumafast	Danisco	<i>Pseudomonas mendocina</i>	<i>Bacillus</i> sp.
Lipolase Ultra	Novozymes	Protein engineered variant of lipolase	<i>Aspergillus oryzae</i>
Lipo Prime	Novozymes	Protein engineered variant of lipolase	<i>Aspergillus oryzae</i>

**Table 13.2** Lipid-based stains commonly found on soiled clothing/laundry.**Food-derived**

Butter and fat  
Edible oils  
Chocolate  
Salad dressing  
Mayonnaise  
Spaghetti sauce

**Body-derived**

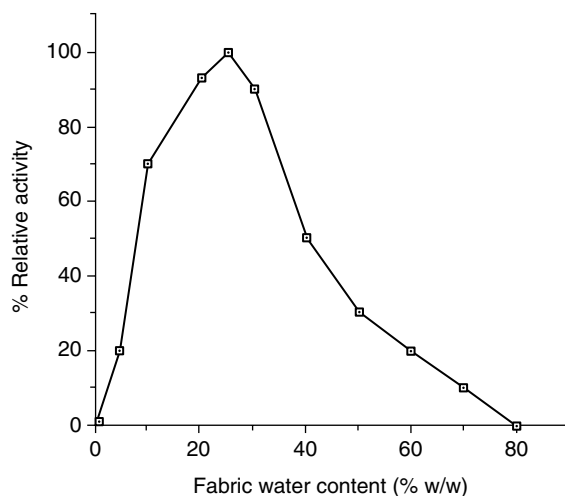
Sebum  
Sweat

**Cosmetics-derived**

Lipstick  
Mascara

even more difficult. Detergent lipases catalytically degrade water-insoluble lipid-based stains, yielding more water-soluble products such as monoglycerides and diglycerides, fatty acids and glycerol. These breakdown products are then more easily removed by detergent action into the washing liquor.

The inclusion of a low amount of a detergent-compatible lipase (usually added up to levels of 0.1% of total detergent) has proven effective in removing such lipid-based stains. However, the cleaning action does not become evident at the end of the first wash, the lipid-based stain only being completely removed after one (or sometimes more) subsequent wash

**Figure 13.3** The effect of water content of fabric on the activity of the detergent lipase Lipolase.

cycles. This phenomenon is known as the multi-cycle effect. It occurs because lipases are generally only poorly active on fully wetted textile. Maximum lipolytic activity (i.e. stain degradation) appears to occur during the spinning/drying of the fabric, when the textile water content has decreased to 10–40% (w/w) (Figure 13.3). As a result the bulk of lipid breakdown products are still present on the fabric after the first wash, and will only be physically removed during a subsequent washing cycle.

Like other detergent enzymes, various lipases have been subject to protein engineering in an attempt to develop more effective products. Targeted end points of such experiments include increasing the enzyme's stability in the presence of detergent constituents, increasing its specific activity or relaxing its substrate specificity (rendering it capable of degrading a greater variety of lipid-based stains). In some instances a non-specific approach was undertaken, for example the substitution of various amino acids at or close to the enzyme's active site, with screening of the variants produced to determine the effect on lipolytic activity. One commercial product (Lipomax, Table 13.1) was identified in this way. In this instance it was discovered that replacement of Met at position 21 with a Leu significantly enhanced wash performance. *In vitro* studies revealed that this substitution increased the enzyme's specific activity by up to 50%.

More specific approaches to protein engineering of lipases have also yielded commercial products. Lipid-based stains are presumed to have a net negative charge (contributed by fatty acids and by anionic surfactants bound to the stain surface). It was thus hypothesized that the replacement of negatively charged amino acid residues on the lipase surface with neutral (or positively charged) amino acids could enhance binding of the enzyme to the stain by minimizing charge repulsion. Novozymes researchers found that replacement of Asp<sup>96</sup> of *H. lanuginosa* lipase (i.e. lipolase) with Leu resulted in an engineered lipase with enhanced cleaning ability. This product was subsequently marketed under the trade name Lipolase Ultra (Table 13.1). Of potential use in designing lipase engineering experiments is the lipase engineering database ([www.led.uni-stuttgart.de](http://www.led.uni-stuttgart.de)), which holds sequence–structure–function information pertaining to a large number of microbial lipases.

### 13.1.2 Lipases in biodiesel production

Petroleum diesel is predominantly composed of saturated (i.e. no double bonds) hydrocarbons, typically ranging from 10 to 20 carbons in length. An example is cetane [*n*-hexadecane; C<sub>16</sub>H<sub>34</sub>, i.e. CH<sub>3</sub>(CH<sub>2</sub>)<sub>14</sub>CH<sub>3</sub>]. A range of biological fats and oils (e.g. vegetable oil and tallow) that are predominantly composed of triglycerides (Figure 13.1) can be converted into biodiesel via a transesterification reaction (Figure 13.4). The energy content and physical and chemical properties of such biodiesel are similar to those of conventional diesel fuel and can be used directly in existing engines. Moreover, biodiesel tends to have low exhaust emissions of particulate matter and greenhouse gasses such as CO, CO<sub>2</sub> and SO<sub>x</sub>.

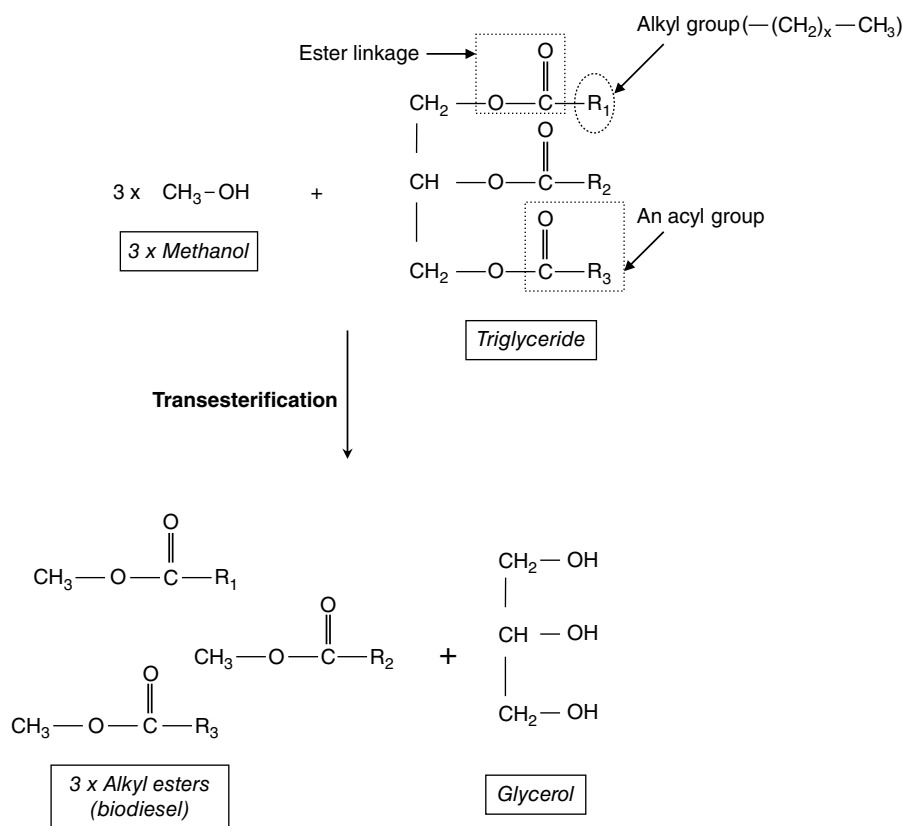
Transesterification of triglycerides can be carried out using different catalytic processes, both chemical and enzymatic. Chemical-based processes can use either alkali, acid or inorganic ions as catalysts, with alkali-based approaches being the most common. However, significant effort has been invested in

the development and refinement of an enzyme-based catalytic approach using lipase. Lipase-based transesterification can achieve high (>90%) biodiesel yields at near neutral pH and at temperatures of 20–50°C. However, lipase cost represents an economic drawback, which is somewhat ameliorated by the use of lipase in immobilized form (hence facilitating reuse). Two major immobilized lipase preparations used in this regard are Novozyme 435 (a lipase derived from *Candida antarctica*, produced by recombinant means in *A. niger* and immobilized on a macroporous acrylic resin) and Lipozyme (a *Mucor miehei*-derived lipase, immobilized on a macroporous ion-exchange resin).

### 13.1.3 Additional applications

The content and structure of constituent triglycerides has a major impact on the nutrition, flavour and sensory value of foodstuffs. Lipases have found application in the alteration or generation of flavour in certain foods. Enzyme-modified cheeses, for example, are produced by controlled proteolysis/lipolysis of traditional cheeses. The resultant production of flavour-rich peptides/free fatty acids generates a more intense flavoured product. Milk-derived cream can be converted into a product known as lipolysed milk fat by treatment with lipases. The free fatty acid mix generated yields a more intense cream/butter-like flavoured product, which in turn can be added to various products to give them such flavours. In bakery application, lipases are added to dough in order to enhance its gas holding-capacity, leading to increased dough stability, increased loaf volume and improvements in crumb structure and softness (Box 13.1). Wheat flour contains approximately 2.5% lipid. Degradation of triglycerides increases the polar lipid content in the dough, which appear responsible for these effects. During baking the high temperatures achieved then subsequently inactivate the added lipase.

Lipases also find increasing use in the pulp and paper industries, to facilitate pitch removal. 'Pitch' is a term used to describe hydrophobic constituents of wood. It is most prevalent in pine and other softwoods, contributing 0.5–3.0% of the wood by



**Figure 13.4** Transesterification reaction, in which methanol is the acyl group acceptor, generating alkyl esters (biodiesel) and glycerol from the original triacylglycerol (triglyceride) molecule. The alcohol most commonly employed is methanol, mainly for economic reasons.

weight. Pitch is largely composed of triglycerides and waxes. These substances lend a sticky property to wood that can cause serious processing problems during paper manufacture. Some of the pitch is removed by pulping and chemical bleaching. An alternative approach entails its enzymatic degradation using lipases. Lipase sourced from *Candida cylindracea* has found most application in this regard, and the enzyme is now employed commercially by some paper manufacturers.

Lipases also show potential in organic synthesis. The stereospecificity of the enzyme is a major advantage in this regard, particularly in the production of some pharmaceutical products/intermediates. Many drugs used in medicine are produced by direct chemical synthesis. If the component synthesized contains a chiral centre, a racemic mixture (equal quantities of D and L isomers) is generally

produced. Usually only one isomer will promote the desired biological effect, with the other isomer being biologically inactive. In some cases, however, this 'other' isomer can exert negative biological activity and must be removed from the final product. Enzymes can potentially be used to separate isomers or to selectively synthesize the required isomer (see also section 13.3, detailing amino acylase). For example, lipases show potential in this context for the production of captopril, an angiotensin-converting enzyme inhibitor.

## 13.2 Penicillin acylase

The discovery of penicillin heralded a revolutionary advance in the medical control of bacterial disease. Subsequently, a variety of other compounds

**Box 13.1 Product case study: Panamore™**

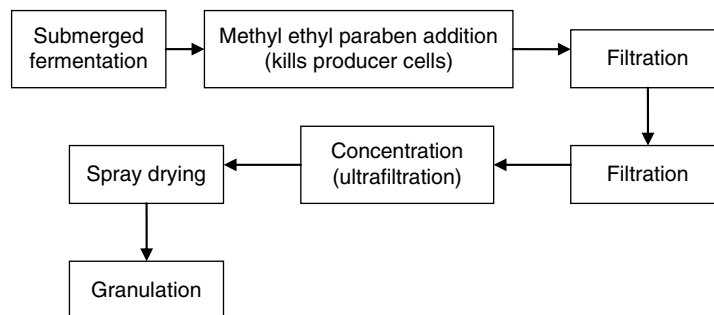
Panamore™ is the trade name given to a recombinant lipase preparation commercialized by DSM. It is applied in the food (bakery) industry in order to enhance the gas-holding capacity of dough, as outlined in the section 13.1.3.

The production organism is a well-characterized *Aspergillus niger* strain, derived ultimately from *A. niger* strain NRRL 3122 by both initial classical mutagenesis and further knowledge-based recombinant manipulation. The producer strain has been engineered in order to prevent it from producing native extracellular glucoamylase, amylase and protease activity. It carries a synthetically produced gene whose sequence is based on preprolipase gene sequences characteristic of several *Fusarium* species. The resulting preprolipase is 346 amino acids in size, while the processed (mature) lipase product is a 247 amino acid, 28.7-kDa glycoprotein.

Product manufacture is via submerged feed-batch aerobic fermentation in a stirred tank

fermenter as overviewed schematically below. The fermentation medium contains glucose, yeast extract, a cocktail of inorganic salts (potassium chloride, manganese sulfate, zinc sulfate, copper sulfate, magnesium sulfate and iron sulfate), as well as phosphoric acid and the antifoam agent ethoxylated propoxylated glycerol oleate. The fermenter is continuously fed with aseptically introduced sterilized fermentation media.

Fermentation is stopped by addition of methyl ethyl paraben under conditions that effectively kill the production organism. The enzyme is recovered (separated from the biomass) via a membrane filtration step. A second filtration step is undertaken to ensure the complete removal of any remaining viable producer cells. The enzyme solution is then concentrated via ultrafiltration, spray-dried and granulated using wheat flour as a granule filling agent. Approximately 10% of enzyme activity is lost during spray-drying.

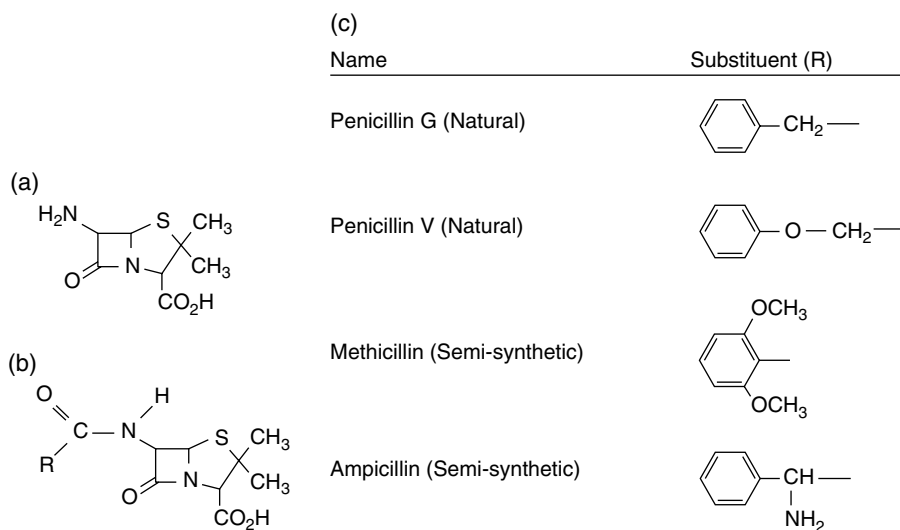


exhibiting antimicrobial activity have been introduced into the clinical arena. The penicillins and the structurally related cephalosporins remain the most popular antibiotics prescribed by the medical community, commanding combined annual sales value in the region of \$15 billion. The passage of time has witnessed an increasing number of bacterial populations resistant to the antimicrobial action of penicillin. Resistance has been counteracted in part by the development of semi-synthetic penicillins, to which many such resistant strains

remain sensitive. The enzyme penicillin acylase plays an essential role in the production of such semi-synthetic penicillins, and hence is the subject of significant industrial demand.

The chemical structures of some naturally occurring and semi-synthetic penicillins are illustrated in Figure 13.5. All contain an identical core ring structure termed 6-amino-penicillanic acid. Different penicillin types differ in their attached side chains. Semi-synthetic penicillins may be produced by the enzymatic removal of the side chain of



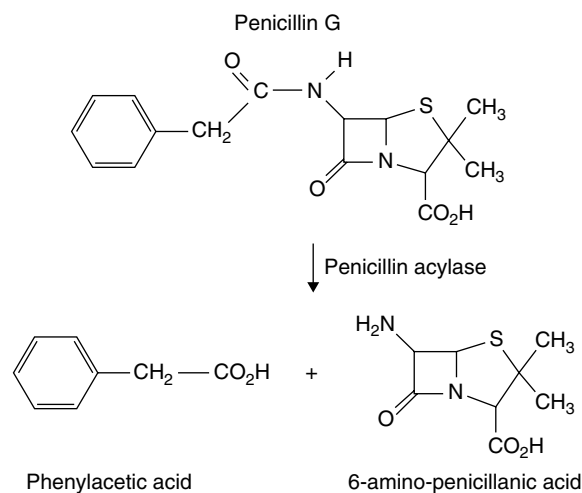


**Figure 13.5** Structure of 6-amino-penicillanic acid (a), generalized penicillin structure (b) and the side groups present in two natural penicillins and two semi-synthetic penicillins (c).

native penicillins, with subsequent attachment of a novel side chain to the resultant 6-amino-penicillanic acid core. The removal of the side chain from penicillin G is illustrated diagrammatically in Figure 13.6. The reaction catalysed by penicillin acylase (penicillin amidase or penicillin amidohydrolase) is generally reversible. The hydrolytic reaction is catalysed under alkaline conditions, while the acylation (biosynthetic) reaction is favoured at neutral/acidic pH (4–7). In this way penicillin acylase may be used to convert native penicillins to semi-synthetic products. Annual global production of the 6-amino-penicillanic acid intermediate is estimated to be in the region of 7000 tonnes.

Quite a number of microorganisms produce penicillin acylase (Table 13.3). Among these, the enzyme produced by *Escherichia coli* has received most attention. The enzyme is a heterodimer, composed of a 20–23 kDa subunit that houses the (penicillin) side chain-binding site and a 60–69 kDa subunit housing the catalytic site. The latter contains a serine residue essential to activity. Although it is possible to utilize the free enzyme, an immobilized form is generally used in the production of semi-synthetic penicillins as this is more attractive economically, facilitating reuse of the enzyme over many production runs.

The increasing incidence of bacterial resistance to natural penicillins renders crucial the continued



**Figure 13.6** Action of penicillin acylase on penicillin G, a natural penicillin produced in large quantities by fermentation. The reaction proceeds in the direction indicated under alkaline conditions. As one of the reaction products is an acid, the pH must be continually adjusted to maintain alkaline values. On completion of the conversion, the pH may be adjusted downwards to a value of 4.2–4.3. At this pH, 6-amino-penicillanic acid precipitates from solution and thus may be harvested. Some penicillin acylase preparations are also capable of catalysing the reverse reaction under mildly acidic conditions. Such enzyme preparations may thus be employed in the synthesis of semi-synthetic penicillin from 6-amino-penicillanic acid and the relevant side-chain group.

**Table 13.3** Various microorganisms that produce penicillin acylase.

---

<i>Escherichia coli</i>
<i>Pseudomonas</i> spp.
<i>Proteus rettgeri</i>
<i>Proteus morgani</i>
<i>Brevibacterium</i> spp.
<i>Bacillus</i> spp.
<i>Flavobacterium</i> spp.
<i>Streptomyces</i> spp.
<i>Aerobacter</i> spp.
<i>Kluyvera</i> spp.

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production of semi-synthetic penicillins. In addition to overcoming the problem of resistance, several semi-synthetic penicillins exhibit improved clinical properties. Many inhibit a greater variety of bacterial pathogens than, for example, penicillin G. Others are more acid-stable and hence are particularly suited for oral administration.

The advent of genomics and recombinant DNA technology has not left untouched the discipline of antibiotic production. These technologies have facilitated the detailed molecular elucidation of biosynthetic pathways for  $\beta$ -lactam antibiotics, such as penicillins and cephalosporins. A number of genes coding for specific enzymes involved in  $\beta$ -lactam biosynthesis have been cloned and characterized. Introduction of additional copies of such genes into producing microorganisms facilitates logical strain improvement. Such genetic manipulations have led to enhanced production of certain industrial strains of antibiotic-producing fungi. An increased understanding of  $\beta$ -lactam biosynthetic pathways could also make possible alteration of such pathways at the molecular level in order to produce novel antibiotic molecules.

### 13.3 Amino acylase and amino acid production

Unlike many microorganisms, most animals cannot synthesize all the naturally occurring amino acids required for protein synthesis. Those essential amino acids that animals are incapable of producing must be procured from dietary sources. Nutritionists

have long recognized that protein dietary constituents must contain a well-balanced amino acid composition, in terms of essential amino acids, in order to ensure optimal animal growth.

Both lysine and threonine constitute essential amino acids in the mammalian diet. They also generally represent the first and second limiting amino acid for pigs and poultry fed a cereal-based diet. Maximal utilization of cereal-derived protein requires addition of almost 4 kg of crystalline lysine and almost 2 kg of threonine per tonne of feed. Other essential amino acids which may become limiting, in particular if animals are fed diets high in soybean, include the sulfur-containing amino acids cysteine and methionine, and also tryptophan.

The problem of attaining a well-balanced dietary amino acid complement could be overcome by generating transgenic animals capable of synthesizing their own essential amino acids. An alternative strategy entails the generation of transgenic plants capable of producing proteins with altered amino acid composition. The major proteins present in soybean are deficient in methionine and cysteine. A number of alternative seed proteins rich in methionine have been identified. Production of these proteins in transgenic soybean could negate the necessity to supplement soybean-based rations with methionine, and as cysteine can be synthesized from methionine it would also not be required. However, such projects are longer term in nature and require a degree of public acceptance of this approach that is not yet attained in some global regions. In the mean time, nutritionists must ensure that animals are fed rations containing an optimal amino acid balance. This often necessitates supplementation of the diet with specific amino acids.

In addition to the amino acids required in bulk quantities by the animal feed industry, a number of other amino acids are required in bulk for a variety of additional industrial processes. Glutamic acid, for example, is widely used in the food industry. Inclusion of its sodium salt (monosodium glutamate) enhances the natural flavour associated with many food types. Hundreds of thousands of tonnes of this amino acid are now produced annually. The development of the sweetener aspartame

(Chapter 12) has substantially increased the industrial demand for its two amino acid constituents, phenylalanine and aspartic acid. Other amino acids are also required in moderate quantities for medical, research and other specialist purposes.

Amino acids may be produced either chemically or enzymatically. Chemical production has traditionally been the method of choice, mainly on economic grounds. However, chemical production yields a racemic mixture consisting of the D and L isoforms of the amino acids. As only the L form is biologically utilizable by most higher species, separation of L from D isoforms is pursued subsequent to chemical synthesis. This is most readily achievable by utilizing the enzyme L-amino acylase.

The racemic amino acid mixture is firstly chemically acetylated, usually by reaction with acetyl chloride or acetic anhydride. The mixture is then passed over a bed of immobilized L-amino acylase. This enzyme will deacylate only the L-form of the acylated amino acids, yielding a free L-amino acid and the intact N-acetyl-D-amino acid (Figure 13.7). These can be conveniently separated via ion-exchange chromatography or crystallization. The N-acetyl-D-amino acid can then be re-racemized either chemically (by addition of acetic anhydride under alkaline conditions) or enzymatically (using a racemase). The racemate can then again be passed over the immobilized amino acylase. This process, now used to produce several hundred tonnes of enantiomerically pure L-amino acids each year, was first commercialized in 1969. The amino acylase

used industrially is sourced from *Aspergillus oryzae*. It is a 73-kDa, dimeric, zinc-containing metalloprotein that is activated by cobalt ions. It displays a pH optimum of 8.5 and is stable up to temperatures approaching 60°C. Various immobilization methods have been evaluated for industrial use, including immobilization via ionic attraction on ion-exchange (DEAE Sephadex) media, covalent attachment to iodoacetylcellulose and gel entrapment. Immobilization on DEAE Sephadex has found traditional industrial favour because:

- preparation of the immobilized enzyme is easy;
- the activity of the immobilized enzyme remains high and stable (maintaining 60–70% of initial activity after continuous operation at 50°C for 30 days);
- regeneration of the immobilized column with fresh enzyme is straightforward;
- the carrier (i.e. DEAE Sephadex) is itself safe and stable (displaying an industrial working life counted in years).

A more thermostable amino acylase has been isolated from *Bacillus stearothermophilus*. It exhibits an activity optimum at 70°C and has been expressed in various recombinant systems. D-amino acylases may be used to produce enantiomerically pure amino acids, although demand for the D-isomer is very significantly lower than that for L-amino acids. D-amino acylases have been purified from various species of *Pseudomonas*, *Streptomonas* and *Streptomyces*.

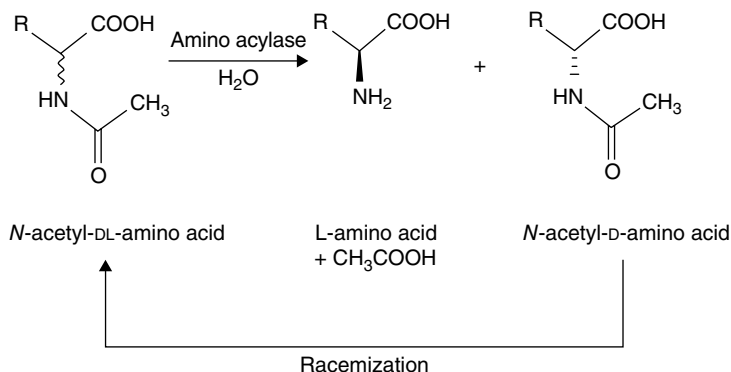


Figure 13.7 Reaction catalysed by amino acylase.

## 13.4 Cyclodextrins and cyclodextrin glycosyltransferase

Cyclodextrins are cyclic oligosaccharides enzymatically derived from starch. Three main cyclodextrin types have been identified (Figure 13.8).  $\alpha$ -Cyclodextrins are composed of six glucose

molecules,  $\beta$ -cyclodextrins are composed of seven glucose molecules, while  $\gamma$ -cyclodextrins consist of eight glucose molecules. In each case, individual glucopyranose units are linked to each other via  $\alpha 1 \rightarrow 4$  glycosidic linkages characteristic of linear starch molecules.

Cyclodextrins were first studied over 100 years ago, when their presence was noted in bacterial digests of starch. However, it is only within the

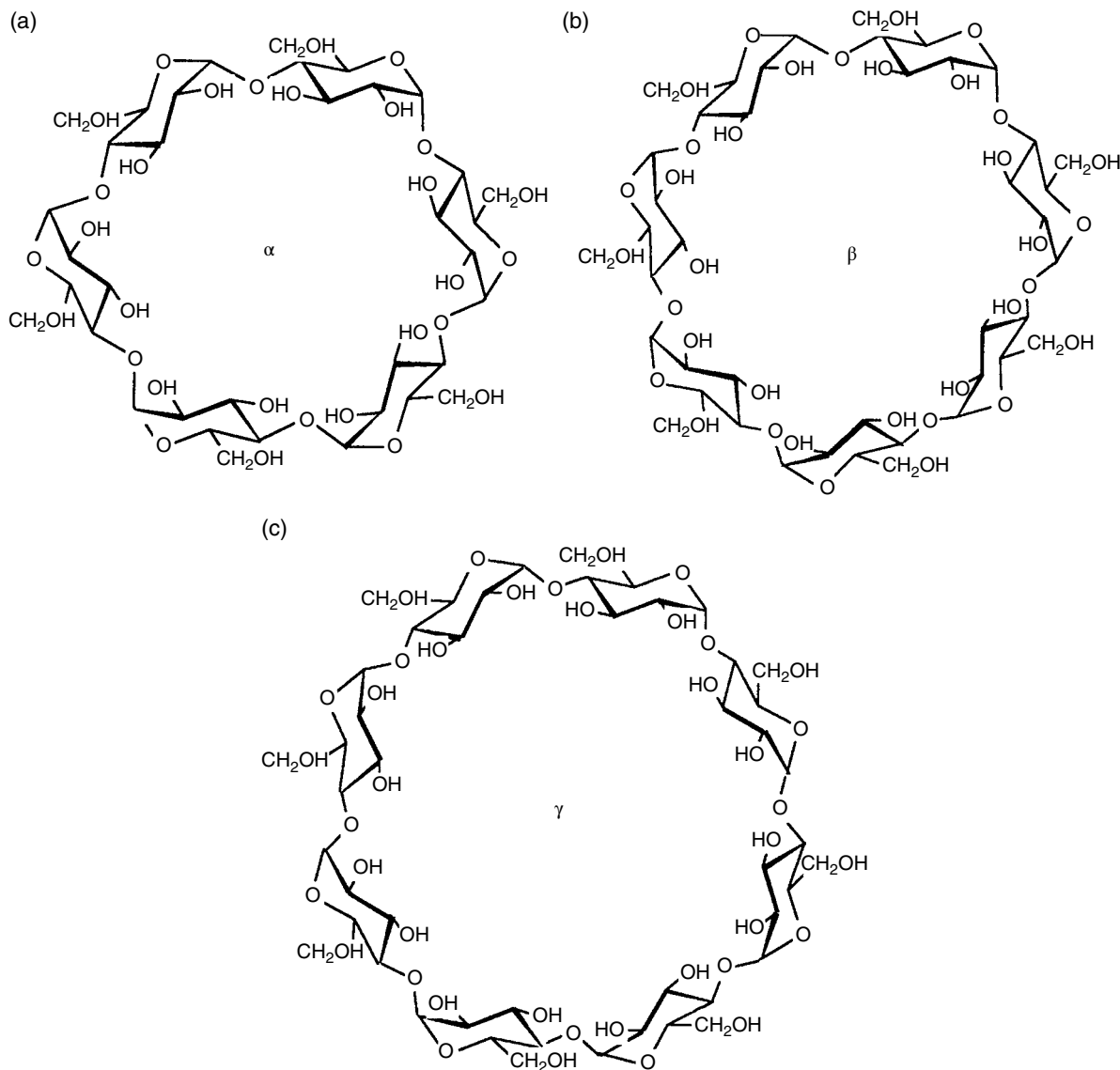


Figure 13.8 Structures of  $\alpha$ ,  $\beta$  and  $\gamma$  cyclodextrins.

recent past that these substances have begun to enjoy widespread industrial demand. They are utilized in the pharmaceutical industry as well as in the food, cosmetic and allied industries.

Cyclodextrins are doughnut-shaped molecules, the outer surface of which is hydrophilic in nature while their internal cavity is apolar. When dissolved in aqueous media, the internal cavity is occupied by water molecules. The polar nature of water renders this energetically unfavourable. Added substances that are less polar than water, and which are of appropriate molecular dimensions, will replace the water as 'guest molecules' within the cyclodextrin cavity. Such guest molecules are retained within the cavity solely by non-covalent interactions. The overall guest molecule-cyclodextrin structure is termed an inclusion complex.

Only molecules of an appropriate size will form stable inclusion complexes. The internal cavity of  $\alpha$ -cyclodextrins is obviously the smallest. This cavity will only accommodate molecules/elements of low molecular mass, such as chlorine, bromine or iodine. Much larger molecules such as steroids or antibiotics may be accommodated in the cavity of  $\gamma$ -cyclodextrin molecules. Specific side groups present in macromolecules may also interact with the cyclodextrin cavity and, in this way, form a complex.

Of the three basic cyclodextrins, the  $\beta$  form is by far the most commonly employed on an industrial scale. The internal dimensions of the  $\beta$ -cyclodextrin cavity make it ideally suited for a variety of applications.  $\beta$ -Cyclodextrins are also economically attractive as they are the least expensive to produce. However, native  $\beta$ -cyclodextrins exhibit poor solubility characteristics. A 14% solution of  $\alpha$ -cyclodextrins and a 23% solution of  $\gamma$ -cyclodextrins are readily achievable in aqueous media. However, the maximum solubility of  $\beta$ -cyclodextrin in water is of the order of 1.8 g/dL, i.e. 1.8%. Such poor solubility characteristics limit the industrial potential of the native  $\beta$ -cyclodextrin molecule.

This limitation has been overcome by the introduction of modified  $\beta$ -cyclodextrins. Substitution of the cyclodextrin hydroxyl groups with a variety of alkyl, ester or other residues dramatically improves solubility while having no effect on its complex-forming ability. Species such as hydroxypropyl

$\beta$ -cyclodextrin and hydroxyethylated  $\beta$ -cyclodextrin are now subject to increasing industrial demand.

Interaction of suitable substances with cyclodextrins effectively results in their molecular encapsulation. Encapsulation may be advantageous for a number of reasons. The guest molecule is protected from a wide variety of chemical and other reactants that might otherwise lead to its destruction. Such protected molecules are less likely to undergo polymerization or autocatalytic reactions. Complex formation may also mask undesirable tastes or odours normally associated with the guest molecule. Volatile compounds may be effectively stabilized by complexation with appropriate cyclodextrins. For example, volatile aroma concentrates sensitive to oxygen, light or heat are often stabilized in this way.

Crystallization of cyclodextrin complexes can effectively convert into powder guest molecules originally present in liquid form. This is often of particular significance in the pharmaceutical industry. Another significant consequence of complex formation is the solubilization of hydrophobic substances in aqueous solution. A variety of medically important drugs are hydrophobic in nature and are known to form stable inclusion complexes. Such hydrophobic drugs can be safely transported through the bloodstream and gastrointestinal tract in such a format. The hydrophobic drug is then released at the cell surface due to the hydrophobic nature of the plasma membrane's lipid bilayer. Cyclodextrins are therefore used medically to improve the bioavailability of a range of poorly soluble medicinal substances. This can often allow administration of lower dosage levels with consequent economic, therapeutic and other benefits.

Cyclodextrins may also be used to stabilize certain proteins. The macromolecular structure of the smallest polypeptide precludes complete inclusion-complex formation. However, virtually all proteins comprise amino acid subunits containing non-polar or hydrophobic side chains. Cyclodextrins may freely interact with such side chains and in this way become intimately associated with the protein molecule. In some instances, and at high concentrations, cyclodextrins may actually destabilize or denature proteins. Under most circumstances, however,

protein–cyclodextrin interactions serve to enhance protein stability. Cyclodextrins have been shown to reduce loss of enzymatic activity due to chemical or physical influences such as heating, freeze-drying, storage or the presence of oxidizing agents.

On standing in aqueous solution, many proteins undergo limited aggregation. Powdered protein preparations, such as freeze-dried products, also sometimes form aggregates on reconstitution in an aqueous media. Aggregate formation prevents intravenous administration of any preparation. Formulation of biopharmaceutical products in order to minimize or eliminate protein–protein interactions does not always yield satisfactory results. Many important biopharmaceutical products, such as growth hormone, urokinase and interleukin-2, still exhibit a marked tendency towards aggregate formation on reconstitution. In many instances, inclusion of a suitable cyclodextrin preparation may potentially eliminate such undesirable intermolecular interactions.

The increasing industrial demand for cyclodextrins is reflected in an increased demand for cyclodextrin glycosyltransferase (CGTase) preparations. CGTase is the enzyme used in the synthesis of cyclodextrins from substrate starch molecules. CGTases belong to family 13 of the glycoside hydrolases ( $\alpha$ -amylase family). An active-site aspartate and glutamate residue are generally directly involved in catalytic transformation. Cyclodextrins may also be synthesized chemically but their enzymatic production is technically and economically more attractive.

Three major types of CGTase have been identified:  $\alpha$ ,  $\beta$  and  $\gamma$ . As the name suggests,  $\alpha$ -CGTase predominantly yields  $\alpha$ -cyclodextrins, whereas  $\beta$ - and  $\gamma$ -CGTase yield  $\beta$ - and  $\gamma$ -cyclodextrins respectively. In all cases, however, prolonged reaction times result in the formation of a mixture of all three cyclodextrin types, with  $\beta$ -cyclodextrin representing the predominant reaction product. CGTases can also produce larger cyclodextrins. So-called 'large ring cyclodextrins' most often contain between 9 and 32 glucopyranose residues, although these are produced at very low yields.

Most CGTases characterized to date are derived from various *Bacillus* species (Table 13.4). The genes coding for many of these enzymes have been

**Table 13.4** Some bacterial producers of cyclodextrin glycosyltransferase.

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<i>Bacillus stearothermophilus</i>
<i>Bacillus megaterium</i>
<i>Bacillus circulans</i>
<i>Bacillus subtilis</i>
<i>E. coli</i> (recombinant)

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identified, sequenced and expressed in a variety of recombinant systems. Elevated levels of heterologous CGTase have been produced in host species such as *E. coli* and *B. subtilis*. Increased production capacity of CGTases should help reduce the overall cost of cyclodextrin preparations, which in turn is likely to promote increased utilization of cyclodextrins in a variety of industrial applications.

## 13.5 Enzymes and animal nutrition

Higher organisms have developed sophisticated digestive systems by which they procure nutrients from ingested matter. Degradation of polymeric nutrients, such as proteins, carbohydrates and lipids, is usually a prerequisite to efficient nutrient assimilation. Most such degradative events are mediated by specific digestive enzymes. Evolutionary pressure has ensured the development of an efficient digestive system and the advent of modern intensive livestock production places added pressure on this digestive process. Biotechnological intervention can serve to redress any digestive imbalance caused by such modern production methods.

Weaning of young animals such as piglets serves as a good example. Modern production practices transform weaning from a gradual process to an abrupt event. The sudden alteration in dietary composition from a milk-based feed to one with a more complex nutritional composition frequently causes digestive upsets in young animals. Young piglets often display a physiological deficiency in gastric acid production. Stomach pH may therefore be above values required for optimal digestive function.

In addition, the overall digestive capability of such animals may not have fully developed at the

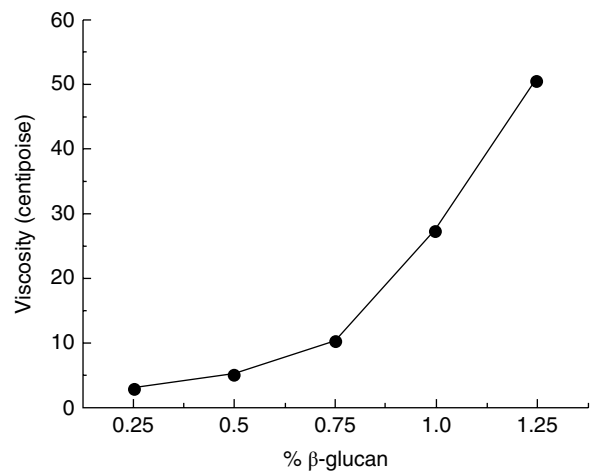
time of weaning. Poorly digested food results in suboptimal nutrient assimilation by the weaned animal and incompletely digested matter also promotes vigorous growth of many microorganisms in the large intestine. Such factors contribute to digestive upsets and an increased incidence of post-weaning scours.

Digestive difficulties associated with weaning may be averted by feeding specially formulated, readily digestible rations, and the inclusion of acidifiers such as citric acid in the diet to generate a low stomach pH. The addition of exogenous enzymes capable of hydrolysing more complex components of the weaned animal's rations also promote increased feed digestibility. Proteolytic enzymes, in addition to cellulases/hemicellulases, have all been used as digestive aids for weanlings. Not surprisingly, the beneficial effects of exogenous enzyme addition is most noticeable when animals are fed rations containing more complex dietary components.

### 13.5.1 Removal of anti-nutritional factors

Various enzymatic preparations have also been used to bring about the removal of specific anti-nutritional factors from animal feeds. The addition of  $\beta$ -glucanase to barley-containing poultry feed is perhaps the most noted such example. The inclusion of xylanase in wheat-based poultry diets serves as an additional example. Incorporation of phytase in cereal-based animal feedstuffs not only removes a major anti-nutritional factor but may also considerably reduce the polluting effect of animal wastes (as discussed later).

$\beta$ -Glucan is a non-starch polysaccharide associated in particular with barley. Structurally it consists of glucose units linked via  $\beta 1 \rightarrow 3$  and  $\beta 1 \rightarrow 4$  linkages. The level of  $\beta$ -glucans present in barley can vary considerably and is influenced by factors such as soil type, growing conditions and time of harvesting. Although it may be present at levels below 2–3%, values in excess of 10% of grain content have also been recorded. When ingested, the  $\beta$ -glucans present in barley become solubilized in the gut. Animals are



**Figure 13.9** The effect of increasing  $\beta$ -glucan concentrations on viscosity in an aqueous solution.

devoid of endogenously produced digestive enzymes capable of hydrolysing the  $\beta$ -glucan molecule.

Soluble  $\beta$ -glucans form highly viscous solutions (Figure 13.9), and thus their presence in the diet promotes formation of highly viscous digesta. This has a particularly negative effect on the digestive function of poultry. Maximum nutrient utilization is impeded and the animals develop difficulty in passing faeces. Because of its viscous nature, excreted faeces adhere to the birds' feathers, to their bedding and to the eggs of laying hens. The multiple negative effects associated with ingested  $\beta$ -glucans has traditionally limited the level of incorporation of barley in poultry feed, although on a food cost basis it may appear attractive to do so.

Some microbial populations produce enzymes termed  $\beta$ -glucanases. These enzymes are capable of hydrolysing  $\beta$ -glucans and incorporation of  $\beta$ -glucanase preparations in poultry diets now facilitates the inclusion of increased levels of barley in such diets. On ingestion, the microbially derived  $\beta$ -glucanase degrades  $\beta$ -glucans present, thus destroying their anti-nutritive properties and providing sugars for energy. This can significantly increase animal growth performance (Table 13.5).

Wheat and indeed other cereals contain xylans, which can have an anti-nutritive effect, particularly in poultry. The digestive complement of animals is incapable of hydrolysing xylans and, as in the case of

**Table 13.5** Effect of supplementing barley-based diets with  $\beta$ -glucanase on performance of poultry. The wheat-based diets serves as a control. Bird performance may be assessed by the feed to weight gain ratio. The lower this value, the more efficiently the bird has utilized the feed provided.

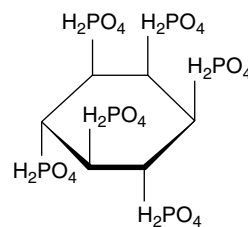
Dietary treatment	Feed consumption per bird (g) per day	Weight gain per bird (g) per day	Feed-gain ratio
Wheat-based diet	102.3	49.9	2.05
Barley-based diet	91.5	41.5	2.2
Barley and $\beta$ -glucanase	96.3	50.2	1.92

$\beta$ -glucans from barley, solubilization of some xylans in the gastrointestinal tract results in increased viscosity of digesta. Addition of microbial xylanase preparations to wheat-based diets will destroy these anti-nutritional factors.

### 13.5.2 Phytase and phytic acid

Over 75% of the total phosphorus present in most cereals is in the form of phytic acid, also known as phytin or *myo*-inositol hexaphosphate (Figure 13.10). The phosphate groups present in phytic acid are biologically unavailable to non-ruminant animals. Such animals lack the enzymatic activity required to release the phosphate groups from the core ring structure. In ruminant species, phytic acid is degraded by microbial populations within the rumen. Lack of phosphorus availability in monogastric animals, such as pigs and poultry, renders likely the possibility of dietary phosphorus deficiency. This scenario is generally avoided by the addition of inorganic phosphorus in the form of dicalcium phosphate to such animal feeds. Phytic acid is also an anti-nutritional factor as it binds a range of essential minerals such as calcium, zinc, iron, magnesium and manganese within the digestive tract and hence renders these unavailable for absorption.

The digestive complement of animals is devoid of an enzymatic activity capable of degrading phytic acid. Several such phytase enzymes (*myo*-inositol hexaphosphate hydrolases) are expressed in plants



**Figure 13.10** Structure of *myo*-inositol hexaphosphate (phytic acid or phytin).

and in a variety of biological systems. Phytase activity has been detected in a wide range of plants, particularly in germinating seeds. These sources include cereals such as wheat, corn, barley and triticale as well as beans (e.g. navy, mung and dwarf beans). Wheat, rye and triticale are among the richest known sources of plant phytase. Phytase is also produced by various bacteria, including *E. coli*, *Bacillus*, *Klebsiella* and *Pseudomonas*. Bacterial phytases are generally produced at low levels, are intracellular and display activity optima at neutral to alkaline pH. The only bacterium known to produce extracellular phytase is *B. subtilis*. The extracellular phytase of *B. subtilis* (strain natto N-77) has been purified to homogeneity using a combination of gel filtration and ion-exchange (DEAE) chromatography. The active enzyme is a monomer, displaying a molecular mass of 36 kDa, an isoelectric point of 6.25 and optimum activity at 60°C.

Unlike bacteria, fungi produce a range of extracellular (as well as intracellular) phytases. *Aspergillus* species are the most prolific producers, particularly *A. niger*, *A. flavus* and *A. candidus*. Various species of *Rhizopus* and *Penicillium* also produce phytase activity. The extracellular fungal phytases tend to exhibit molecular masses ranging from 35 to 100 kDa, isoelectric points of 4–6.5, optimum temperatures of 35–65°C and pH optima ranging from 2.5 to 7. Various yeasts such as *Saccharomyces cerevisiae* also produce phytase. Synthesis of most microbial phytases is repressed by high levels of inorganic phosphate in the microbial growth media.

Addition of microbial phytase to dietary rations has been shown to promote degradation of phytic acid within the digestive tract. Such enzyme-mediated degradation has several associated beneficial effects. Degradation of phytate destroys



**Table 13.6** Typical effects of phytase on phosphorus balance in pigs. Phytase diet was similar to control diet but was supplemented with phytase. Both phosphorus intake and absorption were monitored. Inclusion of phytase resulted in increases in absorbed and retained phosphorus. All differences recorded were found to be statistically significant.

Parameter	Phytase diet	Control diet
Phosphorus intake (g/day)	8.3	7.3
Phosphorus absorbed (g/day)	5.4	3.5
Per cent absorption	65	48
Per cent retained	60	47

its anti-nutritive effect, thus promoting increased mineral absorption. The phosphate groups released from the *myo*-inositol ring are rendered biologically available to the animal (Table 13.6), and this facilitates a significant reduction in the quantities of supplemental inorganic phosphorus that must be added to the diet.

Phosphate pollution derived from the animal production sector has long been a source of concern. It is estimated that over 100 million tonnes of animal manure is generated in the USA each year. Such quantities would contain in excess of 1 million tonnes of phosphorus. Although animals fail to digest phytic acid, many microorganisms present in faeces display appreciable levels of phytase activity. This form of phosphorus may therefore greatly contribute to the polluting effect of animal slurry.

Early phytase preparations used in animal trials were sourced from various strains of phytase-producing fungi. Initial screening studies identified *A. niger* NRRL 3135 (formally known as *A. ficuum*) as the microbial strain producing phytase in greatest quantities, and hence phytase was mainly obtained from this source. *Aspergillus niger* 3135 produces two phytases, A and B. Phytase A (Phy A) is an 85-kDa, 448 amino acid glycoprotein containing a total of nine glycosylation sites. The enzyme displays activity over a pH range of 2–7, with two distinct activity peaks at pH 2.5 and 5.5. Phytase B (Phy B), on the other hand, displays activities over a narrower pH range (2–3). *Aspergillus niger* NRRL 3135 also produces a third extracellular phosphatase, but this

is devoid of phytase activity (i.e. it dephosphorylates phosphorylated substances other than phytic acid).

Phytase production often entails fermentation of *A. niger*, either by surface culture (e.g. using wheat bran-based media) or sometimes by submerged fermentation (e.g. using a corn starch medium). Low natural production levels coupled with phosphate repression of enzyme synthesis renders this product expensive. Although traditional mutational and selection techniques has generated mutants producing increased quantities of enzyme, the vast bulk of phytase now added to animal feed is produced by recombinant means.

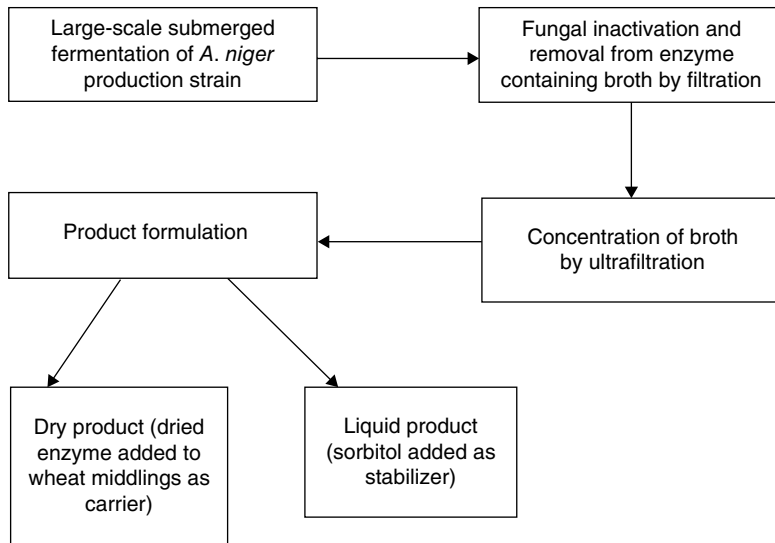
Natuphos is the trade name given to recombinant phytase A (originally) derived from *A. niger* NRRL 3135. Developed by DSM, the coding sequence for the enzyme was placed under a powerful promoter (the glucoamylase promoter sourced from an industrial strain of *A. niger* used by DSM to produce glucoamylase at levels of several grams per litre). Multiple copies of the construct were inserted into *A. niger* (self-cloning). High-level expression (and removal of phosphate repression) renders possible large-scale economic production of the product (Figure 13.11). The phytase A was chosen as it appears to best fulfil various criteria characterizing an enzyme suitable for in-feed application (as discussed later).

A phytase gene obtained from *A. niger* has also been expressed in transgenic tobacco seeds. The recombinant enzyme was expressed as 1% of the soluble protein in the mature seed. Animal trials confirmed that direct application of these seeds to animal feed promoted enhanced phytic acid phosphorus utilization by recipient animals.

### 13.5.3 Factors affecting feed enzyme efficacy and stability

The choice of an enzyme best suited for feed application is not straightforward, as such enzymes must display several characteristics, of which the more important include the following.

- *Enzyme thermostability.* Virtually all animal feed is heat-treated prior to sale. Heat treatment can form part of the feed pelleting process, where



**Figure 13.11** Overview of the process used by DSM to produce their recombinant phytase product Natuphos.

moist heat is applied followed by mechanical pressing. Heat treatment is essential for both pelleted and non-pelleted feed in order to diminish or eliminate the possibility of accidental transmission of pathogens via infected feed. The process typically heats the feed to temperatures of 75–90°C for a period of up to 2–3 minutes. Enzymes added to animal feed must therefore be thermostable. (It is possible to apply an enzyme to feed post heat treatment by spraying. However, the spraying equipment is expensive and uneven enzyme application can sometimes occur.)

- *Activity vs. temperature profile.* The enzyme must display significant activity at 37°C, the temperature at which it is destined to function when ingested by the animal.
- *pH vs. activity profile.* The enzyme must be active at pH values typical of one or more regions of the digestive tract. Most fungal-derived feed enzymes display maximal activity at acidic pH values and hence are likely to be most active in the stomach and upper portion of the small intestine (the duodenum). Conversely, bacterial enzymes (e.g. bacterial amylases or proteases) exhibit appreciable activity at neutral or near-neutral pH values, rendering the small intestine the most likely site of activity for these.

- *Stability in the presence of gastrointestinal tract influences.* Enzymes added to animal feed must remain stable in regions of the digestive tract up to (and including) the area in which they promote their intended effect. In all cases this means that the enzyme must be stable under the acidic conditions encountered in the stomach (pH can drop as low as 1.5), and must be resistant to digestive proteases such as pepsin.

The majority of enzymes added to animal feed display optimum activity at temperatures of 50–60°C. When heated in buffer to temperatures in excess of 70°C, such enzymes rapidly lose activity. However, when present in feed they appear significantly more thermostable, probably due to a protective effect exerted by the bulk feed constituents, and the lower water content of the system. Bulk feed constituents may well also exert a protective influence on enzymes in the stomach environment.

#### 13.5.4 Detection of enzymes after their addition to feed

Regulatory requirements, as well as concerns relating to the effect of pelleting on enzyme activity for example, renders necessary the development of

**Table 13.7** Enzymes most often added to animal feed and the natural substrates that are most often used in their assay.

Enzyme	Substrate	Reaction product monitored
Phytase	Phytic acid	Inorganic phosphate
$\beta$ -Glucanase	$\beta$ -Glucan or lichenan*	Reducing sugars
Xylanase	Xylan	Reducing sugars
Cellulase	Cellulose/modified cellulose (e.g. carboxymethylcellulose)	Reducing sugars
Protease	Haemoglobin, casein, albumin	Amino acids/peptides
Amylase	Starch	Monitoring of starch disappearance

\* Lichenan is an inexpensive glucan obtained from *Cetraria islandica* which, like  $\beta$ -glucan, consists of glucose molecules linked via  $\beta 1 \rightarrow 3$  and  $\beta 1 \rightarrow 4$  glycosidic linkages.

suitable assays to allow detection and quantification of enzyme activity after addition to feed. Assay procedures entail initial extraction of the supplemental enzyme from the feed by agitating it in the presence of buffer, with subsequent assay of the buffer contents. A number of methodological hurdles make development of such feed assays technically challenging. Enzymes are generally added to feed in low amounts (often as little as 50 g of enzyme per 1000 kg feed, which equates to very low activity levels per kilogram of feed). The low initial additional levels and subsequent extraction procedures produce very dilute enzyme solutions, requiring sensitive assays and prolonged incubation times.

Enzymes are often most conveniently assayed by incubation in the presence of their substrate, with subsequent quantification of the amount of product formed over a given time (Table 13.7). All feedstuffs naturally contain high levels of the reaction products that feed enzymes normally produce. These substances co-extract with the enzyme into the extraction buffer. In effect this generates enzyme blank values so high as to render this assay approach impractical.

Various alternative assay strategies have been adopted to overcome this difficulty, the most successful of which involve the use of chromogenic substrates. These are synthetic or semi-synthetic substances containing a coloured group that is

**Table 13.8** Synthetic chromogenic substrates that may be used to assay the enzymes indicated.

Enzyme	Chromogenic substrate
$\beta$ -Glucanase	Azo $\beta$ -glucan
Xylanase	Xylan coupled to Remazol Brilliant Blue (RBB)
Cellulase	RBB-cellulose
Protease	Azoalbumin
Amylase	Starch coupled to Ostazin Brilliant Red

released by action of the appropriate enzyme. The colour released can be quantified spectrophotometrically subsequent to termination of the assay. A number of chromogenic substrates suitable for assaying enzymes extracted from feed are available (Table 13.8). An alternative approach can entail physical separation of the enzyme from its natural product (e.g. via diafiltration) in the feed extracts, prior to enzyme assay.

## 13.6 Enzymes in molecular biology

Many of the basic techniques underpinning molecular biology rely on the use of specific enzymes. The isolation of specific sequences of chromosomal DNA and the reinsertion of isolated DNA into vectors requires the use of the two enzyme types, restriction endonucleases and DNA ligases. The enzyme reverse transcriptase plays a central role in the generation of complementary DNA (cDNA) from a mRNA template, while DNA polymerase allows the catalytic amplification of specific DNA segments via the polymerase chain reaction (PCR). While not sold in the same bulk quantities as the industrial enzymes discussed thus far, these technical enzymes nonetheless represent a significant niche market.

### 13.6.1 Restriction endonucleases and DNA ligase

Most microorganisms have developed a variety of mechanisms by which they protect themselves

from invading viral and other pathogens. One such method relies on specific microbial enzymes termed restriction endonucleases. These enzymes are capable of cleaving foreign double-stranded DNA and hence prevent its replication. Host microbial DNA may be modified, usually by methylation of specific DNA sequences, and in this way is protected from destruction by endogenous restriction endonucleases.

Well over 1000 restriction endonucleases have been identified thus far. They belong to one of three types: type I and III restriction enzymes are complex, consisting of a number of subunit types and requiring a variety of cofactors to maintain activity. These enzymes cleave DNA at sites removed from the DNA sequence which the enzyme recognizes. Type II restriction endonucleases are less complex, generally consisting of a single subunit, require only  $Mg^{2+}$  for activity and cleave DNA at the sequence which the enzyme recognizes and binds. Type II restriction endonucleases

have thus found wide application in both pure and applied molecular biology.

Restriction endonucleases recognize, bind and cut DNA sequences that exhibit a defined base sequence (Table 13.9). These sequences normally exhibit a twofold symmetry around a specific point and are usually four, six or eight base pairs in length. Such areas are often termed palindromes. In general, the larger the recognition sequence, the fewer such sequences present in a given DNA molecule and hence the smaller the number of DNA fragments that will be generated. Depending on the specific restriction endonuclease utilized, DNA cleavage may yield blunt ends or staggered ends; the latter are often referred to as 'sticky ends'.

Each microorganism expresses one or more restriction endonuclease of defined recognition sequence. Restriction endonucleases used either alone or in combination find a ready market in research and industrial applications relying on DNA manipulation. Some are biologically labile

**Table 13.9** Some commercially available restriction endonucleases, their sources, DNA recognition sites and cleavage points.

Restriction enzyme	Source	DNA recognition sequence and cleavage site
Bcl I	<i>Bacillus caldolyticus</i>	5' - T ↓ GATCA - 3' 3' - ACTAG ↑ T - 5'
Bgl II	Recombinant <i>E. coli</i> carrying <i>Bgl</i> /II gene from <i>Bacillus globigii</i>	5' - A ↓ GATCT - 3' 3' - TCTAG ↑ A - 5'
Bsa AI	Recombinant <i>E. coli</i> carrying <i>Bsa</i> AI gene from <i>B. stearothermophilus</i> A	5' - PyAC ↓ GTPu - 3' 3' - PuTG ↑ CAPy - 5'
Bsa JI	<i>B. stearothermophilus</i> J	5' - C ↓ CNNGG - 3' 3' - GGNC ↑ C - 5'
Bsi EI	<i>B. stearothermophilus</i>	5' - CGPuPy ↓ CG - 3' 3' - GC ↑ PyPuGC - 5'
Eco RV	Recombinant <i>E. coli</i> carrying <i>Eco</i> Rv gene from the plasmid J62 plg 74	5' - GAT ↓ ATC - 3' 3' - CTA ↑ TAG - 5'
Mwo I	Recombinant <i>E. coli</i> carrying cloned <i>Mwo</i> I gene from <i>Methanobacterium wolfeii</i>	5' - GCNNNN ↓ NNGC - 3' 3' - CGNN ↑ NNNNCG - 5'
TSP 509 I	<i>Thermus</i> sp.	5' - ↓ AATT - 3' 3' - TTAA ↑ - 5'
Xba I	Recombinant <i>E. coli</i> carrying <i>Xba</i> I gene from <i>Xanthomonas badvii</i>	5' - T ↓ CTAGA - 3' 3' - AGATC ↑ T - 5'
Xho I	Recombinant <i>E. coli</i> carrying <i>Xho</i> I gene from <i>X. holcicola</i>	5' - C ↓ TCGAG - 3' 3' - GAGCT ↑ C - 5'

G, Guanine; C, Cytosine; A, Adenine; T, Thymine; Pu, any purine; Py, any pyrimidine; N, either a purine or pyrimidine. Arrow indicates site of cleavage.

and must be transported and stored at temperatures below  $-20^{\circ}\text{C}$ . Many such enzymes are commercially available. Restriction endonucleases are also used to cleave chromosomal DNA in the first step of restriction fragment length polymorphism (RFLP) analysis.

DNA ligases catalyse the formation of phosphodiester bonds in DNA. As such they are mainly used in recombinant DNA technology to seal or 'ligate' a target DNA fragment into a chosen vector as part of a DNA cloning/expression procedure. Ligases available commercially include T4 ligase, a 55-kDa, 487 amino acid enzyme sourced from the phage T4, and *E. coli* ligase, a 73-kDa, 671 amino acid enzyme. Thermostable DNA ligases such as Taq DNA ligase are also commercially available. Taq ligase is sourced from a recombinant strain of *E. coli* containing a ligase gene isolated from the thermophile *Thermus aquaticus*.

### 13.6.2 DNA polymerase

DNA polymerases catalyse the synthesis of new DNA strands and thus function naturally to promote DNA replication and (in some instances) DNA repair. The enzymes find application in molecular biology for purposes such as the amplification of specific fragments of DNA via PCR (see also Chapter 2, Box 2.3). DNA polymerases most suited to this application should be heat-stable as the PCR process entails repeated alternating cycles of (DNA polymerase-mediated) polynucleotide synthesis and heat denaturation ( $95^{\circ}\text{C}$ , used to separate DNA double strands). Taq DNA polymerase was the first such thermostable DNA polymerase made commercially available. The enzyme, sourced from *Thermus aquaticus*, displays optimum activity at  $75^{\circ}\text{C}$ , but is quite stable at much higher temperatures. It displays a half-life of 90 minutes at  $95^{\circ}\text{C}$ . Subsequently a number of alternative thermostable DNA polymerases have come on the market (Table 13.10). These are sourced from various hyperthermophiles but are invariably produced by recombinant DNA technology, usually via heterologous expression in *E. coli*. These enzymes display half-lives of the

**Table 13.10** Some DNA polymerases available commercially.

Trade name	Source
Taq DNA polymerase	<i>Thermus aquaticus</i>
Tli DNA polymerase	<i>Thermococcus litoralis</i>
Pfu DNA polymerase	<i>Pyrococcus furiosus</i>
Tfl DNA polymerase	<i>Thermus flavus</i>
Tth DNA polymerase	<i>Thermus thermophilus</i>
Pwo DNA polymerase	<i>Pyrococcus woesei</i>
Vent DNA polymerase	<i>Thermococcus litoralis</i>
Deep Vent DNA polymerase	<i>Pyrococcus</i> sp.
Phusion DNA polymerase	<i>Pyrococcus</i> sp.

Note that the sources listed are the microorganisms which naturally produce the indicated enzymes. However, the commercial products are usually produced by recombinant means in *E. coli*. Most of the listed enzymes display molecular masses in the region of 90–95 kDa.

order of hours at  $95^{\circ}\text{C}$  (e.g. Deep Vent DNA polymerase exhibits a half-life of 23 hours at  $95^{\circ}\text{C}$  and 8 hours at  $100^{\circ}\text{C}$ ).

Various DNA polymerases have also been engineered or chemically modified in some way so as to enhance their performance in PCR reactions. For example, so-called Phusion polymerase is a fusion product in which a double-stranded DNA-binding domain has been fused to a *Pyrococcus*-like polymerase. The binding domain obviously increases the affinity of the engineered product for double-stranded DNA, allowing for increased polymerase reaction speeds.

During initial PCR set-up, PCR primers can potentially bind to partially complementary sequences within the DNA template. This can result in the subsequent amplification of undesired (non-specific) DNA fragments. This can potentially be overcome by undertaking 'hot-start' PCR. Hot-start PCR protocols usually use DNA polymerases modified to remain inactive at temperatures below  $70\text{--}75^{\circ}\text{C}$ . This is achieved in practice by inclusion of a blocking agent (both chemical agents and monoclonal antibody-based agents have been developed) which resides at or near the active site at low temperatures, thus inhibiting enzyme activity. At temperatures above  $70\text{--}75^{\circ}\text{C}$  the blocking agent detaches from the polymerase, allowing the amplification reaction to proceed.

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# Chapter 14

## Non-catalytic industrial proteins

The preceding three chapters have focused on industrial-grade enzymes. A variety of non-catalytic bulk proteins are also produced in very significant quantities by the biotech sector (Table 14.1). These proteins, which are largely derived from food sources and/or find application in the food sector, are the basis of this chapter. Such proteins are added to food either for obvious nutritional reasons or more commonly because they exhibit specific desirable functional properties.

### 14.1 Functional properties of proteins

Protein functionality may be broadly defined as the non-nutritive properties of a protein that affect its utilization as a food ingredient. The properties influenced are often responsible for or affect the appearance, structure/texture, mouthfeel and flavour retention of food. The major functional properties of proteins which render them of interest for food application are summarized in Table 14.2. Most such attributes are

either hydration-related properties (e.g. solubility, dispersibility, viscosity and gelation) or surface-related properties (e.g. foaming and emulsification).

The physicochemical properties of proteins that affect their functional properties are diverse and include amino acid composition and sequence, protein size and conformation, as well as isoelectric point (pI) and molecular flexibility. Although the physicochemical characteristics of the major proteins added to food are characterized in detail, it is still not possible to automatically predict the exact effects of their addition to a given foodstuff. This is likely because:

- food processing can lead to protein denaturation, altering their physicochemical characteristics;
- varying methods of production/extraction of food proteins can result in variation of product composition, particularly with regard to minor 'contaminants' (e.g. the presence of lipid in milk protein products);
- the added protein will interact with other food ingredients thereby influencing the functional effects observed.

**Table 14.1** Principal non-catalytic proteins that find bulk industrial application. Although most are used as, or added to, human food/animal feed, some also have non-food applications in the pharmaceutical and cosmetics sectors for example.

**Animal-derived**

Casein  
Whey proteins  
Gelatin  
Egg proteins

**Plant-derived**

Soy protein  
Wheat protein

**Table 14.2** Functional properties exhibited by some proteins which renders attractive their addition to selected foods.

Functional property	Protein (example)	Food (example)
Viscosity	Gelatin	Soups, gravies, desserts
Gelation	Milk and egg proteins	Confectionery, some meat products
Cohesion	Whey and egg proteins	Bakery products, pasta, sausages
Elasticity	Cereal products	Bakery products, meats
Fat/flavour binding	Milk, egg, cereal proteins	Confectionery, bakery products
Emulsification	Milk and egg proteins	Sausages, soups, cakes
Foaming	Milk and egg proteins	Cakes, ice creams, whipped toppings

### 14.1.1 Viscosity and thickening

Various foodstuffs (e.g. soups and gravies) usually contain added viscosity/thickening agents. Soluble polymers of high molecular mass (e.g. polysaccharides and proteins) generate viscous solutions when dissolved in water, even at low concentrations. The level of viscosity depends on properties such as polymer size and shape as well as level of hydration and range/extent of intermolecular interactions formed. Viscosity generally increases with increasing molecular mass, and randomly coiled/extended polymeric structures exhibit greater viscosity than tightly folded polymers of the same molecular mass.

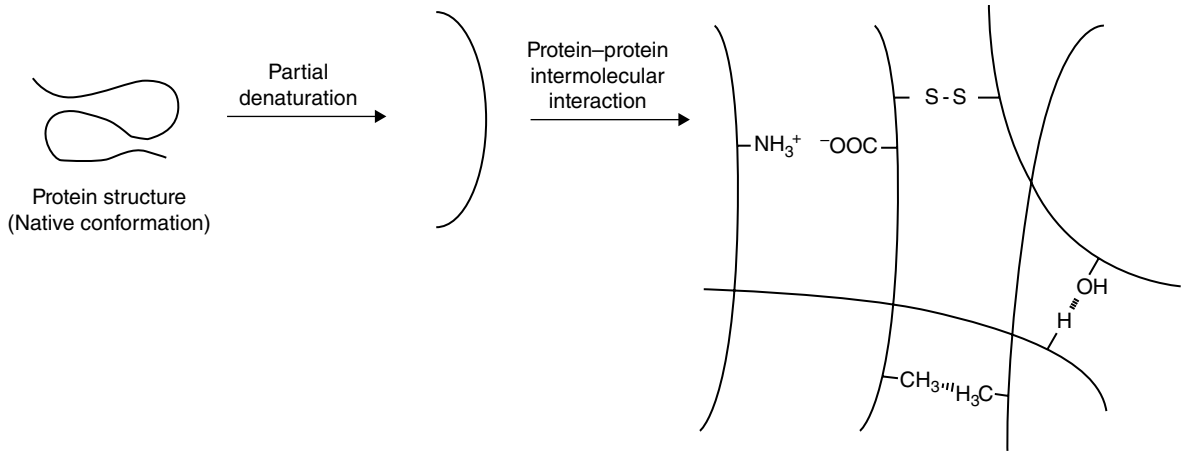
As such, various carbohydrates, gums and proteins of extended (as opposed to highly globular) structure (e.g. gelatin or partially denatured globular polypeptides) can be used as thickening agents in the food sector.

### 14.1.2 Gelation: cohesion and elasticity

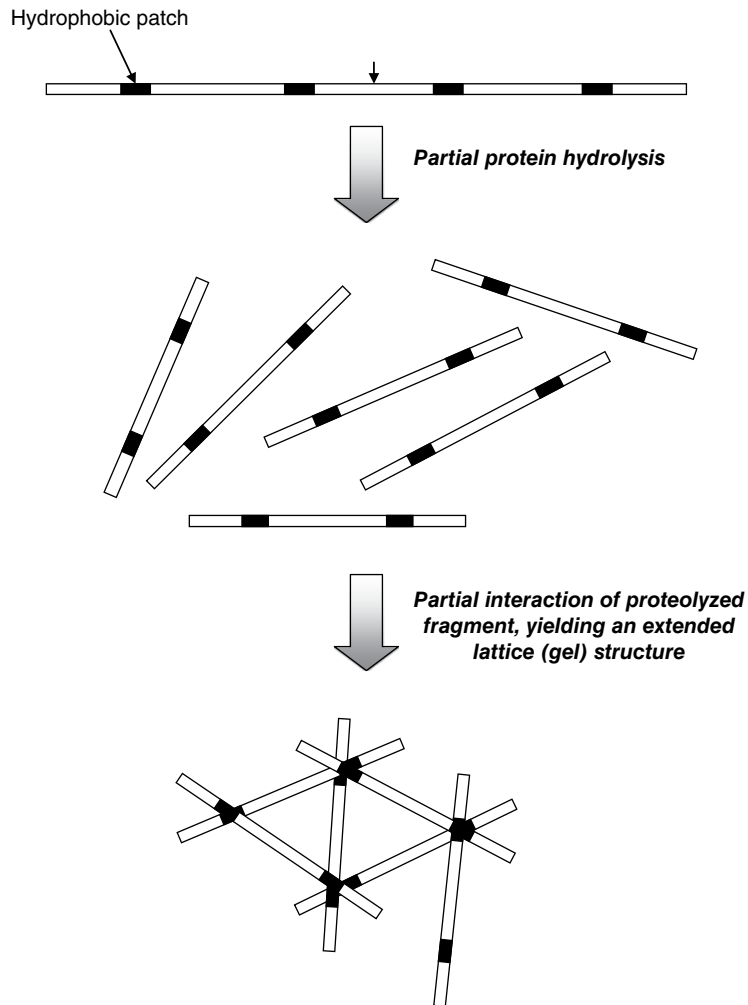
Most gels are composed of polymers that are cross-linked, thereby forming an interconnected three-dimensional network structure, immersed in a liquid medium. Food-based gels almost always consist of cross-linked carbohydrates or proteins (or sometimes a mixture of both) immersed in water. Partial polymer unfolding or denaturation is a prerequisite to gel formation for most food proteins. This is most often achieved by heating but can also be attained by mechanical agitation or by altering the protein solution's pH or ionic strength. The unfolded/extended protein structures then interact with each other, forming the extended three-dimensional network. The range and relative importance of the intermolecular forces that stabilize the gel structure depends on the protein type used. Both covalent (disulfide and  $\gamma$ -glutamyl) bonds and non-covalent forces (hydrogen bonding, electrostatic and hydrophobic interactions) all contribute to gel stability (Figure 14.1).

In most instances proteolysis of protein substrates capable of forming gels decreases gel-forming ability, as the polymer (i.e. the protein) molecular mass is reduced and the introduction of additional charged groups at the points of hydrolysis increases the hydrophilicity of the protein mix. However, limited hydrolysis of some proteins actually increases their gel-forming ability. Soy protein and whey protein, at concentrations as low as 2%, can form gels when partially hydrolysed by some proteases under specific conditions (usually at elevated temperatures, about 50°C, and at neutral pH). The mechanism(s) driving gel formation in such instances remain to be clarified but likely centres in part or in full on the formation of a limited number of intermolecular hydrophobic interactions (Figure 14.2).

Cohesion and elasticity describe functional properties that also depend on intermolecular interactions



**Figure 14.1** Overview of the process of protein-based gel formation. Although not shown, the gel structure will also be influenced/stabilized by hydrogen bonding between the protein strands and the solvent water molecules.



**Figure 14.2** Partial hydrolysis of protein substrate molecules, with subsequent lattice formation of proteolysed fragments via hydrophobic interactions, thereby forming a gel.

between individual (sometimes partially unfolded) protein molecules. Cohesive forces are by and large non-covalent (i.e. hydrogen bonds, ionic and hydrophobic interactions) which hold a loose network of protein molecules together. The non-covalent nature of these attractions allow the cohesive forces to be broken relatively easily. The functional property of elasticity depends on the formation of both covalent and non-covalent intermolecular linkages between entangled protein monomers.

### 14.1.3 Fat and flavour binding, emulsification and foaming

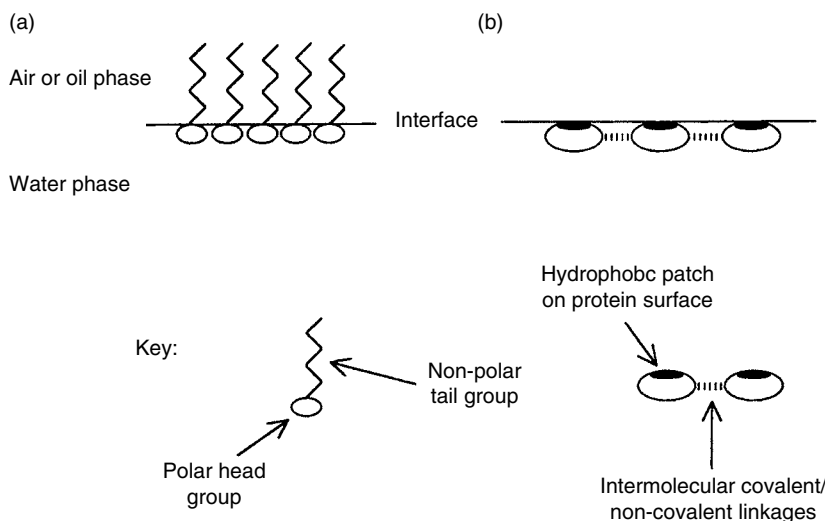
Many flavour and aroma compounds present naturally in (or added to) food are hydrophobic molecules of relatively low molecular mass. These can be retained/stabilized in food by binding to hydrophobic patches on the surface of proteins, as well as hydrophobic stretches exposed on partially denatured proteins. Food/aroma molecules displaying a polar character may also bind food proteins via hydrogen bonds or electrostatic attractions.

The amphipathic nature of most proteins (i.e. they contain both hydrophobic and hydrophilic regions) facilitates their use as emulsifying agents or foam-stabilizing agents in the food industry. Most foods contain an emulsified hydrophobic and

hydrophilic ingredient mix. Examples include milk, butter, ice creams and sausages. Many other foods are in fact foams or started out as foams (systems in which tiny air bubbles are dispersed in an aqueous phase, examples including whipped cream, soufflés, mousses, marshmallows and cakes). Both product types are characterized by an apolar phase (i.e. lipid or air) being dispersed in an aqueous-based phase.

Such oil–water or air–water interfaces display high interfacial tension. They collapse as soon as they are formed unless a stabilizing agent (an emulsifier) is added to the system. The emulsifier (surfactant/foaming agent), having hydrophobic and hydrophilic regions, will align along the interface such that its hydrophobic groups are in contact with the apolar phase and its hydrophilic groups are in contact with the polar phase. This reduces the interfacial tension and stabilizes the emulsion.

Low-molecular-mass surfactants (e.g. mono-glycerides and diglycerides, sorbitan monostearate and phospholipids) and high-molecular-mass surfactants (proteins and some gums) find widespread application in the food industry as emulsifiers and foaming agents. Lower-molecular-mass surfactants tend to be more effective at lowering interfacial tension. They pack more tightly along the interface than can proteins (the larger size of proteins causes steric hindrance; Figure 14.3).



**Figure 14.3** The interaction of (a) low-molecular-mass and (b) protein-based surfactants with an air or oil–water interface. Refer to text for details.

However, protein surfactants generally generate more stable foams and emulsions. In addition to lowering interfacial tension, the continuous film they form around the oil–air droplet is further stabilized by intermolecular disulfide and non-covalent linkages. Congregation at an aqueous–apolar interface also promotes partial denaturation of most proteins.

## 14.2 Milk and milk proteins

The principal constituents of milk are presented in Table 14.3. Milk is secreted by the females of all mammalian species, of which there are in excess of 4000. In addition to the major constituents, milk contains several hundred minor constituents, including various vitamins and minerals, flavour components, etc. Milk is a very variable substance in terms of exact composition. Interspecies differences are clearly evident. However, the breed, health and nutritional status of animals within a single species can also affect the composition of the milk. Indeed, milk proteomics has become an active area of research over the last decade or so.

The disaccharide lactose represents the major sugar present in milk. Traces of other sugars (e.g. glucose and fructose) are also often present. The milk of most species contains 2.5–5.0% (w/v) lactose, although no lactose is present in the milk of some seals and sea lions. The milk of humans and monkeys displays higher than average lactose levels (7–10%). Lactose is the major constituent of dried milk and whey powder.

**Table 14.3** Average composition (% w/v) of the milk of humans and various agriculturally important species.

Species	Lactose	Fat	Protein	Total solids
Human	7.0	3.8	1.0	12.0
Cow	5.0	3.7	3.3	12.5
Sheep	4.8	7.5	4.5	19.0
Goat	4.0	4.5	3.0	12.3

The lipid content of milk can also vary very significantly. The milk of most agriculturally important species displays a fat content of 3–7%, although the milk of dolphins can contain up to 33% fat while that of the harp seal contains over 50% fat. As the major biological function of milk fat is to provide a source of energy for the neonate, it is not surprising that mammals inhabiting cold/marine environments secrete more fat in their milk. The major biological function of most milk proteins (discussed in detail in later sections) is to provide a dietary source of amino acids for neonatal protein synthesis, although some have alternative functions (e.g. the protective function of milk immunoglobulins).

From a physicochemical standpoint, milk is a complex fluid, containing three major phases. Lactose and most of the minor constituents are in true aqueous solution. Proteins are dispersed in this solution, some almost completely (e.g. whey proteins) while others (caseins) form large colloidal aggregates, often with diameters of up to 0.5  $\mu\text{m}$ . The lipid fraction exists in an emulsified state, generally as globules with diameters of up to 20  $\mu\text{m}$ . Milk and milk proteins have an extremely wide range of food uses (Table 14.4); however, I now focus specifically on the biochemistry, industrial production and applications of the major milk proteins, namely:

- caseins;
- $\alpha$ -lactalbumin;
- $\beta$ -lactoglobulin;
- serum albumin;
- immunoglobulin;
- lactophorin;
- lactoferrin;
- various enzymes (e.g. peroxidase, lysozyme).

### 14.2.1 Caseins

#### 14.2.1.1 Biochemistry

Bovine milk contains four different casein types (four related but distinct gene products). These are termed  $\alpha_{s1}$ -,  $\alpha_{s2}$ -,  $\beta$ - and  $\kappa$ -casein. The total casein concentration in such milk is approximately 25 g/L and the four caseins listed occur in the approximate ratio of 4 : 1 : 4 : 1. The caseins interact to form

**Table 14.4** The major dairy-based products, how they are produced and their uses.

Process	Primary product	Further products
Centrifugal separation	Cream	Butter, butteroil, ghee Creams: various fat content (HTST pasteurized or UHT sterilized, coffee creams, whipping creams, dessert creams), cream cheeses
	Skim milk	Powders, casein, cheese, protein concentrates
Concentration: thermal evaporation or ultrafiltration		In-container or UHT -sterilized concentrated milks Sweetened condensed milk
Concentration and drying		Whole milk powders; infant formulae; dietary products
Enzymatic coagulation	Cheese	1000 varieties; further products, e.g. processed cheese, cheese sauces, cheese dips, cheese analogues
	Rennet casein	Whey powders, demineralized whey powders, whey protein concentrates, whey protein isolates, individual whey proteins, whey protein hydrolysates, neutraceuticals. Lactose and lactose derivatives
	Whey	
Acid coagulation	Cheese	Fresh cheeses and cheese-based products
	Acid casein	Functional applications, e.g. coffee creamers, meat extenders; nutritional applications
	Whey	Whey powders, demineralized whey powders, whey protein concentrates, whey protein isolates, individual whey proteins, whey protein hydrolysates, neutraceuticals
Fermentation		Various fermented milk products, e.g. yoghurt, buttermilk, acidophilus milk, bioyoghurt
Freezing		Ice-cream (numerous types and formulations)
Miscellaneous		Chocolate products

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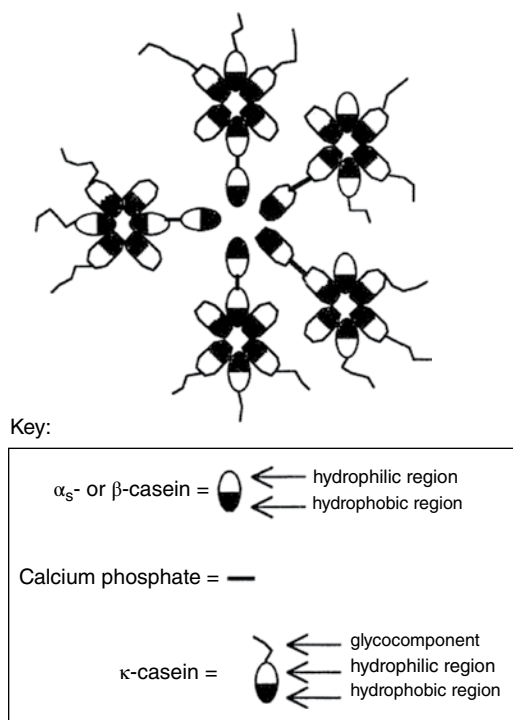
casein micelles, particles with diameters ranging from 50 to 250 nm, which also contain appreciable levels of bound calcium phosphate. Caseins have the following biochemical properties.

- They display molecular masses of approximately 20–25 kDa:  $\alpha_{s1}$  is a 23.5-kDa, 199 amino acid protein;  $\alpha_{s2}$  is a 25.2-kDa, 207 amino acid protein;  $\beta$ -casein is a 24-kDa, 209 amino acid protein; and  $\kappa$ -casein is a 19-kDa, 169 amino acid protein.
- They are phosphoproteins: the R groups of several of their serine residues are substituted with phosphate groups. These groups bind  $\text{Ca}^{2+}$  ions strongly.
- They are predominantly hydrophobic proteins (hydrophobic residues in their amino acid backbones generally occur in clusters). This is particularly true for the hydrophobic  $\beta$ -casein, where the majority of hydrophobic residues occur from residue 40 to 209, and  $\kappa$ -casein, where residues 1–105 are predominantly hydrophobic

while the remainder of the molecule is highly hydrophilic.

- They all contain unusually high amounts of proline residues.
- $\kappa$ -Casein molecules display variable levels of glycosylation (up to half of  $\kappa$ -casein molecules remain unglycosylated). The other casein subtypes are devoid of a glycocomponent.

It appears that individual casein molecules do not possess a rigid three-dimensional conformation (a state conducive with high proline content), but interact with each other to form a roughly spherical micellar structure. The exact arrangement of casein molecules in the micelle remains the subject of debate. Individual caseins likely interact via hydrophobic interactions, as well as via hydrogen bonding and electrostatic interactions. The calcium phosphate present also likely provides stabilizing interactions with polar/charged species present in the caseins.



**Figure 14.4** Schematic diagram of a likely model of the structure of casein micelles in milk. Variations from this model have also been proposed. The arrangement of submicelles and the elongated glycochain of the  $\kappa$ -casein molecules would explain the 'hairy raspberry' appearance of many micelles as visualized by electron microscopy. Fox and McSweeney, 1997. Reproduced with kind permission from Springer Science+Business Media B.V

Unlike  $\alpha_{s1}$ - and  $\beta$ -caseins,  $\alpha_{s2}$ - and  $\kappa$ -caseins each contain (two) cysteine residues and are therefore capable of forming intermolecular disulfide linkages, which also likely stabilizes overall micellar structure.

It has been established that the vast majority of  $\kappa$ -casein molecules are present on the micelle surface (much lower quantities of the  $\alpha_s$ -caseins are also found on the surface, but the highly hydrophobic  $\beta$ -caseins are buried in the micelle's centre). The  $\kappa$ -casein molecules are mainly oriented such that their hydrophilic glycosylated C-terminal ends are protruding outwards from the micelle, while their hydrophobic N-terminal ends protrude inwards, interacting with internal caseins (presumably mainly via hydrophobic interactions). The amphipathic nature of  $\kappa$ -casein in particular therefore plays the

major role in stabilizing micellar structure in its normal aqueous environment (Figure 14.4).

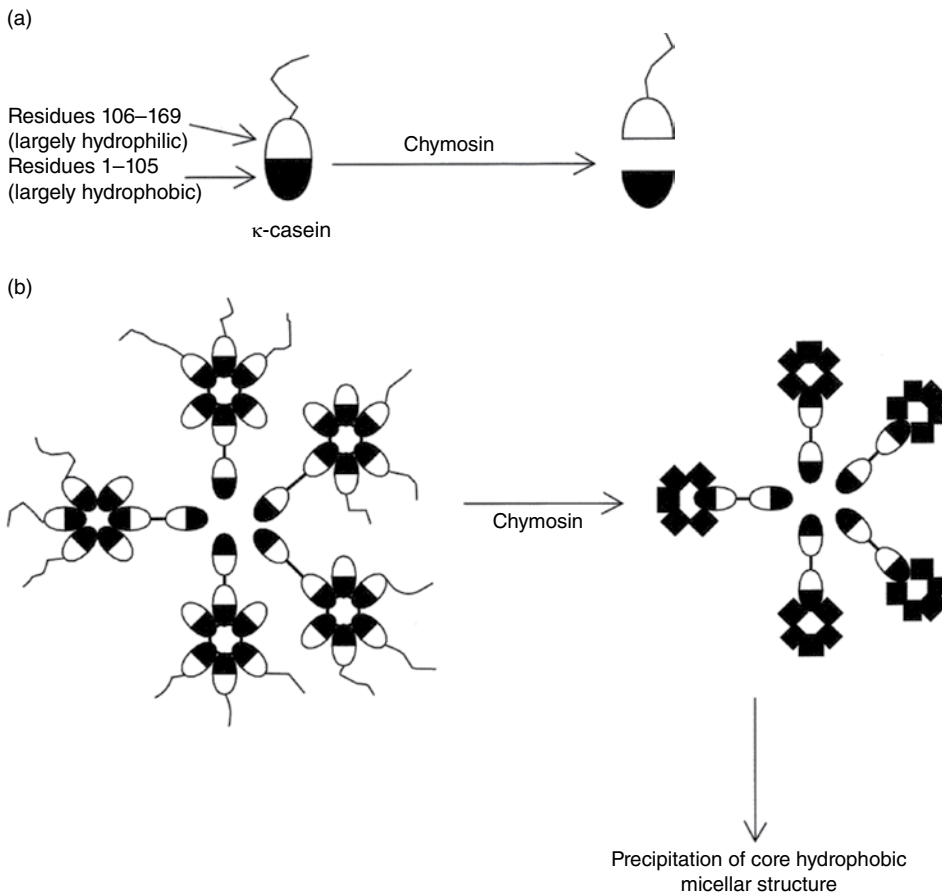
The structure of the casein micelle also explains why the addition of chymosin to milk destabilizes micellar structure, causing aggregation and thus curd formation (refer to chymosin and cheesemaking in Chapter 12). Chymosin selectively cleaves the Phe<sup>105</sup>—Met<sup>106</sup> peptide bond of  $\kappa$ -casein. This releases the hydrophilic component of  $\kappa$ -casein, responsible for micellar stabilization, resulting in aggregation of the modified micelles (Figure 14.5). Biologically, caseins appear to simply play a nutritive function, providing the recipient neonates with a source of amino acids. In addition, casein micelles also serve as a source of calcium, phosphate and small amounts of sugars (derived from the glyco-component of  $\kappa$ -casein molecules).

#### 14.2.1.2 Industrial production and uses

Caseins can be prepared on a laboratory scale from whole/skimmed milk by a number of means (Table 14.5). However, technical and economic considerations have largely limited its industrial-scale production to isolation, precipitation or enzymatic (chymosin-based) coagulation (Figure 14.6). Casein has been produced commercially for almost 100 years and several hundred thousand tonnes of caseins are produced annually. Production begins by removal of the fat from whole milk by centrifugation, yielding skim milk. Prior fat removal is undertaken in order to avoid the subsequent development of off-flavours in dried casein production due to oxidation of contaminant lipid.

Product recovery by isoelectric precipitation entails acidification of the milk, producing 'acid caseins'. Acidification (to pH 4.6) can be achieved either by direct addition of mineral acids (usually HCl) or, more slowly, by fermentation using lactic acid bacteria. The latter method usually involves addition of a *Lactococcus* starter culture with subsequent incubation at 22–25°C for 14–16 hours (the bacteria metabolize some of the milk lactose, producing lactic acid).

When the required pH is reached, the milk is heated to 50°C by steam injection. This encourages maximal casein precipitation (i.e. curd formation).

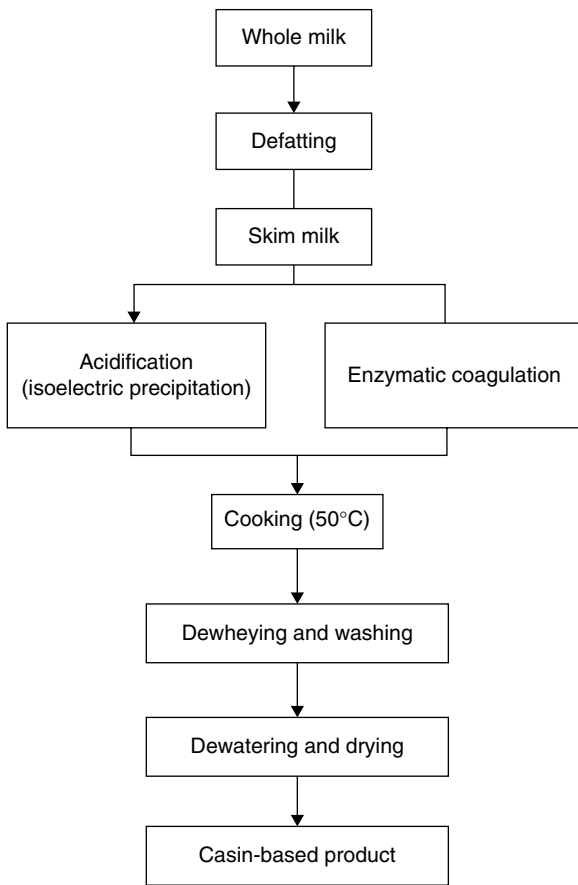


**Figure 14.5** Effect of chymosin (rennin) on individual  $\kappa$ -casein molecules (a) and the resulting destabilizing effect on the casein micelles leading to their aggregation (b).

**Table 14.5** Methods by which micellar caseins may be extracted from milk on a laboratory scale.

Method	Details
Centrifugation	Micelles can be sedimented by centrifugation at $100,000 \times g$ for 1 hour. Prior addition of $\text{CaCl}_2$ (0.2 mol/L) causes more extensive aggregation of micelles, allowing their recovery at lower centrifugal speeds
Isoelectric precipitation	Acidification of milk to pH 4.6
Salting out	Addition of ammonium sulfate (to a concentration of 260 g/L) precipitates casein (and also some whey proteins)
Ethanol precipitation	Addition of ethanol to a final concentration of 40% selectively precipitates casein from solution
Cryoprecipitation	Cooling of milk to $-10^\circ\text{C}$ precipitates casein
Ultrafiltration	Ultrafiltration of whole milk will concentrate/retain both casein and whey proteins
Treatment with chymosin	Aggregated casein micelles precipitate and are easily recovered from milk. $\kappa$ -Casein component is obviously modified
Chromatographic purification	Methods such as ion-exchange and gel filtration chromatography may be used to partially/fully separate caseins from other milk proteins

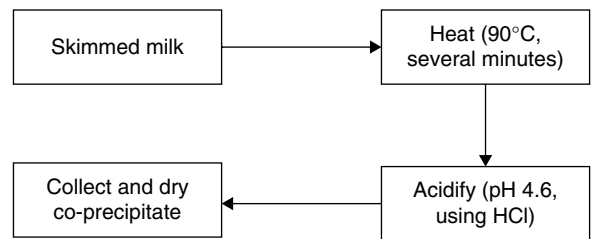




**Figure 14.6** Overview of the major methods by which casein is produced on an industrial scale. Fox and McSweeney, 1997. Reproduced with kind permission from Springer Science+Business Media B.V

The curd is then separated from the residual liquid (the whey), either by filtration or low-speed centrifugation. Before drying, the casein product is normally washed with hot water in order to remove residual whey constituents. The drying process begins with mechanical pressing (or sometimes centrifugation). The product is then fully dried (to a moisture content of below 12%). This is usually achieved by placing the casein on vibrating perforated stainless steel trays/conveyors and passing warm air up through the perforations. The product is then milled (ground) before final packaging in order to reduce and standardize the casein particle size.

Enzyme-based casein production differs from the above process only by the means in which casein is



**Figure 14.7** Overview of industrial-scale co-precipitate production. Refer to text for details.

precipitated from the skim milk. This process entails addition of chymosin to the milk without prior adjustment of the pH (which is about 6.7). The enzyme-containing milk is then held at a temperature of about 30°C for a period usually in excess of 1 hour, during which time coagulation occurs (see also Figure 14.5). This so-called rennet casein product is then further processed by means similar to that used on acid casein (Figure 14.6).

Acid (and rennin) caseins are largely insoluble in water. Soluble casein products (caseinates) are produced industrially by solubilizing acid casein in alkali, with subsequent spray-drying of the product. Solubilization with NaOH is usually undertaken, yielding sodium caseinate. However, KOH, Ca(OH)<sub>2</sub> or NH<sub>3</sub> are sometimes used, yielding potassium, calcium or ammonium caseinate.

The acid and enzyme-based methods of casein production yield a relatively pure casein product. Another milk-protein based product manufactured commercially is termed 'co-precipitate'. Co-precipitate production is outlined in Figure 14.7. Initial heating of skimmed milk to 90°C results in the denaturation of most non-casein milk proteins. The denatured whey proteins form a complex with casein, and the entire complex is then precipitated from solution by acidification to pH 4.6. Such casein-whey protein co-precipitate contains in excess of 90% of total milk protein.

Caseins produced commercially obviously consist of a mixture of the four casein subtypes. Although they may be fractionated chromatographically, this is not required in the context of their industrial use. Casein/caseinates have found widespread application in the food industry (Table 14.6). Their emulsifying properties are the functional characteristic that renders them most useful industrially. Emulsions

**Table 14.6** Major applications of milk-derived proteins in the food industry.

**Bakery products**

*Caseins/caseinates/co-precipitates*

Used in: bread, pastries/cookies, breakfast cereals, cake mixes, pastries, frozen cakes and pastries, pastry glaze

Effect: nutritional, sensory, emulsifier, dough consistency, texture, volume/yield

*Whey proteins*

Used in: bread, cakes, muffins, croissants

Effect: nutritional, emulsifier, egg replacer

**Dairy products**

*Caseins/caseinates/co-precipitates*

Used in: imitation cheeses (vegetable oil, caseins/caseinates, salts and water)

Effect: fat and water binding, texture enhancing, melting properties, stringiness and shredding properties

Used in: coffee creamers (vegetable fat, carbohydrate, sodium caseinate, stabilizers and emulsifiers)

Effect: emulsifier, whitener, gives body and texture, promotes resistance to feathering, sensory properties

Used in: cultured milk products, e.g. yoghurt

Effect: increase gel firmness, reduces syneresis

Used in: milk beverages, imitation milk, liquid milk fortification, milk shakes

Effect: nutritional, emulsifier, foaming properties

Used in: high-fat powders, shortening, whipped toppings and butter-like spreads

Effect: emulsifier, texture enhancing, sensory properties

*Whey proteins*

Used in: yoghurt, Quarg, Ricotta cheese

Effect: yield, nutritional, consistency, curd cohesiveness

Used in: cream cheeses, cream cheese spreads, sliceable/squeezable cheeses, cheese fillings and dips

Effect: emulsifier, gelling, sensory properties

**Beverages**

*Caseins/caseinates/co-precipitates*

Used in: drinking chocolate, fizzy drinks and fruit beverages

Effect: stabilizer, whipping and foaming properties

Used in: cream liqueurs, wine aperitifs

Effect: emulsifier

Used in: wine and beer industry

Effect: fines removal, clarification, reduce colour and astringency

*Whey proteins*

Used in: soft drinks, fruit juices, powdered or frozen orange juices

Effect: nutritional

Used in: milk-based flavoured beverages

Effect: viscosity, colloidal stability

**Dessert products**

*Caseins/caseinates/co-precipitates*

Used in: ice-cream, frozen desserts

Effect: whipping properties, body and texture

Used in: mousses, instant puddings, whipped toppings

Effect: whipping properties, film former, emulsifier, imparts body and flavour

*Whey proteins*

Used in: ice-cream, frozen juice bars, frozen dessert coatings

Effect: skim-milk solids replacement, whipping properties, emulsifying, body/texture

**Confectionery**

*Caseins/caseinates/co-precipitates*

Used in: toffee, caramel, fudges

Effect: confers firm, resilient, chewy texture; water binding, emulsifier

Used in: marshmallow and nougat

Effect: foaming, high-temperature stability, improve flavour and brown colour

*Whey proteins*

Used in: aerated candy mixes, meringues, sponge cakes

Effect: whipping properties, emulsifier

**Table 14.6** (Continued)**Pasta products**

Used in: macaroni, pasta and imitation pasta

Effect: nutritional, texture, freeze-thaw stability, microwaveable

**Meat products**

*Caseins/caseinates/co-precipitates*

Used in: comminuted meat products

Effect: emulsifier, water binding, improves consistency, releases meat proteins for gel formation and water binding

*Whey proteins*

Used in: frankfurters, luncheon meats

Effect: pre-emulsion, gelation

Used in: injection brine for fortification of whole meat products

Effect: gelation, yield

**Convenience foods**

Used in: gravy mixes, soup mixes, sauces, canned cream soups and sauces, dehydrated cream soups and sauces, salad dressings, microwaveable foods, low-lipid convenience foods

Effect: whitening agents, dairy flavour, flavour enhancer, emulsifier, stabilizer, viscosity controller, freeze-thaw stability, egg yolk replacement, lipid replacement

**Textured products**

Used in: puffed snack foods, protein-enriched snack-type products, meat extenders

Effect: structuring, texturing, nutritional

**Pharmaceutical and medical products**

*Special dietary preparations*

Ill or convalescent patients

Dieting patients/people

Athletes

Astronauts

*Infant foods*

Nutritional fortification

'Humanized' infant formulae

Low-lactose infant formulae

Specific mineral balance infant foods

Casein hydrolysates: used for infants suffering from diarrhoea, gastroenteritis, galactosaemia, malabsorption, phenylketonuria

Whey protein hydrolysates used in hypoallergic formulae preparations

Nutritional fortification

*Intravenous feeds*

Patients suffering from metabolic disorders, intestinal disorders for postoperative patients

*Special food preparations*

Patients suffering from cancer, pancreatic disorders or anaemia

*Specific drug preparations*

$\beta$ -Caseinomorphins used in sleep or hunger regulation or insulin secretion

Sulfonated glycopeptides used in treatment of gastric ulcers

*Miscellaneous products*

Toothpastes

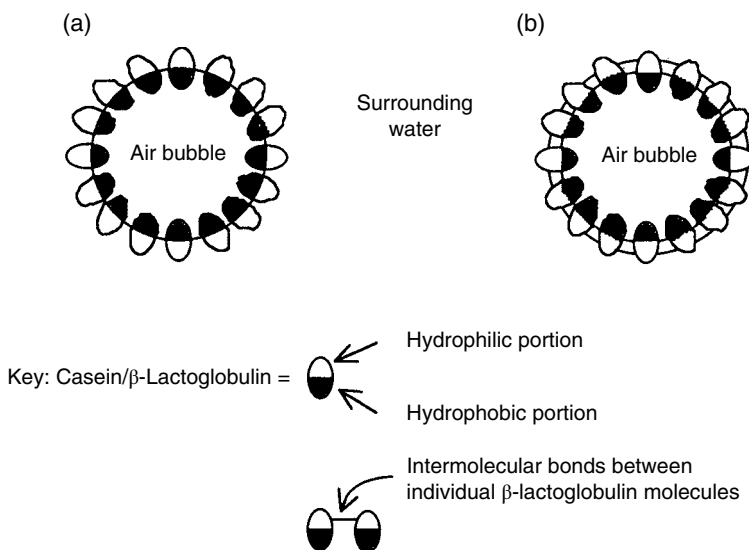
Cosmetics

Wound treatment preparations

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based on caseinates or individual casein molecules are very stable (emulsions based on intact casein micelles are less stable). Their strongly amphipathic nature coupled to their conformational flexibility allow them to congregate in an extended manner

along an oil-water interface. Moreover, their charged nature (mainly due to the fact they are phosphoproteins) ensures that individual emulsified droplets are of like charge and thus repel each other. Caseins are also unusual in that they retain a stable emulsion in



**Figure 14.8** Comparison of (a) casein and (b)  $\beta$ -lactoglobulin-based foams.  $\beta$ -Lactoglobulin-based foam structures are stabilized by the formation of intermolecular non-covalent bonds and disulfide linkages.

the presence of a high concentration of ethanol. Because of this, they also find application as emulsifying agents for cream liquors.

In contrast to their emulsification properties, caseins are less effective as foaming agents. Although casein solutions will foam effectively, the gas-liquid interface of the air cells quickly collapses. This again is a reflection of casein biochemistry. Although they adsorb strongly to the air-liquid interface, the hydrophilic portions of individual casein molecules protruding inward to the aqueous phase do not interact with each other. This lack of a stabilizing intermolecular interaction results in a mechanically weak interface (Figure 14.8). For this reason, caseins are not widely used on their own as foaming agents.

## 14.2.2 Whey proteins

### 14.2.2.1 Biochemistry

The solution that remains after casein is precipitated from milk is termed whey. Acid whey is obtained after isoelectric precipitation of casein, while rennet (i.e. 'sweet') whey is produced by rennet-mediated coagulation of casein. Whey is therefore a major

**Table 14.7** Composition (%) of rennin-derived whey.

Lactose	4.77
Protein	0.82
Fat	0.07
Lactic acid	0.15
Calcium	0.05
Potassium	0.13
Sodium	0.07
Phosphorus	0.06
Water	93.0

byproduct of cheese manufacture. The typical composition of rennet-derived whey is provided in Table 14.7. The composition of acid whey would be similar, although the exact concentration of individual constituents could differ slightly. Approximately 20% of total (whole) milk proteins are retained in whey. Collectively, these are termed 'whey proteins', 'serum proteins' or 'non-casein nitrogen'. The major whey proteins include  $\beta$ -lactoglobulin,  $\alpha$ -lactalbumin, serum albumin and immunoglobulins (mainly IgG), as summarized in Table 14.8.

$\beta$ -Lactoglobulin constitutes about 50% of total whey protein (about 12% of total milk protein).

**Table 14.8** Some physicochemical characteristics of the major (bovine) whey proteins.

	$\beta$ -Lactoglobulin	$\alpha$ -Lactalbumin	Serum albumin	IgG
Concentration (g/L)	2–4	0.5–1.5	0.4	0.5–1.0
Molecular mass (kDa)	18	14	66	~150
No. of amino acid residues	162	123	582	~1360
No. of cysteine residues	5	8	35	16
Isoelectric point (pI)	5.2	4.2–4.8	4.7–4.9	5.5–8.3
Glycosylated?	No	No	No	Yes

Four genetic variants of this 18-kDa protein have been characterized. Each differs from the others by one or two amino acid substitutions.  $\beta$ -Lactoglobulin is particularly rich in cysteine residues and X-ray diffraction studies reveal a compact globular structure consisting of 10–15%  $\alpha$ -helix and 45%  $\beta$  conformation. The protein is relatively heat-labile, being denatured at temperatures above 65°C. This exposes highly reactive sulfhydryl groups, which is important in terms of its food functionality properties. The quaternary structure of  $\beta$ -lactoglobulin is pH dependent. At pH values below 3.5 and above 7.5, it exists in monomeric form. At pH values between 3.5 and 5.5 it forms an octameric structure, while at pH 5.5–7.5 it exists as a dimer.

Although present in the milk of most agriculturally significant species,  $\beta$ -lactoglobulin is absent from the milk of some mammals, including humans. There is still considerable debate with regard to its natural biological function, although it is known to bind vitamin A tightly in a hydrophobic crevice close to the protein's surface.

$\alpha$ -Lactalbumin is the second most abundant protein in whey derived from the milk of cows and many other mammals. It has been purified from a range of mammalian milks and sequence studies reveal a highly conserved primary structure. The milk of most species of cattle contains a single  $\alpha$ -lactalbumin variant ( $\alpha_{1a}$ ), although some species produce two variants ( $\alpha_{1a}$  and  $\alpha_{1b}$ ). These differ from each other by a single amino acid:  $\alpha_{1a}$  contains a glutamate residue at position 10, whereas  $\alpha_{1b}$  contains an arginine in this position.

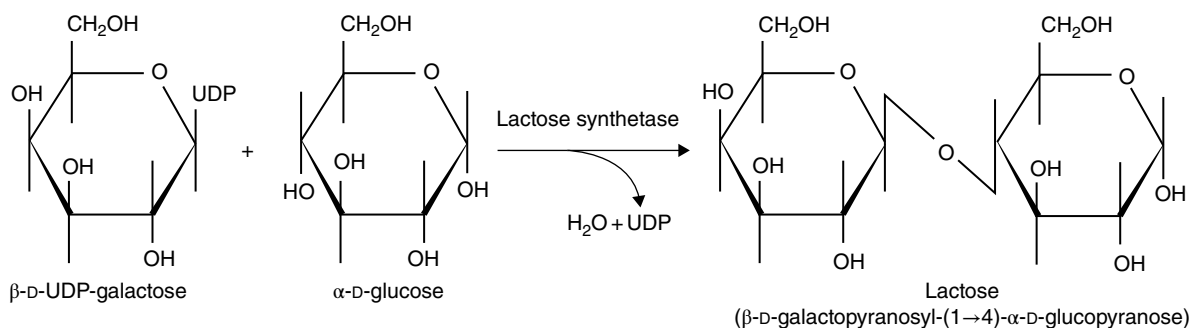
$\alpha$ -Lactalbumin contains higher than average levels of tryptophan and sulfur-containing amino acids (cysteine and methionine). Its isoelectric point is in

the region of 4.8, at which pH it is least soluble. As in the case of  $\beta$ -lactoglobulin,  $\alpha$ -lactalbumin displays a compact globular structure, consisting of 25%  $\alpha$ -helix and 14%  $\beta$  conformation. Its tertiary structure is very similar to that of lysozyme's, which is a reflection of the considerable sequence homology that exists between these two proteins.

$\alpha$ -Lactalbumin appears more heat-stable than  $\beta$ -lactoglobulin. It is denatured at temperatures much in excess of 60°C, although it renatures on cooling. The protein binds calcium and other divalent cations tightly at a surface cleft which houses three asparagine residues, and removal of bound calcium renders it more thermolabile.

Biologically,  $\alpha$ -lactalbumin plays a direct and novel role in the synthesis of lactose. The final step in lactose synthesis, involving the condensation of UDP-galactose and D-glucose, is catalysed by lactose synthetase (Figure 14.9). This enzyme is a heterodimer, consisting of A and B subunits. The A subunit catalyses the transfer of galactose from UDP-galactose to the acceptor molecule. In the absence of subunit B, a wide range of sugars can act as the acceptor. The presence of the B protein, however, confers specificity to the reaction, rendering glucose as the only acceptor molecule used, and thus ensuring only the synthesis of lactose. The concentration of lactose in milk is directly proportional to the concentration of  $\alpha$ -lactalbumin present. The milk of animals devoid of this protein is also devoid of lactose (e.g. many marine mammals).

Cows' milk is also found to contain (bovine) serum albumin (BSA), at concentrations usually between 0.1 and 0.4 g/L. The protein is identical to BSA found in blood serum, and probably enters the milk by leakage from the vascular system. The 66-kDa molecule is significantly larger than  $\beta$ -lactoglobulin



**Figure 14.9** The final step in the biosynthesis of lactose as catalysed by lactose synthetase. Overall, milk lactose is synthesized from blood-derived glucose via a four-step enzymatic pathway. One of the enzymes is an epimerase which converts UDP-glucose into UDP-galactose.

or  $\alpha$ -lactalbumin. Its amino acid sequence has been determined and it exhibits a globular structure featuring three defined domains. It displays 17 intramolecular disulfide linkages as well as one cysteine with a free thiol (SH) group. Incubation under reducing conditions renders the protein less conformationally stable by breaking disulfide linkages. Heating a BSA solution to temperatures in excess of 40°C results in its precipitation, most likely caused by protein unfolding with subsequent formation of intermolecular hydrophobic interactions. BSA also undergoes acid denaturation at lower pH values (~4). This is likely caused by mutual repulsion of positively charged amino acid residues at such pH values (of its 582 amino acids, BSA contains 59 lysine residues, 23 arginines and 17 histidines). Although BSA plays a well-known transportational role in blood, its functional significance in milk is unclear.

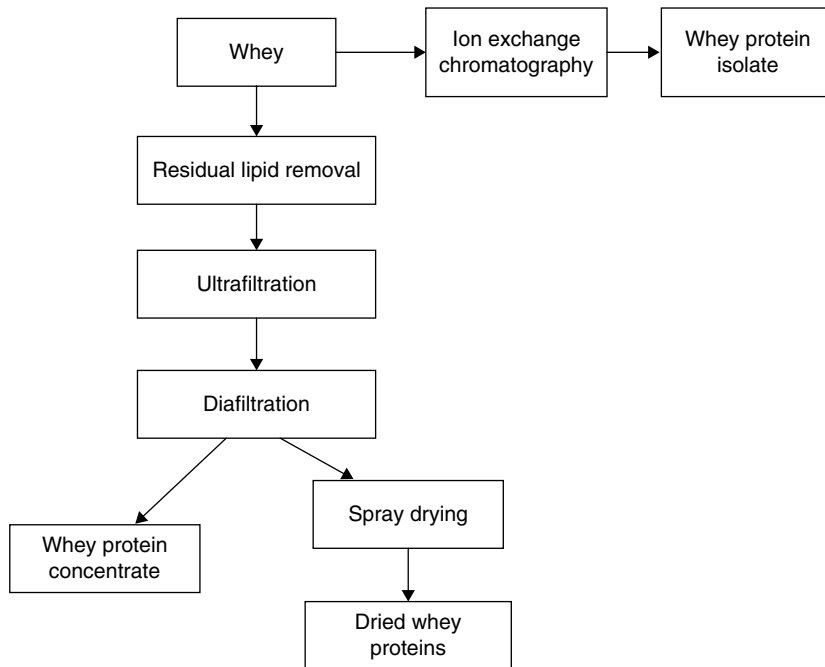
The fourth major protein type found in milk is the immunoglobulins. IgG is the predominant immunoglobulin subtype found in bovine milk whereas IgA predominates in human milk. The biological function of these proteins is obviously an immunological one, serving to provide neonates with early and immediate immunological protection. In isolated form, immunoglobulins display a high unfolding temperature. However, they are far more thermolabile in the presence of other whey proteins. This may be due to the formation of intermolecular disulfide linkages with  $\beta$ -lactoglobulin and/or BSA. Additional minor whey proteins include several which display antimicrobial actions.

These include the enzymes lysozyme and lactoperoxidase as well as lactoferrin.

#### 14.2.2.2 Industrial production and uses

The worldwide manufacture of cheese results in the annual production of about 200 million tonnes of whey, which contains in the region of 1.2 million tonnes of whey protein. The low protein concentration initially rendered whey protein production relatively expensive, but the development of ultrafiltration in particular has helped reduce processing costs. Whole whey protein-based products are manufactured essentially by concentration of the whey, often with subsequent drying. Concentration can be achieved by evaporation but ultrafiltration is now the method of choice (Figure 14.10). The application of ultrafiltration in diafiltration mode (see Chapter 3) also allows separation of low-molecular-mass constituents (e.g. lactose and minerals) from the whey proteins.

The initial step in whey processing invariably entails the removal of residual lipid (in order to prevent fouling of ultrafilter membranes and the development of lipid-based off-flavours in the final product). This is generally achieved by the addition of  $\text{CaCl}_2$  to whey, with pH adjustment to 7.3 and heating to 50°C. Under these conditions, calcium phospholipoprotein complexes are formed and precipitate out of solution. They (along with, for example, casein fines) can then be removed by microfiltration or low-speed centrifugation. A combination of ultrafiltration and diafiltration, using membranes with molecular



**Figure 14.10** Overview of an ultrafiltration process used to produce a whey protein-based product. Refer to text for further details.

mass cut-off values of 10–50 kDa, results in the production of whey protein concentrate. Concentrates containing 30–75% (w/v) protein are most often produced commercially. If required, the concentrates can be dried fully, most often by spray-drying.

Whey protein isolates usually contain greater than 90% protein. The manufacturing process involves the use of ion-exchange chromatography. The whey pH is initially adjusted to a value lower than 4.6 (if cation-exchange resins are used) or greater than 4.6 (if anion-exchange resins are used). Application of the whey results in binding of the protein fraction. A subsequent washing step removes unbound material (e.g. lactose and minerals). Appropriate subsequent readjustment of the pH then results in elution of the whey proteins. This whey protein isolate may also be spray-dried if required.

In addition to whey protein isolates and concentrates, several additional whey protein-based products are of actual or potential interest, including:

- $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin-rich fractions (for use in infant food formulations);

- whey protein hydrolysates (partially proteolysed whey protein, mainly for food use);
- modified whey protein (generally heat-induced whey protein polymers, which can form gels at low temperatures, and hence are used as ‘cold set’ agents).

Whey protein products are finding increasing use as animal feed additives (mostly whey protein concentrates of less than 55% protein content), for human nutrition, or are used in the food industry for their functional properties (see Table 14.6). From a functional point of view,  $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin are the most significant whey protein constituents, being responsible for surface active functional properties (emulsifying and foaming) as well as hydration and gelling properties. Whey proteins are also increasingly being used to form edible films or coatings (a coating is a thick film) in order to improve food appearance and/or preservation. Edible films and coatings are basically dried polymer gels usually derived from either polysaccharides or proteins. Whey protein-based films and coatings have

better mechanical and barrier properties compared with most protein/carbohydrate-based products, particularly when plasticizers (sorbitol or glycerol) are added in order to avoid brittleness and improve resistance to moisture transfer across the film/coat.

## 14.3 Animal-derived proteins

### 14.3.1 Collagen

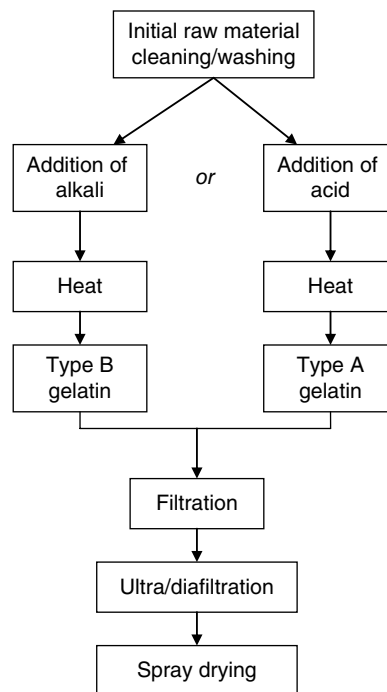
Collagen represents the principal constituent of connective tissue, being found in greatest quantities in tendons, cartilage, bones, blood vessels and skin. The basic structural unit of collagen, tropocollagen, consists of a triple helix of individual collagen polypeptides, each containing about 1000 amino acids. Collagen has an unusual amino acid composition, containing high levels of glycine (23%). It also contains high levels of proline and modified proline (3-hydroxyproline and 4-hydroxyproline) as well as another modified amino acid, 5-hydroxylysine. The collagen triple helix is stabilized by intermolecular covalent and non-covalent bonds. The covalent linkages are mainly formed between the modified amino acids mentioned above. Individual tropocollagen molecules are held together by yet more intermolecular linkages in the overall collagen fibril-based structure.

Although not produced in true 'bulk' quantities, collagen-based products have found important niche application in cosmetic surgery, where it is used as dermal filler to reduce wrinkles, repair skin defects or augment lips. After its injection the collagen forms a soft cohesive network of fibres, responsible for restoring/generating skin contour to the desired level. Typically repeat administration ('touch up implantations') is required (usually in 6-monthly to 2-yearly intervals) in order to maintain the desired level of correction, as the injected collagen is subject to normal body protein turnover. Collagen products, either currently used or in clinical trials, can be derived from various sources. Bovine- and porcine-derived collagens are both used, while human collagen can be derived from

cadavers or via fibroblast culture. Collagen preparations can be chemically cross-linked by using glutaraldehyde, which allows the generation of higher concentrations of collagen product. Cross-linking also likely protects, at least partially, the collagen product from collagenase activity *in vivo*, thereby producing longer-lasting implant products.

### 14.3.2 Gelatin

Gelatin is partially degraded/unstructured collagen. Typical industrial-scale gelatin production processes are outlined in Figure 14.11. The raw material (usually skin/hides, bone, tendons and loose connective tissue) is first stripped of non-collagenous compounds and washed (initial preparation). This is followed by the addition of either acid or alkali and heating. Acid is favoured in processes utilizing less cross-linked collagenous raw materials such as pig skin, fish skin and bone, while alkali treatment is



**Figure 14.11** Overview of common processes by which gelatin is manufactured on an industrial scale. Refer to text for further details.



usually characteristic of highly cross-linked collagens, mainly bovine hide. Acid or alkali promotes substrate swelling. The material is then heated (usually stepwise, from temperatures ranging from about 40°C up to boiling in some cases), which promotes extraction of the collagen and its conversion to gelatin. Conversion entails disassembly of the highly ordered fibril structure and significant collapse of the tropocollagen triple helix. Limited cleavage of the backbone of some individual collagen polypeptides may also occur. Gelatin produced by acid treatment is termed type A gelatin, and displays an isoelectric point between 6 and 9. Alkali-generated product is termed type B gelatin. The alkaline pH, particularly at high temperatures, promotes the hydrolysis of the side-chain amide groups of asparagine and glutamine residues in the collagen, forming aspartic and glutamic acid residues, respectively. This in turn results in type B gelatins displaying lower isoelectric points (typically 4–5).

The gelatin extract solution, however generated, is then usually filtered (to remove suspended elements, intact fragments of collagen, etc.). Subsequently, the solution is usually diafiltered to remove inorganic salts and to facilitate adjustment of pH to 5.0–5.8. Finally, the product is usually spray-dried and milled to give a flaky/ground granular product.

Gelatin finds extensive application in the food industry. Because of its unbalanced amino acid composition (Table 14.9), it is a poor nutritional source. Its application in foods stems instead from its functional properties. The most notable functional property of gelatin is its ability to form a gel structure. Gelatin readily dissolves in hot water yielding random collagen-based coils. As the temperature is allowed to decrease to 35–40°C, some of the individual collagen polypeptides begin to interact with one another, forming short segments of triple helix. These junction zones effectively cross-link gelatin polypeptides in a three-dimensional lattice structure, thereby forming the gel. Reheating to temperatures above 40°C reverses these interactions, to yield a molten product once again. A gelatin solution can go through many such cycles of melt and solidification without damage. Gelatin finds most extensive application in the food sector as a gelling agent

**Table 14.9** Amino acid composition (%) of gelatin.

Glycine	23.0
Alanine	7.8
Valine	2.25
Leucine	2.9
Isoleucine	1.26
Cysteine	0.09
Methionine	0.9
Phenylalanine	2.0
Proline	16.2
Hydroxyproline	12.7
Serine	0.36
Threonine	1.7
Tyrosine	0.45
Aspartate	6.0
Glutamate	10.2
Arginine	7.3
Lysine	3.7
Histidine	0.72

(e.g. gelatin-based desserts). The protein also displays weak amphipathic properties and, as such, can help stabilize emulsions and foams. It is on this basis that gelatin is often added to ice creams and marshmallow products.

Gelatin also finds application in non-food areas, particularly in the pharmaceutical and medical sectors. Pharmaceutically, it is used to produce gelatin-based capsules as well as a thickening agent in some paste-based medicinal products. It is also used as a gelling agent for the manufacture of suppositories and pessaries. Its ability to absorb five to ten times its own weight of water forms the basis for its use in some surgical procedures, as gelatin sponges can mop up significant quantities of blood. Gelatin that is more extensively degraded (by, for example, prolonged heating) displays adhesive properties. Such degradation products form the basis of many animal-based glues and binders. A summary of the major industrial applications of gelatin is presented in Table 14.10.

Because of demand, annual global gelatin production levels are estimated to exceed 300,000 tonnes. Almost half of this is derived from pigin, skin,

**Table 14.10** Major food and non-food applications of collagen-derived gelatin.**Food applications**

Gelling agent  
 Stabilizer  
 Thickener  
 Emulsifier  
 Foaming agent

**Pharmaceutical/medical**

Gelatin-based capsules  
 Thickening agents for pastes  
 Gelling agent for suppositories  
 Gelatin-based surgical sponges

**Other uses**

Glue manufacture  
 Binder in match heads  
 Sizing agent for paper/textile manufacture  
 Photographic films  
 Manufacture of rubber substitutes

with bovine hide and bones representing the majority of the remainder. In some world regions where fish processing is a major industry, gelatin is also extracted from fish skin and bones. The cited advantage of this source is that it is not associated with pathogens (particularly prions) that could cause human disease. However, the standard gelatin manufacturing process would likely inactivate any such pathogens even if they were initially present.

### 14.3.3 Egg proteins

Eggs form part of the staple diet in many world regions and represent a good source of dietary protein. Layer hens typically produce in the region of 250 eggs per annum. Exact egg weight will vary depending on breed, age and nutritional status of the bird, but will usually be in the region of 55–60 g. In terms of composition, whole eggs consist of approximately 10% shell, 60% egg white and 30% yolk. Many processed foods contain egg white/yolk as an ingredient. Although contributing to its nutritive value, they are most often added to food due to their functional characteristics.

The initial step of egg processing for food application entails mechanical breaking followed by sieving (to remove eggshell particles). This is carried

out at refrigeration temperatures in order to discourage microbial growth. If whole egg is required, homogenization follows. Alternatively, mechanical separation of egg white and yolk can be undertaken, followed by independent homogenization/blending. Pasteurization is next undertaken. For whole egg/egg yolk, this is usually carried out by holding at 64.4°C for 150 seconds. The lipid content of the yolk exerts a protective effect on the thermolabile proteins (mainly egg white proteins) present. Isolated egg white is pasteurized at lower temperatures (~57°C), although the natural presence of lysozyme helps prevent microbial spoilage in this product. After pasteurization is completed, the processing temperature is reduced and the product is either frozen, spray-dried, salted or acidified in order to prevent microbial spoilage. Further processing to yield purified egg protein is not undertaken.

Egg white proteins contribute significantly to the overall functional characteristics of egg-based products. Egg white consists of approximately 86% water and 11% protein, with lower levels of carbohydrate, fat and minerals being present. The major proteins found in egg white are presented in Table 14.11. Ovalbumin is found at highest concentration in egg white. It is a 385 amino acid, 45-kDa phosphoglycoprotein. The carbohydrate component is attached to the polypeptide backbone by an N-glycosyl linkage involving Asn<sup>292</sup>. Three variant forms of ovalbumin can be separated by isoelectric focusing for example. These represent unphosphorylated, monophosphorylated and diphosphorylated forms of the protein. The phosphate groups reside on either Ser<sup>68</sup> or Ser<sup>344</sup> or both. Ovalbumin is also acetylated at its N-terminus and contains four cysteine residues.

Ovalbumin, along with some other egg white proteins, undergo denaturation and coagulation when heated. A gel-like structure results that is stabilized by intermolecular hydrophobic interactions. Gel formation impacts on food texture and water-holding capacity. Ovalbumin also contributes to the foaming properties of egg white, although when compared with proteins such as gelatin and casein it displays only modest foamability. Whipping of ovalbumin solutions induces congregation of the protein molecules at the air–water interface. This induces their partial denaturation, exposing sulfhydryl groups

**Table 14.11** Major protein types found in egg white. Immunoglobulins are found in egg white and yolk. Refer to text for further details.

Protein	Per cent of egg white	Molecular mass (kDa)	pI
Ovalbumin (egg albumen)	52	45	4.6
Ovotransferrin (conalbumin)	13	78	6.6
Ovomucoid	11	28	4.0–4.3
Lysozyme	3.5	14	10.7
Ovomucin ( $\alpha$ and $\beta$ )	1.5	18 ( $\alpha$ ), 400 ( $\beta$ )	
Immunoglobulins (various)	<1.0	Vary	Vary

normally buried internally in the protein. These are readily oxidized, forming intermolecular disulfide linkages that help stabilize the foam. Non-covalent interactions also play an important role in ovalbumin-based foam stability.

Ovomucoid and ovomucin represent additional proteins found in egg white. Ovomuroid is a 28-kDa trypsin inhibitor. It is a relatively thermostable glycoprotein whose conformation displays three separate domains linked by intradomain disulfide linkages. Ovomucin is a sulfated glycoprotein that displays viscous/gel-forming properties and plays a central role in the foaming ability of egg white. It consists of two subunits,  $\alpha$  and  $\beta$ , of molecular mass 18 and 400 kDa, respectively. Both subunits are glycosylated and approximately 50% of the  $\beta$ -subunit's molecular mass is contributed by the carbohydrate component.

## 14.4 Plant-derived proteins

A number of plant-derived proteins find actual or potential use in the food industry for functional purposes. Prominent among these are soy proteins, as well as rapeseed/canola proteins.

### 14.4.1 Soy proteins

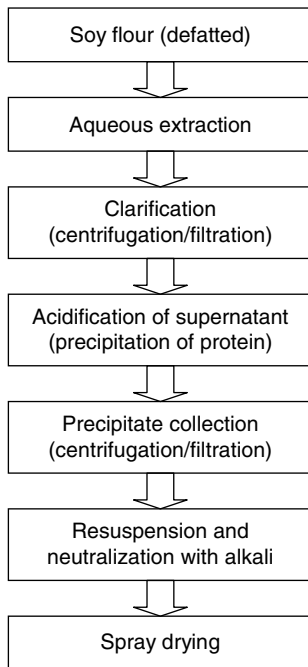
Soy proteins constitute an important group of functional proteins used in the food industry. Seed proteins derived from soybean, and indeed other legumes, are classified as globulins or albumins.

**Table 14.12** The major globulin-based seed proteins found in soybean.

7S Globulins
$\beta$ -Conglycinin, a 150–200 kDa glycoprotein
$\gamma$ -Conglycinin, a 170 kDa glycoprotein
Basic 7S globulin, a 168 kDa glycoprotein
11S Globulins
Glycinin, a 300–380 kDa protein

Globulins can account for up to 90% of the protein fraction and serve a storage function. Prominent globulin proteins found in soybean are summarized in Table 14.12. Glycinin and  $\beta$ -conglycinin in particular demonstrate gel-forming functional properties. Soy proteins also display emulsification properties and are used to help stabilize oil in water emulsions of soup, sausages and coffee whitener.

Soybean processing plants can generate three different soy protein-containing products. Soy flour (~50% protein content) is generated by simply milling soybeans into a fine powder. It is generally added directly to baked goods, thereby increasing their nutritional and protein content and potentially improving product texture. Soy protein concentrate (~70% protein) is typically produced by treatment of soy flour with solvents such as ethanol or dilute acid, under conditions where the protein fraction becomes insoluble, thereby allowing the extraction of still soluble carbohydrate and other flour components. Concentrates are sometimes added to foods simply in order to increase their nutritional value. They are also added to selected foods (e.g. meat- and poultry-based products) in order to increase



**Figure 14.12** Overview of the production of soy protein isolate.

water and fat retention. Production of soy protein isolate, which is at minimum 90% soy protein, is overviewed in Figure 14.12. Isolates are typically added to foods as an emulsifier or to improve texture or moisture retention.

### 14.4.2 Rapeseed/canola proteins

Rapeseed, including canola which is a variety of rapeseed, is a prominent agricultural crop, grown as an animal feed and as a source of vegetable oil (for human consumption and, more recently, as a biodiesel). The seeds typically contain 40% oil and 20% protein. Rapeseed meal is a byproduct of rapeseed oil extraction and typically consists of 50% protein. The protein content can be increased significantly by further processing steps, including aqueous-based extraction with subsequent protein precipitation. The major proteins present in rapeseed meal include the napins and cruciferins (storage proteins) as well as oleosin (a structural protein associated with the oil fraction).

Rapeseed-derived proteins are of interest in food processing as they are available in large quantities, are inexpensive and display a well-balanced amino acid content. They display some functionally useful attributes, including water- and fat-absorbing capacity, gelling ability and emulsifying properties. However, the actual application of these proteins has been limited by the presence of various phenols, glucosinolates and phytates, which display anti-nutritional properties as well as potentially undesirable tastes and the capacity to interact with some foodstuffs, producing coloured products.

## 14.5 Sweet and taste-modifying proteins

The production of glucose and fructose syrups and their use as sweeteners has been reviewed in Chapter 12. Sweetness, however, is not a sensation on which sugar-based substances have a monopoly. Several proteins (and peptides) have been identified which, if tasted, are perceived as being intensely sweet. The sweetest natural substances thus far discovered are in fact two plant proteins, thaumatin and monellin (Table 14.13). These, and the majority of other sweet proteins thus far characterized, are derived from the fruit or berries of selected African plants. These fruit and berries have long been consumed as food sweeteners by indigenous African populations. Their intense sweetness renders them attractive as, for example, low-calorie sweeteners. Unlike sugars, they do not promote tooth decay and can be safely used as sweeteners for diabetics.

Thaumatococcus is a protein found in the berries of the West African plant *Thaumatococcus daniellii*. Although its existence was documented in the 1850s, it was first isolated, characterized and used commercially in the 1970s. Two variants of thaumatin are known to exist, thaumatin I and II. The former exhibits a molecular mass of 22,209 Da while the latter exhibits a molecular mass of 22,293 Da. Both are 207 amino acid polypeptides differing in sequence by only five residues. Their three-dimensional structure is globular and they display several significant stretches of  $\beta$  conformation. They are extremely

**Table 14.13** Some sweet/taste-modifying proteins and their sources.

Protein	Source	Molecular mass (kDa)	Sweetness (relative to sucrose on a weight basis)
Thaumatococin	Fruit of <i>Thaumatococcus daniellii</i>	22.2	3000
Monellin	Fruit of <i>Dioscoreophyllum cumminsii</i>	11.1	3000
Mabinlin	Seeds of <i>Capparis masaikai</i>	14.0	400
Pentadin	Berries of <i>Pentadiplandra brazzeana</i>	12.0	500
Miraculin	Berries of <i>Richadella dulcifica</i>	28.0	Tasteless
Curculin	Fruits of <i>Curculigo latifolia</i>	27.8	550

thermostable, retaining their sweet taste even after boiling for 1 hour. The presence of eight disulfide linkages likely exerts a major stabilizing influence, but the proteins may also undergo partial unfolding during boiling, only to refold on subsequent cooling. Thaumatococins trigger their sweet sensation by binding specific taste bud receptors on the tongue. They are 3000 times sweeter than sucrose on a weight basis, which equates to 100,000 times sweeter on a molar basis.

Thaumatococins were first commercialized in the 1970s by the Tate and Lyle company under the tradename Talin. The natural producer grows poorly and produces little berries outside its native Africa, and Talin production entails local harvest of the berries with shipping to the final production site after freezing. Up to 50% of the berry mass may be accounted for by the protein, which is extracted in water after crushing. The soluble extract is then concentrated by ultrafiltration and spray-dried/freeze-dried yielding the final 'purified' product.

The thaumatococin genes have been isolated and expressed in various recombinant microbial systems, including *Escherichia coli*, *Bacillus subtilis*, *Streptomyces levidans*, *Saccharomyces cerevisiae* and *Kluyveromyces lactis*. The thaumatococin genes have also been expressed in transgenic plants and extracts from such plants have exhibited a sweet taste. Production by recombinant means exhibits advantages in overcoming problems of source availability. It also allows the production of variant forms exhibiting altered amino acid sequences. This may allow identification of even sweeter thaumatococins or ones displaying altered taste properties.

Thaumatococin is GRAS listed and at concentrations below its sweetness threshold it acts as a flavour enhancer. It has found commercial application as a sweetener/flavour enhancer in animal feed, human food and beverages, chewing gum and breath fresheners. At high levels, it also imparts a strong liquorice-like taste to foods and this property has somewhat limited its widespread food application.

Monellin was first purified in 1972 from the fruit of the West African plant *Dioscoreophyllum cumminsii*. It is a heterodimer consisting of a 44 amino acid residue A chain and a 50 amino acid residue B chain, held together by non-covalent interactions. The overall molecular mass is 11 kDa and the protein displays a pI value of 9.0–9.4. Monellin displays little sequence homology to thaumatococin, although polyclonal antibodies raised against one protein cross-react with the other. Monellin displays a similar level of sweetness to thaumatococin. It is less stable than thaumatococin and the sweet taste is not perceived for several seconds after consumption. The sweetness sensation then increases slowly followed by a slow decline, taking up to an hour. For such reasons, it is unlikely that monellin will gain the commercial acceptability that thaumatococin has. Pentadin is yet another sweet-tasting protein. It was first extracted in small quantities from the berries of the African plant *Pentadiplandra brazzeana* in 1989. The protein displays a taste profile somewhat similar to that of monellin.

Curculin is a homodimeric protein displaying a molecular mass of about 28 kDa. It is produced in the fruit of the herb *Curculigo latifolia*, which grows in western Malaysia. The protein is over 500 times

sweeter than sucrose on a weight basis but also has taste-modifying properties. Finally, miraculin is a 191 amino acid, 28-kDa glycoprotein originally isolated from the berries of the West African shrub *Richadella dulcifica*. This protein, which by itself is tasteless, has the ability to convert sour-tasting substances into sweet-tasting ones. Lemons, for example, when consumed after chewing miraculin-containing berries, taste like sweet oranges. Again, a disadvantage of this protein from an applied perspective is that it remains bound to taste receptors for a prolonged period. The sweet taste perceived can therefore last for up to 2 hours.

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