Part I

Protein Analytics
Investigation of the structure and function of proteins has already kept scientists busy for over 200 years. In 1777 the French chemist Pierre J. Macquer subsumed under the term *Albumins* all substances that showed the peculiar phenomenon of change from a liquid to a solid state upon warming. Chicken egg white, casein, and the blood component globulin already belonged to this class of substances. Already as early as 1787 (i.e., about the time of the French Revolution) the purification of egg white-like (coagulating) substances from plants was reported. In the early nineteenth century many proteins like albumin, fibrin, or casein were purified and analyzed. It soon became apparent that these compounds were considerably more complicated than other organic molecules known at that time. The word protein was most probably introduced by the Swedish chemist Jöns J. von Berzelius in about 1838 and was then published by the Dutch Gerardus J. Mulder. Mulder also suggested a chemical formula, which at that time was regarded as universally valid for all egg white-like materials. The homogeneity and purity of these purified proteins did not correspond of course to today’s demands. However, it became clear that proteins are different and distinct molecules.

At that time purification could succeed only if one could use very simple steps, such as extraction for enrichment, acidification for precipitation, and spontaneous crystallization. Already in 1889 Hofmeister had obtained chicken albumin in crystalline form. Although Sumner in 1926 could already crystallize enzymatically active urease, the structure and the construction of proteins remained unknown up to the middle of the twentieth century. Only by the development of efficient purification methods, which allowed single proteins to be isolated from complicated mixtures, accompanied by a revolution in analysis techniques of the purified proteins, was today’s understanding of protein structures possible.

This chapter describes fundamental purification methods and also touches on how they can be used systematically and strategically. It is extremely difficult to look at this subject in general terms, because the physical and chemical properties of single proteins may be very different. However, this structural diversity, which in the end determines also the function of the various proteins, is biologically very meaningful and necessary. Proteins – the real tools and building materials of a cell – have to exercise a plethora of different functions.

### 1.1 Properties of Proteins

**Size of Proteins** The size of proteins can be very different. From small polypeptides, like insulin, which consists of 51 amino acids, up to very big multifunctional proteins, for example, to the apolipoprotein B, a cholesterol-transporting protein which contains more than 4600 amino acid residues, with a molecular mass of more than 500 000 Dalton (500 kDa). Many proteins are composed of oligomers from the same or different protein chains and have molecule masses up to some millions Daltons. Quite in general it is to be expected that, the greater a protein is, the more

The molar mass \( (M) \) – often wrongly called molecular weight – is not a mass but is defined as the mass of a substance divided by the amount of substance:

\[
M = \frac{m}{n} = N_A \cdot m_M
\]

The unit is g mol\(^{-1}\).

Absolute molecule mass \( (m_M) \) is the molar mass of a molecule divided by the number of molecules in one mol \( (\text{Avogadro constant, } N_A) \):

\[
m_M = \frac{m}{N_A}
\]

The unit is g.

The relative molecular mass \( (M_r) \) is defined as the mass of one molecule normalized to the mass of \(^{12}\text{C} \) (carbon 12), which by definition is equal to 12.

\[
M_r = \frac{12}{m_M}
\]

It is dimensionless, but it has been given the “unit” Dalton (Da) (formerly atomic mass unit).
difficultly its isolation and purification will be. This has its reason in the analytical procedures which show very low efficiencies with big molecules. Figure 1.1 shows the separation capacity (the maximum number of analytes, which can be separated under optimal condition) of individual separation techniques against the molecule mass of the analytes.

It is evident that for small molecules like amino acids or peptides some chromatographic procedures are clearly able to distinguish more than 50 analytes in a single analysis. In the area of proteins ($M_r > 10^4$ Da) one recognizes that of the chromatographic techniques actually only ion exchange chromatography is able to separate efficiently more complicated mixtures. In the molecular mass area of proteins electrophoretic methods are by far more efficient. That is why in proteome analysis (e.g., the analysis of all proteins of a cell), where several thousand proteins have to be separated, electrophoretic procedures (linear and two-dimensional) are very often used. From the figure is also evident that almost no efficient separation procedures exist for large molecules, for example, for protein complexes with molecular masses greater than 150 kDa, or for organelles.

The separation efficiency of a method is not always the relevant parameter in a protein purification. If selective purification steps are available the separation capacity is no longer significant and the selectivity becomes the crucial issue. Consequently, an affinity purification, which is based on the specific binding interaction of a substance to an affinity matrix, for example, an immune precipitation or an antibody affinity chromatography, has a quite low separation capacity of 1, but has an extremely high selectivity. Due to this highly selectivity a protein can easily be isolated even from a complex mixture in a one-step procedure.

With the most common purification techniques, electrophoresis and chromatography, the analytes must be present in a dissolved form. Thus, the solubility of the protein in aqueous buffer media is a further important parameter when planning a protein purification. Many intracellular proteins, located in the cytosol (e.g., enzymes), are readily soluble while structure-forming proteins like cytoskeletal proteins or membrane proteins most often are much less soluble.

Especially difficult to handle in aqueous solutions are hydrophobic integral membrane proteins, which are usually surrounded by lipid membranes. Without the presence of detergents such proteins will aggregate and precipitate during the purification.
Available Quantity  The quantity available in the raw material plays a crucial role in determining the effort that must be invested for a protein purification. A protein intended for the isolation is present perhaps only as a few copies per cell (e.g., transcription factors) or as a few thousand copies (e.g., many receptors). On the other hand, abundant proteins (e.g., enzymes) can constitute percentage shares of the total protein of a cell. Overexpressed proteins of proteins are often present in in clearly higher quantities (>50% in a cell) as well as some proteins in body fluids (e.g., albumin in plasma >60%). Purification with higher quantities of a protein is usually much simpler. Especially with the isolation of rare proteins different sources of raw material should be checked for the content of the protein of interest.

Acid/Base Properties  Proteins have certain acidic or basic properties because of their amino acid composition, properties that are used in separations via ion exchange chromatography and electrophoresis. The net charge of a protein is dependent on the pH of the surrounding solution. At a low pH-value it is positive, at high pH negative, and at the isoelectric point it is zero. Positive and negative charges compensate at the latter pH.

Biological Activity  The purification of a protein is often complicated by the fact that a particular protein often can be detected and localized among the various other proteins only due to its biological activity and location. Hence, one must take into account at every stage of protein isolation the preservation of this biological activity. Usually the biological activity is based on a specific molecular and spatial structure. If it is destroyed, one speaks of denaturation. This often is irreversible. To avoid denaturation, one must exclude in practice the application of some procedures.

The biological activity is often stable to different extents under different environmental conditions. Too high or too low buffer concentrations, temperature extremes, contacts with artificial surfaces such as glass or missing cofactors can change biological characteristics of proteins. Some of these changes are reversible: small proteins in particular are, after denaturation and loss of activity, often able to renature under certain conditions, regaining the biologically active form. For larger proteins, this is rarely the case and often results in only a poor yield. Measurement of the biological (e.g., enzymatic) activity makes it possible to monitor the purification of a protein. With increasing purity a higher specific activity is measured. In addition, the biological activity itself can be utilized for the purification of the protein. The activity often goes hand in hand with binding properties to other molecules, such as enzyme–substrate or cofactor, receptor–ligand, antibody, antigen, and so on. This binding is very specific and can be used to design affinity purifications. These are characterized by high enrichment factors and may achieve great efficiency that is difficult to obtain by other techniques.

Stability  When proteins are extracted from their biological environment they are often markedly impaired in their stability. They may be degraded by proteases (proteolytic enzymes) or associate into insoluble aggregates, which almost always leads to an irreversible loss of biological activity. For these reasons, protease inhibitors are often added in the first steps of an isolation and the purification is carried out quickly and generally at low temperatures.

Considering the diversity of the characteristics of proteins it immediately becomes obvious that a protein separation cannot be performed under a single schematic protocol. For a successful isolation strategy a realistic judgement of the behavior of a protein in different separation and purification methods, a minimal understanding of the solubility and charge properties of the protein to be purified, and a clear vision of why the protein is to be purified are absolutely necessary.

Goal of a Protein Purification  Above all the first steps of a purification procedure, the level of purity to be aimed at and also the analytics to be used are highly dependent on the intention behind purifying a certain protein. Thus, far higher demands for cleanness must be made with the isolation of a protein for therapeutic purposes (e.g., insulin, growth hormones, or blood coagulation inhibitors) than for a protein that is used in the laboratory for structural investigations. In many cases one wants to isolate a protein only to make an unequivocal identification or to clarify some amino acid sequence segments. For such purposes usually a tiny amount of protein
(usually in the microgram range) is sufficient. With the sequence information one is able to identify the protein in protein data banks or it provides the information needed to produce oligonucleotides to isolate the gene corresponding to the protein. The protein can then be expressed in a host organism in much larger quantities (up to gram quantities) than was present in the original source (heterologous expression). Then, many of the other investigations are carried out not with the material from the natural source but with the recombinant protein. New strategic approaches to the analysis of biological questions, such as proteomics and other subtractive approaches, require completely new types of sample preparation and protein isolation. Here it is essential not to change the quantitative relations of the single proteins. A major advantage of these new strategies is that the preservation of the biological activity is no longer so important. Although each protein purification is to be regarded as a unique case, one can still find, especially for the first purification steps, some general rules and procedures that have already been applied frequently in successful isolations; they will be discussed in detail below.

### 1.2 Protein Localization and Purification Strategy

The first step in any protein purification aims to bring the protein of interest into solution and remove all particulate and insoluble material. Figure 1.2 shows a scheme for different proteins. For the purification of a soluble extracellular protein, cells and other insoluble components must be removed to obtain a homogeneous solution, which can then be subjected to purification methods discussed in the following sections (precipitation, centrifugation, chromatography, electrophoresis, etc.). Sources of extracellular proteins are, for example, culture supernatants of microorganisms, plant and animal cell culture media, or body fluids such as milk, blood, urine, and cerebrospinal fluid. Often, extracellular proteins are present only in relatively low concentrations and demand as the next step an efficient concentration step.

To isolate an intracellular protein, the cells must be destroyed in a manner that releases the soluble contents of the cell and keeps the protein of interest intact. Cell disruption methods differ mainly according to cell type and amount of cells.

**Membrane Proteins and other Insoluble Proteins** Membrane-associated proteins are usually purified after isolation of the relevant membrane fraction. For this purpose, peripheral membrane proteins that are bound loosely to membranes are separated by relatively mild conditions, such as high pH, EDTA addition, or lower concentrations of a non-ionic detergent. This fraction of peripheral membrane proteins often can then be treated like soluble proteins. Integral membrane proteins that aggregate outside their membrane via hydrophobic amino acid sequence regions and become insoluble can only be isolated from the membrane by using high detergent

![Figure 1.2 Purification scheme for different proteins. According to localization and solubility different purification steps are necessary before any subsequent selective and highly efficient purification steps.](image-url)
concentrations. At present, they present probably the greatest challenge to the isolation and purification techniques.

Proteins that are insoluble in normal aqueous buffers are in general structural proteins (e.g., elastin). Additionally, they are sometimes also crosslinked via post-translationally attached modifications (e.g., functional groups). Here a first and highly efficient purification step is to remove all soluble proteins. Further steps are usually possible only under conditions that destroy the native structure of the proteins. The further processing is often carried out by cleavage of the crosslinking of the denatured proteins and the use of chaotropic reagents (e.g., urea) or detergents.

**Recombinant Proteins** A special situation occurs in the production of recombinant proteins. A rather simple purification is possible by the expression of recombinant proteins in inclusion bodies. These are dense aggregates of the recombinant product, which are present in a non-native state and are insoluble, because the protein concentration is too high, or because the expressed protein in the host environment cannot be correctly folded, or because the formation of the (correct) disulfide bonds in the reducing environment inside the host is not possible. After a simple purification by differential centrifugation (Section 1.5.2), in which the other insoluble cell components are removed, the recombinant protein is obtained in a rather pure form. However, it still needs to be converted into the biologically active state by renaturation.

When the expression of recombinant proteins does not result in inclusion bodies, the protein is present in a soluble state inside or outside of the cell, depending on the vector. Here, further purification is similar to the purification of naturally occurring proteins but with the advantage that the protein to be isolated is already present in relatively large amounts.

Recombinant proteins can be easily isolated by using specific marker structures (tags). Typical examples are fusion proteins in which at the DNA level the coding regions for a tag structure and the desired protein are ligated and expressed as a single protein. Such fusion proteins often can be isolated in a rather pure form in a one-step procedure on applying a specific antibody affinity chromatography against the tag structure. Examples are GST fusion proteins with antibodies against GST or biotinylated proteins using avidin columns. Another frequently used tag-structure is multiple histidine residues, which are attached to the N- or C-terminal end of the protein chain and are easy to isolate by immobilized metal affinity chromatography (IMAC).

### 1.3 Homogenization and Cell Disruption

To purify biological components of intact tissues, the complex cell associations must be disrupted in a first step by homogenization. The result is a mixture of intact and disrupted cells, cell organelles, membrane fragments, and small chemical compounds derived from the cytoplasm and from damaged subcellular compartments. Since the cellular components are transferred to a non-physiological environment, the homogenization media should meet several basic requirements:

- protection of the cells from osmotic bursting,
- protection from proteases,
- protection of the biological activity (function),
- prevention of aggregation,
- minimal destruction of organelles,
- no interference with biological analyses and functional tests.

Normally this is done by isotonic buffers at neutral pH. Often, a cocktail of protease inhibitors is added (Table 1.1).

If you want to isolate intracellular organelles, such as mitochondria, nuclei, microsomes, and so on, or intracellular proteins, the still intact cells have to be disrupted. This is accomplished by mechanical destruction of the cell wall. This procedure releases heat of friction and therefore has to be carried out with cooling. The technical realization of the disruption process varies depending on the starting material and location of the target protein (Table 1.2).
For very sensitive cells (e.g., leukocytes, ciliates) repeated pipetting of the cell suspension or pressing it through a sieve is sufficient to achieve a disintegration by surface shear forces. For the slightly more stable animal cells, the shear forces are generated with a glass pestle in a glass tube (Dounce homogenizer). These methods are not suitable for plant and bacterial cells.

- Cells that have no cell wall and are not associated (e.g., isolated blood cells) can be broken osmotically by being placed in a hypotonic environment (e.g., in distilled water). The water penetrates into the cells and causes them to burst. In cells with cell walls (bacteria, yeasts) the cell walls must be treated enzymatically (e.g., with lysozyme) before an osmolytic digestion can succeed. Such exposure is very gentle and is therefore particularly suitable for the isolation of nuclei and other organelles.
- For bacteria repeated freezing and thawing is often used as a disruption method. By changing the aggregate state the cell membrane is deformed so that it breaks and the intracellular content is released.
- Microorganisms and yeasts can be dried at 20–30 °C in a thin layer for two to three days. This leads to destruction of the cell membrane. The dried cells are then ground in a mortar and can be stored at 4 °C if necessary also for longer periods. Soluble proteins can be extracted with an aqueous buffer from the dry powder in a few hours.
- With cold, water-miscible organic solvents (acetone, –15 °C, ten-times volume) cells can be quickly drained, with the lipids extracted into the organic phase, and thus the cell walls are destroyed. After centrifugation, the proteins remain in the precipitate, from where they can be recovered by extraction with aqueous solvents.
- With stable cells such as plant cells, bacteria, and yeasts a mortar and pestle can be applied for cell disruption, although larger organelles (chloroplasts) may be damaged. The addition of an abrasive (sea sand, glass beads) facilitates the disruption.
- For larger quantities, a rotating knife homogenization can be used. The tissue is cut by a rapidly rotating knife. As this produces considerable heat a way of cooling should be present. For small objects such as bacteria and yeasts, the efficiency of the pulping process is significantly improved by the addition of fine glass beads.
- Vibration cell mills are used for a relatively harsh disruption of bacteria. These are lockable steel vessels in which the cells are vigorously shaken with glass beads (diameter 0.1–0.5 mm). Again, the heat generated must be dissipated. Cell organelles can be damaged in this decomposition method.
- Rapid changes in pressure break cells and organelles in a very efficient manner. Therefore, strong pressure changes are produced in the suspension of a cell material with ultrasonic waves in the frequency range 10–40 kHz through a metal rod. Since in this method much heat is released, only relatively small volumes and short sound pulses with a maximal duration of 10 s should be applied. DNA is fragmented under these conditions.
- In a further disruption method that is particularly suitable for microorganisms, up to 50 ml of a cell suspension are pressed through a narrow opening (<1 mm), where the shear forces destroy the cells (French press).

<table>
<thead>
<tr>
<th>Table 1.1 Protease inhibitors.</th>
<th>Concentration</th>
<th>Inhibitor of</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylmethylsulfonyl fluoride (PMSF)</td>
<td>0.1–1 mM</td>
<td>Serine proteases</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>0.01–0.3 μM</td>
<td>Serine proteases</td>
</tr>
<tr>
<td>ε-Amino-n-caproic acid</td>
<td>2–5 mM</td>
<td>Serine proteases</td>
</tr>
<tr>
<td>Antipain</td>
<td>70 μM</td>
<td>Cysteine proteases</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>1 μM</td>
<td>Cysteine proteases</td>
</tr>
<tr>
<td>Pepstatin A</td>
<td>1 μM</td>
<td>Aspartate proteases</td>
</tr>
<tr>
<td>Ethylenediaminetetraacetic acid (EDTA)</td>
<td>0.5–1.5 mM</td>
<td>Metalloproteases</td>
</tr>
</tbody>
</table>
Depending on the objectives, proteins of interest in soluble form must be subjected to further purification steps. For this purpose, the homogenate is usually roughly separated into different fractions by differential centrifugation (Section 1.5.2).

### 1.4 Precipitation

The precipitation of proteins was one of the first techniques used for the purification of proteins (salting out of proteins was done for the first time over 130 years ago!). The method is based on the interaction of precipitating agents with the proteins in solution. These agents may be relatively non-specific resulting in the precipitation of practically all proteins from a solution. This is one way to obtain the total proteins from a cell lysate and is used frequently in the first steps of a purification process to obtain and concentrate the whole protein content of a cell. The precipitation can also be carried out in such a way that a fractionation of the components of a solution is possible. An example of this is the Cohn-fractionation of plasma, which was formulated in 1946 and still is used for plasma protein production on a large scale. Blood plasma is mixed with increasing amounts of cold ethanol and the proteins that are insoluble at the respective ethanol concentration are precipitated by centrifugation. However, with the exception of the precipitation of antigens with antibodies, precipitation is not protein specific and therefore can only be used for a rough pre-fractionation of protein mixtures.

Depending on the problem and starting material, the precipitation can be performed under different conditions. As well as the efficiency of the precipitation in itself, further aspects also be considered:

- Is the biological activity affected by the precipitant and precipitation conditions?
- Under what conditions can the precipitant be removed?

<table>
<thead>
<tr>
<th>Table 1.2 Biological starting materials and disruption methods.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Material</strong></td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>Bacteria</td>
</tr>
<tr>
<td>Gram positive</td>
</tr>
<tr>
<td>Last</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Gram negative</td>
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<tr>
<td></td>
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<tr>
<td>Yeast</td>
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<td></td>
</tr>
<tr>
<td>Plants</td>
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<td></td>
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<td></td>
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<tr>
<td></td>
</tr>
<tr>
<td>Fibrous tissues</td>
</tr>
<tr>
<td>Non-fibrous tissues</td>
</tr>
<tr>
<td>Higher eukaryotes</td>
</tr>
<tr>
<td>Cells from suspension culture</td>
</tr>
<tr>
<td></td>
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<tr>
<td></td>
</tr>
<tr>
<td>Fibrous tissues</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Muscle tissues</td>
</tr>
</tbody>
</table>

The ability of a salt to precipitate proteins is described in the so-called Hofmeister series (Figure 1.3). Salts further to the left (so-called-antichaotropic or cosmotropic salts) are particularly good and gentle precipitants. They increase hydrophobic effects in the solution and promote protein aggregation via hydrophobic interactions. Salts further to the right (chaotropic salts) reduce hydrophobic effects and keep proteins in solution. The oldest method used to precipitate proteins is the “salting out” of proteins by adding ammonium sulfate. The concentration of the proteins prior to precipitation should be about 0.01–2%. Ammonium sulfate is particularly suitable since in concentrations above 0.5M it also protects the biological activity of sensitive proteins. It is easily removed from the proteins (dialysis, ion exchange) and is also inexpensive. Thus it may be used already in the very first purification steps for the precipitation from larger volumes. Ammonium sulfate added usually under controlled conditions (temperature, pH) in portions to the protein solution, whereby a fractional precipitation and thus an accumulation of the protein of interest can be achieved. Notably, a complete precipitation can take several hours. Ammonium sulfate precipitates are usually tight and easy to centrifuge (100 g, cf. Section 1.5). The only major drawback of ammonium sulfate relates to the precipitation of proteins that require calcium for their activity/structure because calcium sulfate is virtually insoluble and thus it will be removed from the proteins. Therefore, these proteins have to be precipitated with other salts (e.g., acetates).

Precipitation using Organic Solvents  
For over 100 years it has been known that proteins can be precipitated with cold acetone or short-chain alcohols (mainly ethanol). Long-chain alcohols (greater than C₅) have a low solubility in water and are not suitable for precipitation. For the choice of organic precipitant or the optimal temperature no general rules can be given. Ethanol has proven particularly useful in the precipitation of plasma proteins. For protein solutions that contain lipids, acetone is often used, since in addition to the precipitation of the proteins at the same time the lipids are extracted. To avoid too high a local concentrations of the organic solvent, which may result in the denaturation of the proteins, the solvent should be added slowly. Good cooling and slow addition are also useful, since by the addition of organic solvent (e.g., ethanol to water) heat may be generated, which could lead to unwanted denaturation. The precipitate can be pelleted by centrifugation (see below) and again dissolved in aqueous buffers. In a frequently used protocol for acetone precipitation a fivefold volume excess of cold acetone (–20 °C) is added to the protein solution and incubated overnight at –20 °C. Then, it is centrifuged for 30 min at 20,000 g. This precipitation usually provides excellent results even for very small quantities of protein. The recovery of protein (yield of the precipitation) must be monitored with analytical methods (SDS-gel electrophoresis or activity tests, etc.).

Precipitation using Trichloroacetic Acid  
One method commonly used to precipitate proteins from solutions is the precipitation with 10% trichloroacetic acid, wherein a final concentration of 3–4% should be reached. After centrifugation, the precipitate is resuspended in the desired buffer for further purification steps, wherein the pH of the solution should be checked. This method denatures the proteins and is therefore primarily used for the concentration for gel electrophoretic separation or prior to enzymatic cleavages. The minimum sample concentration should be 5 μg ml⁻¹.

Precipitation of Nucleic Acids  
Protein solutions of cell lysates, especially bacteria and yeasts, contain a large amount of nucleic acids (DNA and RNA) which can interfere with protein purification and therefore usually has to be removed. Because nucleic acids are highly negatively charged polyanions they can be precipitated with strong alkaline substances (e.g., polyamines, polyethylenimines or anion exchange resins) or very basic proteins (protamines). Optimization of the nucleic acid precipitation – and washing conditions – must avoid also precipitating the...
proteins of interest, either by the precipitating agents used or due to binding to the precipitated nucleic acids (e.g., histones, ribosomes).

1.5 Centrifugation

Centrifugation is not only one of the oldest techniques for the removal of insoluble constituents, it is also used for cell fractionation and isolation of cell organelles. It is based on the movement of particles in a liquid medium by centrifugal forces. The central part of a centrifuge, the rotor, serves to receive the sample cup and is driven by a motor to high rotational speed. There are various construction designs of rotors, such as fixed-angle rotors, vertical or swinging bucket rotors (Figure 1.4), which are available in various sizes and materials. They allow separations of a few microliters to several liters and can be operated according to the task in hand with various adjustable rotational speeds. Usually, cooled centrifuges are used for working with biological materials. High-speed centrifuges, ultracentrifuges, are operated consistently under vacuum to avoid frictional heat that occurs at high speeds due to air resistance. In the operation of

![Figure 1.4 Rotors for centrifugation. Fixed angle rotor, vertical rotor, and swing-out rotor – loading conditions (a); under centrifugation at start of separation (b); during separation (c); slowing down (d) and finish of centrifugation (e); protein containing fractions in dark grey.](image-url)
centrifuges certain safety precautions must be followed, in particular opposite sample containers must be well balanced to avoid any imbalance that could destroy the centrifuge.

1.5.1 Basic Principles

The physical principle of centrifugation is a separation according to size and density. On a particle being moved at a constant angular velocity around an axis of rotation, a centrifugal force is acting, which accelerates the particles outwardly. The acceleration ($B$) is dependent on the angular velocity and on the distance $r$ from the rotation axis:

$$ B = \omega^2 r $$  \hspace{1cm} (1.1)

The acceleration is related to gravitational acceleration $g$ (981 cm s$^{-2}$) and expressed as relative centrifugal force (RCF) in multiples of the acceleration due to gravity ($g$):

$$ RCF = \omega^2 r / 981 $$  \hspace{1cm} (1.2)

The relationship between the angular velocity and the rotational speed in rotations per minute (rpm) is given by:

$$ \omega = \pi \times \text{rpm} / 30 $$  \hspace{1cm} (1.3)

then, by substitution:

$$ RCF = 1.118 \times 10^{-5} r (\text{rpm})^2 $$  \hspace{1cm} (1.4)

Notably, during centrifugation usually the distance of the particle from the axis of rotation and thus the RCF changes. Therefore, for the calculations the mean distance is often used.

The sedimentation rate of spherical particles in a viscous fluid is described by Stokes’ law, given below, where, $v$ is the sedimentation rate, $g$ is the relative centrifugal acceleration, $d$ is the diameter of the particle, and $\rho_p$ and $\rho_m$ are the density of the particle or the liquid, respectively, and $\eta$ is the viscosity of the medium. The sedimentation rate increases with the square of the particle diameter and the difference in densities between particle and medium, and decreases with the viscosity of the liquid. Now, if the sedimentation takes place in a medium such as, for example, 0.25 M sucrose, which is less dense than all the particles, and also has a low viscosity, the diameter of the particles is the dominant factor for the sedimentation velocity.

Stokes’ law:

$$ v = \frac{d^2 (\rho_p - \rho_m) g}{18 \eta} $$  \hspace{1cm} (1.5)

The sedimentation coefficient ($s$) is the sedimentation velocity under geometrically specified conditions of the centrifugal field. It is specified in Svedberg units ($S$):

$$ s = v / r \omega^2 $$  \hspace{1cm} (1.6)

1 S corresponds to $10^{-13}$ s. This order of magnitude is valid for different biological molecules. The Svedberg unit of a biomolecule sometimes appears in its name (e.g., 18S-rRNA), which then allows a conclusion to be made as to the size of the particle. Table 1.3 gives the size and conditions for the purification by centrifugation of cells and a few cellular compartments. A good overview is given by the representation of the particles in a density/sedimentation coefficient diagram (Figure 1.5) or in a density/g-values chart. From Stokes equation, the various techniques of centrifugation can be easily understood.

1.5.2 Centrifugation Techniques

Differential Centrifugation  Differential centrifugation exploits the different sedimentation rates of different particles. It is carried out in a fixed-angle rotor and assumes that the sedimentation rates are sufficiently different. The large and heavy nuclei sediment relatively quickly (1000 g for 5–10 min) and can be found even at low centrifugation speed in the precipitate (pellet). At higher RCF mitochondria (10 000 g, 10 min) and microsomes (100 000 g for 1 h) sediment. However, the individual fractions are not pure because slow particles that were geometrically close to the bottom of the centrifuge tube are contaminated by fast particles that were near the surface and must travel a longer distance. Differential centrifugation is not only used
for the enrichment of particles but also for concentration. Thus, for example, from one liter of bacterial cell culture the cells can be pelleted by centrifugation in 15 min at 2000 g and then can be resuspended in a smaller volume.

Zonal Centrifugation  If the sedimentation rates of molecules do not differ sufficiently, the viscosity and density of the medium can be used to generate selectivity. In the zonal centrifugation a preformed flat density gradient, mostly from sucrose, is used and the sample layered over the gradient (see below). The particles which at the beginning of the centrifugation – in contrast to differential centrifugation – are present in a narrow zone are now separated according to the sedimentation velocity. The density gradient, in addition to the minimization of convection, also has the effect that at increasing density and viscosity those faster particles are slowed down that would otherwise sediment with the increasing RCF caused by increasing distance from the rotor axis. This gives an approximately constant rate of sedimentation of the particles. Zonal centrifugation, which is usually carried out at relatively low speeds with swinging-or vertical

<table>
<thead>
<tr>
<th>Particle</th>
<th>Diameter (μm)</th>
<th>Density (g cm⁻³)</th>
<th>S-Value</th>
<th>Sedimentation in sucrose gradient at</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells</td>
<td>5–15</td>
<td>1.05–1.2</td>
<td>10²–10⁸</td>
<td>1000g/2 min</td>
</tr>
<tr>
<td>Nuclei</td>
<td>3–12</td>
<td>&gt;1.3</td>
<td>10⁵–10⁷</td>
<td>600g/15 min</td>
</tr>
<tr>
<td>Nuclear membrane</td>
<td>3–12</td>
<td>1.18–1.22</td>
<td></td>
<td>1500g/15 min</td>
</tr>
<tr>
<td>Plasma membrane</td>
<td>3–20</td>
<td>1.15–1.18</td>
<td></td>
<td>1500g/15 min</td>
</tr>
<tr>
<td>Golgi-apparatus</td>
<td>1</td>
<td>1.12–1.16</td>
<td></td>
<td>2000g/20 min</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>0.5–4</td>
<td>1.17–1.21</td>
<td>1 x 10⁴–5 x 10⁴</td>
<td>10 000g/25 min</td>
</tr>
<tr>
<td>Lysosomes</td>
<td>0.5–0.8</td>
<td>1.17–1.21</td>
<td>4 x 10³–2 x 10⁴</td>
<td>10 000g/25 min</td>
</tr>
<tr>
<td>Peroxisomes</td>
<td>0.5–0.8</td>
<td>1.19–1.4</td>
<td>4 x 10³</td>
<td>10 000g/25 min</td>
</tr>
<tr>
<td>Microsomes</td>
<td></td>
<td></td>
<td></td>
<td>100 000g/1 h</td>
</tr>
<tr>
<td>Endoplasmic reticulum</td>
<td>0.05–0.3</td>
<td>1.06–1.23</td>
<td>1 x 10³</td>
<td>150 000g/40 min</td>
</tr>
<tr>
<td>Ribosomes</td>
<td></td>
<td>1.55–1.58</td>
<td>70–80</td>
<td></td>
</tr>
<tr>
<td>Soluble proteins</td>
<td>0.001–0.01</td>
<td>1.2–1.7</td>
<td>1–25</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1.5  Density and sedimentation coefficients of some cell compartments. The figure shows the distribution of different cell components in terms of their density and their sedimentation coefficients. ER, endoplasmic reticulum.
rotors, is an incomplete sedimentation; the maximum density of the medium must not exceed the lowest density of the particles. The centrifugation is stopped before the particles pellet.

**Isopycnic Centrifugation** The previously discussed techniques of differential and zonal centrifugation are especially suitable for the separation of particles that differ in size. These techniques are not well suited for particles having a similar size but different densities. For these cases, isopycnic centrifugation (also known as sedimentation equilibrium centrifugation) is used. Here centrifugation is performed for long periods at high speed in a density gradient until equilibration. According to Stokes’ equation, particles remain in the floating state when their density and the density of the surrounding medium are equal \( \nu = 0 \). Particles in the upper part of the centrifuge tube sediment until they reach the state of suspension and cannot sediment further because the layer below has a greater density. The particles in the lower region rise accordingly up to the equilibrium position. In this type of centrifugation, the gradient density must exceed the density of all particles to be centrifuged.

**Density Gradient** To generate the density gradient, which can be continuous or discontinuous (in stages), various media are used, which have been found for the different application areas as appropriate:

- **CsCl solutions** can be prepared with densities up to 1.9 g ml\(^{-1}\). They are of very low viscosity, but have the drawback of high ionic strength, which can dissociate some biological materials (chromatin, ribosomes). In addition, CsCl solutions have high osmolality, which makes them unsuitable for osmotically sensitive particles such as cells. CsCl gradients are particularly suitable for the separation of nucleic acids.

- **Sucrose** is often used for the separation of subcellular organelles in zonal centrifugation. The inexpensive and easy to prepare solutions are nonionic and relatively inert to biological materials. The low density of isotonic sucrose solutions \((<9\% \text{ w/v})\) often prevents the centrifugation of cells. In isopycnic centrifugation the high viscosity of highly concentrated sucrose solutions leads to poor resolution.

Because of the high osmolality of natural sucrose, high molecular weight polysaccharides such as glycogen, dextran, or synthetic polysaccharides such as Ficoll are used to generate the gradients. Although these polysaccharides have a better osmolality than sucrose, their high viscosity leads to longer centrifugation times and poorer separations. The polysaccharides are used for zonal centrifugation and isopycnic centrifugation. After centrifugation one may dispose of the polysaccharides by dilution and subsequent precipitation of biological particles.

Colloidal silica particles coated with polymer may also be used as density gradient media. They show lower osmolality, but higher viscosity than the cesium salts. Percoll consists of polyvinylpyrrolidone (PVP) coated silica particles about 15–30 nm in diameter, which are available as a suspension with a density of 1.130 g ml\(^{-1}\). During centrifugation density gradients form quickly by itself due to the colloidal nature of Percoll. The profile, however, changes during centrifugation. An isotonic density gradient can be formed by addition of sucrose or salts to Percoll.

Today a class of iodinated media is used for the formation of a stable, inert, and non-toxic density gradient; they were originally developed as contrast media for X-ray structure analysis. The most widely used representative of these media is Nycodenz, which is particularly suitable for centrifugation of cells and membrane bound particles.

**Fractionation of the Separated Bands** After centrifugation the separated bands have to be recovered from the centrifuge vessel. If discontinuous gradients were used, the fractions of interest are sometimes visible to the density limits and can be gently aspirated with a Pasteur pipette. For a continuous gradient in which the fractions are often not clearly visible, fractionation is performed by making a hole in the bottom of the centrifuge tube and then collecting the gradient dropwise in sample containers. Another method is to introduce a dense liquid to the bottom of the tube which can displace the entire gradient upwards. With a suitable device at the top of the tube, the gradient can be delivered into a fraction collector. Then a solution that exceeds the density of the gradient is pumped through the glass tube to the bottom of the centrifuge vessel. The gradient is lifted and collected at the fraction collector.
1.6 Removal of Salts and Hydrophilic Contaminants

In the course of protein purification often solutions are obtained that are inappropriate in ionic strength or buffer composition for the next purification step. For example, after hydrophobic interaction chromatography the salt content of the sample is too high for direct subsequent ion exchange chromatography. In many cases, through good planning of the purification strategy, it will be possible to avoid additional steps for desalting. Especially in the last purification step prior to further analytical investigations the sample should be salt-free by using a volatile solvent system. On the other hand, there are several ways of buffer changing and desalting, which afford good yields at least with highly soluble proteins.

**Dilution**  Often it is very easy to reach the ionic strength required for the next purification step by simply diluting the sample with distilled water. For the next step in the purification usually a concentration step, such as ion exchange chromatography or affinity purification, is necessary. If dilution is insufficient to lower the salt concentration in the desired manner, desalting techniques need to be applied, which will be described next.

**Dialysis**  The longest known desalting method is dialysis. The most important part of dialysis is the dialysis membrane, where small molecules can diffuse freely, while larger molecules are retained. There are various membranes available, which differ mainly in pore size (molecular weight cut-off threshold, called the cut-off value). The cut-off value usually indicates the molecular weight of proteins that are excluded by 90% of the membrane. These values are determined with dextrans or globular proteins and are expressed in the membrane description. But, in addition, the hydration and the charge of a protein also play an important role in passage through the membrane. The cut-off value does not give a sharp molecular weight cut-off but may only give a clue as to which molecular size can pass through the membrane relatively unhindered. The protein solution is placed in a dialysis tube, consisting of a dialysis membrane. It is recommended that prior to dialysis the tubing, which is usually made of regenerated cellulose and contains significant amounts of heavy metals, is boiled for a few minutes in distilled water and rinsed with distilled water extensively to remove impurities. Since the volume of the sample solution can significantly increase due to water migration during dialysis, the dialysis tube should only be filled to no more than two-thirds full. The so-filled dialysis tube, largely free of air and sealed by nodes at both ends, is suspended in a beaker with the desired buffer. The rate of diffusion through the membrane is determined by the concentration gradient of the diffusible particles, the diffusion constants of these particles, the membrane surface, and the temperature. For effective desalting the buffer has to be stirred and changed several times to obtain the largest possible concentration gradient across the membrane surface. For the stability of proteins, dialysis is normally performed in a cold room. The progress of the desalting can be checked by measuring the conductivity of the buffer. Normally, two-to-three buffer exchanges with a 4–6 h equilibration period each are sufficient.

In an extensive desalting (dialysis against water) it must be noted that proteins can partially precipitate because of a low ionic strength. The precipitates may be centrifuged and often can be dissolved in a small volume of a solution with a slightly higher ionic strength.

Dialysis is a very simple, relatively slow technique that is limited to very small amounts of sample (micrograms) by adsorption losses. For these small amounts and small volumes (<500 μl) instead of dialysis tubes various special constructions are used that have a small sample chamber of a few microliters (e.g., Eppendorf vessels or the lid of the Eppendorf vessel) and can be sealed on one side against the buffer with the dialysis membrane.

With a dialysis tube it is also possible to concentrate samples. The dialysis tubing is not to be put in a buffer tank, but in a hygroscopic material, for example, Sephadex G100. This draws the liquid and small molecules through the membrane wall. The material will be changed if it becomes wet.
Ultrafiltration and Diafiltration  For rapid concentration of protein solutions ultrafiltration is suitable. Asymmetric membranes, having different pores size at the top and bottom and various exclusion limits, of cellulose, cellulose esters, polyethersulfone, or poly(vinylidene fluoride) (PVDF) have been developed. Ultrafiltration is not like dialysis, which is driven by a concentration gradient, but works according to the flow rate of the solvent through the ultrafiltration membrane. The salts (or other molecules with molecular weights significantly below the cutoff) are pressed together with the water through the membrane. For this purpose, pressure, vacuum, or centrifugation can be used. Ultrafiltration is usually performed in disposable containers of different sizes, depending on the sample volume. The sample volume is thereby reduced without a significant change in the salt concentration.

Diafiltration is based on the same principle, in which the sample is desalted in a similar way to dialysis. Here the volume of sample is kept constant by continuously feeding fresh dialysis buffer to the sample to replace the volume that is removed to the filtrate during ultrafiltration. The time needed for a particular diafiltration can be estimated from the volume of the filtrate. For a reduction in salt concentration by a factor of 10 the volume of the collected filtrate must be 2.3 times the initial volume of the sample, for a 100-fold reduction in salt concentration 4.6-times the initial sample volume must be collected. In a variant of the technique the sample may be diluted after the filtration volume reduction and ultrafiltered again. This is repeated until the desired salt concentration is reached.

Gel Chromatography  During gel chromatography the substances present in the sample are separated according to their size. Salts thereby elute after the passage of about one column volume. Gel chromatography has some drawbacks that make it less favorable for large quantities and large volumes, as often occur at the beginning of a purification procedure:

- The sample volume shall not exceed 5% of the column volume, otherwise the separation between proteins and salts is no longer sufficient.
- Gel chromatography columns are easily overloaded with large amounts of protein, which leads to a mixture of protein and salt ranges.
- The separation is better at low flow rate, resulting in a relatively long analysis time.
- In gel chromatography the elution volume is at least triple that of the initial sample volume, which usually necessitates a concentration or a concentrating next step in a separation procedure. Today, for small sample volumes and small amounts of protein several smaller spin columns are on the market, which contain gel chromatography material and show excellent recoveries in protein desalting.

As with dialysis, concentration, desalting, or salt exchange should be performed at 4 °C. In addition, in ultrafiltration there is a risk that small amounts of protein in very dilute solutions (<20 μg ml⁻¹) adsorb to the membranes or the vessel walls. Small amounts of glycerol or detergents (e.g., Triton X-100) at concentrations below the critical micellar concentration (CMC, Section 1.8.1) added to the protein solution may sometimes help to minimize the losses caused by adsorption.

Reversed-Phase Chromatography  Especially for the late steps in a purification protocol a possibility for desalting of relatively hydrophilic proteins is reversed-phase chromatography. Additionally, this often results in a further separation of the applied sample. Using volatile solvents such as 0.1% trifluoroacetic acid in water, larger volumes of salt containing samples can be desalted, since the hydrophilic salts are not bound by reversed-phase material, while proteins are bound via their hydrophobic regions onto the column. With a gradient of an organic solvent (usually acetonitrile in 0.1% trifluoroacetic acid), the proteins can be eluted again. For the desalting of proteins a rather hydrophilic, large pore reversed-phase material should be used since hydrophobic, fine-pore material that is very suitable for peptide separations often gives very poor yields in the elution of proteins. The protein containing fractions are collected and subsequently the volatile organic solvent (e.g., acetonitrile) is evaporated in a vacuum centrifuge or with nitrogen. The disadvantages of desalting with reversed-phase chromatography are a possibility of protein denaturation, a generally poor recovery of hydrophobic proteins, and the high price of the column material. Proteins exhibit a special retention behavior when bound to reversed-phase material.
Above a certain concentration of the organic modifier the binding of the protein to the chromatographic material changes from a typical reversed phase mechanism to a normal phase behavior. This allows a desalting of proteins with an inverse gradient. The sample is applied in substantially pure organic solvent onto the reversed-phase column. The protein binds according to a normal phase mechanism, while salts and less hydrophobic impurities are eluted. The protein can now be eluted with a gradient to 0.1% trifluoroacetic acid in water.

For sample preparation/desalting of peptides for mass spectrometry disposable pipette tips containing a few microliters reversed-phase material are often used. When applied to such small columns, the peptides bind to the reversed-phase material and are concentrated there, where they can be washed and desalted at the same time using aqueous solutions (e.g., 0.1% trifluoroacetic acid). The bound proteins are eluted by small amounts of highly concentrated organic solvents. It is important that during the sample application no organic solvent is present in the sample, as this would interfere with the binding of the peptides on the reversed-phase material. If organic solvent from previous purification steps is present, its concentration must be reduced by evaporation or dilution to a maximum of 5%.

Immobilization on a Membrane An excellent possibility to prepare and also store proteins for further analyses (e.g., sequencing, amino acid analysis, mass spectrometry) is to transfer them to a chemically inert membrane (immobilization). The salt-containing protein solution can be applied onto a hydrophobic membrane (e.g., PVDF), in portions to a small area, and dried. At not too great amounts of salt the hydrophobic binding of proteins to these membranes is strong enough that even intensive washing with water does not remove the proteins from the membrane. Proteins can also be transferred to membranes by electroblotting. This is usually combined with a gel electrophoretic separation as the last step of the purification procedure. The immobilization on a membrane is only recommended for pure proteins as the last purification step of sample preparation for structure determination, since the recovery of the immobilized proteins from the membrane is extremely difficult.

1.7 Concentration

Especially after the first steps of a cleaning cycle one is often confronted with large volumes of dilute protein solutions. Furthermore, also, the ionic strength or pH values often do not fit the conditions needed for the next purification step. Therefore, methods must be used in order to both concentrate solutions and desalt or resalt. For recovery and protein stability reasons each protein purification should be performed in a minimum number of sub-steps. If possible, concentration and salt exchange/desalting should be combined in one step (see also Section 1.6).

Precipitation As discussed above (Section 1.4), precipitation is an effective way to concentrate a protein from its solution and at the same time largely free it of salts. After centrifugation (typically at low temperature), the supernatant is discarded and the pellet dissolved in the desired buffer for further separation or analyses steps. Problems arise in protein solutions containing detergents, since the precipitation generally gives only poor yields.

Dialysis and Ultrafiltration These are used in addition to desalting also for the concentration of samples (Section 1.6). Especially for the concentration of small volumes and smaller amounts of protein in the last purification steps prior to structure analysis, special microconcentrators are often used that exhibit, due to their design, only low adsorption losses.

Binding to the Chromatographic Material An elegant method by which to enrich and concentrate proteins and peptides also from large volumes is to bind them on reversed-phase material. The sample application buffer must be devoid of any substances that provide eluting conditions for the reversed-phase material (e.g., acetonitrile, alcohols, detergents). Alternatively, ion exchange materials can be used to enrich proteins and peptides. This is only successful if the ionic strength of the sample buffer is very low (<10 mM), which can usually be achieved through desalting or dilution.
1.8 Detergents and their Removal

Many proteins and enzymes in their natural environment are not surrounded by water but by hydrophobic lipid layers, the biological membranes. In cell disruption, these proteins remain associated with the membranes. Peripheral membrane proteins, which are only surface-associated to the membrane, usually can be solubilized by relatively mild treatments, without dissolving the membrane. High salt concentrations, extreme pH conditions, high concentrations of chelating agents (10 mM EDTA), or denaturing substances (8 M urea or 6 M guanidine hydrochloride) can be used. But as soon as the proteins are partially or almost completely embedded in the membrane (membrane anchor or integral membrane proteins), attempts to bring them into solution with normal aqueous buffers will fail. Today these proteins represent a major challenge for the purification and analysis techniques. If the lipids are removed from the membrane the proteins will, via their hydrophobic regions, form insoluble aggregates and precipitate or bind very strongly to all types of surfaces that exhibit a certain hydrophobic character. Such proteins can generally be brought and kept in solution only by using detergents in the solution.

1.8.1 Properties of Detergents

Detergents are amphiphilic molecules, that is, they have on the one hand a polar hydrophilic, possibly also ionic, moiety that is responsible for a good water solubility and on the other hand they have a nonpolar, lipophilic moiety capable of interacting with hydrophobic regions of a membrane protein; in this respect they approximately mimic the natural lipid environment of membrane proteins. An important property of detergents is their ability to form micelles. These are aggregates of different detergent molecules in which all the hydrophilic groups point towards the outside and all hydrophobic groups are directed inwardly (Figure 1.6). The size of micelles is dependent on the individual detergent (Table 1.4). Membrane proteins are incorporated as hydrophobic molecules in these micelles and can be brought into solution outside of a biological membrane and often retain their biological activity. The lowest concentration at which detergents can form micelles is called the critical micellar concentration (CMC). The CMC is different for different detergents (Table 1.4) and is a function of parameters such as temperature, ionic strength, pH, and the presence of di- and trivalent cations or organic solvents. The CMC of ionic detergents decreases significantly with higher ionic strength, and is independent of temperature. However, the CMC of non-ionic detergents is relatively independent of the salt concentration, but significantly increases with increasing temperature.

As can be seen from Table 1.4, several different detergents can be used in the purification and analysis of membrane proteins. Which detergent for a particular protein is optimal must be

![Figure 1.6](https://example.com/figure16.png)

Figure 1.6 Formation of detergent micelles above the CMC and embedding of proteins into the micelles. In the micelle the hydrophilic polar structures face the aqueous phase and the hydrophobic parts are positioned inside. Membrane proteins can insert into such micelles and are kept in solution as a detergent–protein complex.
Table 1.4 Common detergents (n: aggregation number to form micelles).

<table>
<thead>
<tr>
<th>Name</th>
<th>$M_r$ (monomer)</th>
<th>$n$</th>
<th>$M_r$ (micelle) (kDa)</th>
<th>CMC (mM)</th>
<th>Dialyzable</th>
<th>Concentration of use</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium dodecyl sulfate (SDS)</td>
<td>288</td>
<td>60–100</td>
<td>&gt;18</td>
<td>8</td>
<td>–</td>
<td>10 mg mg⁻¹ protein</td>
<td>CMC is strongly dependent on ionic strength</td>
</tr>
<tr>
<td>Desoxycholate</td>
<td>416</td>
<td>10</td>
<td>4</td>
<td>4 (in 50 mM NaCl)</td>
<td>–</td>
<td>0.1–10 mg mg⁻¹</td>
<td>precipitates at low pH in presence of divalent cations, n increases with ionic strength</td>
</tr>
<tr>
<td>Benzyl dimethyl(n-hexadecyl)ammonium chloride (BAC-16)</td>
<td>396</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>cationic detergent, suitable for electrophoresis of membrane proteins</td>
</tr>
<tr>
<td>Cetyltrimethylammonium bromide (CTAB)</td>
<td>365</td>
<td>170</td>
<td>62</td>
<td>1</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triton X-100 poly(ethylene glycol)$_n$-octylphenyl ether</td>
<td>628</td>
<td>140</td>
<td>90</td>
<td>0.2</td>
<td>–</td>
<td>1–5 mM</td>
<td>Absorbs in UV (due to aromatic ring)</td>
</tr>
<tr>
<td>Triton X-114</td>
<td>≈514</td>
<td></td>
<td>0.2</td>
<td></td>
<td>–</td>
<td>5 mM</td>
<td>phase separation at heating to over 22 °C</td>
</tr>
<tr>
<td>Tween 80 (poly(oxyethylene)$_n$-sorbitan monooloate)</td>
<td>≈1310</td>
<td>58</td>
<td>76</td>
<td>0.02</td>
<td>–</td>
<td>&gt;10 mg mg⁻¹ membrane lipid</td>
<td>Polysorbates have been reported to be incompatible with alkalis, heavy metal salts, phenols, and tannic acid. They may reduce the activity of many preservatives</td>
</tr>
<tr>
<td>Octyl glucoside 1-Octyl-$eta$-glucopyranoside</td>
<td>292</td>
<td>30–100</td>
<td>8</td>
<td>15</td>
<td>+</td>
<td>20–30 mM</td>
<td></td>
</tr>
<tr>
<td>CHAPS 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate</td>
<td>615</td>
<td>4–14</td>
<td>6</td>
<td>4</td>
<td>+</td>
<td>6–13 mM</td>
<td>Suitable for protein solubilization for isoelectric focusing/2D electrophoresis.</td>
</tr>
<tr>
<td>Zwittergent 3-12 N-Dodecyl-$N$/-$N$/-dimethyl-3-ammonio-1-propanesulfonate</td>
<td>335</td>
<td>55</td>
<td>18</td>
<td>3.6</td>
<td>+</td>
<td>15–30 mM</td>
<td></td>
</tr>
</tbody>
</table>
determined empirically for each individual case. Generally, detergent concentrations of 0.01–3% are used. The solubilization of proteins is often carried out at a detergent concentration close to the CMC.

Depending on the planned further purification steps, certain properties of individual detergents have to be taken into consideration:

- The high UV absorption of certain detergents (e.g., as in Triton or Nonidet due to the aromatic ring) can interfere with the detection of proteins, for example, in chromatographic analyses.
- Ionic detergents cannot be used in ion exchange chromatography, as they compete with the proteins for the ionic binding sites on the column material.
- Detergents often bind strongly via hydrophobic interactions on reversed-phase columns and also change the elution properties of the reversed phase materials.
- How easily can the detergent be removed from the protein (Section 1.8.2)?
- Especially, non-ionic detergents of the oxyethylene family (Triton, Tween, Nonidet) can easily contain oxidative impurities, particularly peroxides. Since these impurities can modify proteins, these detergent solutions should be as fresh as possible, be stored under nitrogen, and removed with a syringe from the vessel.

Whether a detergent has brought a certain protein in solution can be decided by centrifugation. A soluble protein (or its activity) has to be found in the supernatant after one hour of centrifugation at 100 000 g. If no biological activity can be measured with various detergents, the buffer conditions should be changed or substances should be added that are known to stabilize the protein structure, for example, 20–50% glycerol, reducing agents such as dithiothreitol, chelating agents such as 1 mM EDTA, or protease inhibitors.

### Ionic Detergents

with cationic or anionic hydrophilic groups solubilize proteins well in its monomeric form and also solubilize membrane proteins. However, these detergents have the disadvantage of distorting and denaturing the structure of proteins – thus they almost always lose their biological activity. Probably the most widely applied detergent, sodium dodecyl sulfate (SDS), is used in the electrophoretic separation and molecular weight determination in polyacrylamide gels. The salt concentration significantly affects the CMC of SDS (salt free: 8 mM, in the presence of 0.5 M NaCl: 0.5 mM). The cationic detergent 16-BAC allows good separations of (membrane) proteins in an acidic environment. Thus, base-labile protein modifications remain intact (e.g., protein carboxymethyl esters) that are hydrolyzed in conventional purification methods in acidic or alkaline medium. 16-BAC is also successful as the first dimension of a two-dimensional gel electrophoretic separation of membrane proteins. The second dimension separation is a conventional SDS-gel electrophoresis.

### Nonionic Detergents

These detergents affect less strongly protein–protein interactions and are generally less denaturing than ionic detergents. They are therefore particularly suitable for the isolation of intact protein assemblies, but in integral membrane proteins they do not prevent aggregation and furthermore, due to its low CMC, they are difficult to remove by dialysis. A special position is held by Triton X-114, which is soluble in water at 4 °C, but at temperatures above 20 °C forms water-insoluble micelles and thus shows a separation between the aqueous and detergent phase. Hydrophilic proteins remain in the aqueous phase, while integral membrane proteins are found in the detergent phase. This makes it possible to differentiate soluble proteins and membrane proteins.

### Zwitterionic Detergents

These detergents, which carry positively and negatively charged groups in their polar moiety, exhibit dissociating and denaturing properties between those of ionic and nonionic detergents. They can be applied in ion exchange chromatography or isoelectric focusing.

### 1.8.2 Removal of Detergents

For subsequent analysis (amino acid analysis, amino acid sequence analysis, mass spectrometry, etc.) detergents, which are present in relatively high concentrations, interfere almost always...
and usually must be removed. All processes for the removal of detergents should be considered, however, that the solubilized hydrophobic proteins almost always need the presence of these substances for their solubility and activity. Otherwise, activity losses or adsorption losses to all types of surfaces will occur! The removal of detergents should therefore always be planned as the last step before the actual analysis so that no further sample manipulation takes place.

**Dilution below the Critical Micelle Concentration** Micelles of detergents with a high CMC (e.g., octylglucoside) can be dissolved into their monomeric state by diluting below their CMC and can then be easily removed by dialysis (caution: adsorption of detergent-free proteins to the dialysis membranes). Detergents with a low CMC (most of nonionic detergents) are virtually impossible to remove by dialysis and are best removed by special chromatographic columns (see below).

**Extraction** Different extraction methods are used to remove SDS. Besides the chloroform/methanol extraction, ion-pair extraction is especially commonly used. The dry sample is extracted with a solution of the ion-pair reagent in an organic solvent. Typical systems are acetone/trimethylamine/acetic acid/water or heptane/tributylamine/acetic acid/butanol/water. Sufficient water (about 1%) must be present in order to form the relatively nonpolar alkyl ammonium SDS-ion pair which is extractable with organic solvents. Specimens that are present even in small volumes of aqueous solution can be freed of SDS; only the water is omitted from the extraction solutions. This method can remove in an extraction step up to 95% of SDS. The protein is recovered as a precipitate by centrifugation and residual SDS can then be removed by washing with heptane or acetone. Salts may interfere with the removal of SDS and should be removed prior to extraction. The recovery rates for proteins are often 80% and higher.

**Ionic Retardation** Chromatographic materials from polymerized acrylic acid that includes a strong ion exchange resin (quaternary ammonium groups on a polystyrene–divinylbenzene matrix) are available. SDS–protein complexes, which are chromatographed on these materials, lose almost all of SDS, and the protein is eluted with high yields of 80–90%. These columns are designed as single use columns, since the bound SDS practically cannot be removed. Also with ionic retardation, salts may interfere with the SDS binding to the column material and should be removed beforehand. This can be done by gel filtration or by simply applying a small layer of size exclusion gels at the top of ion retardation column, resulting in delay of the proteins and hence a separation from the buffer salts.

**Gel Filtration in the presence of Organic Solvents** In particular relatively weak hydrophobic proteins can sometimes be kept in solution in a buffer containing organic solvent (e.g., 20% acetonitrile), and thus separated from salts and detergents by a gel permeation chromatography. More hydrophobic proteins must be kept in solution by suitable solvent mixtures having a high content of organic acids, such as formic acid/propanol/water.

**Removal of Detergents by Reversed-Phase HPLC** Membrane proteins can be freed from detergents and salts also by using reversed-phase column chromatography. Short, relatively hydrophilic columns (RP-C4) with a low surface area must be used to achieve satisfactory recoveries. The chromatographic conditions are generally selected in such a way that a compromise between recovery and the quality of separation is obtained. Generally, short, steep gradients using volatile solvent systems with high levels of organic acids (see above) should be used. These methods are mainly used when the primary objective is the structure determination of membrane proteins, since the biological activity is almost always destroyed by the high proportion of organic solvent and acid. For desalting, removal of detergents and hydrophobic components, reversed-phase chromatography has been applied successfully also with an inverse gradient (see Section 1.6, reversed-phase HPLC).

**Special Chromatographic Support Materials for the Separation of Detergents** Removal of detergents by specific, commercially offered materials is based in principle on a hydrophilic column material with such a small pore volume that proteins with a molecular weight of above 10 000 Da cannot enter the interior of the stationary phase and elute in the void volume. The relatively small detergent molecules, however, reach the interior of the column material, where
they interact with hydrophobic binding sites and are bound there. Binding capacities for the different detergents are from 50 to 100 mg detergent per ml of column material. Protein concentrations should be above 50 μg ml⁻¹, because otherwise significant losses due to nonspecific adsorption to the column material can be found.

Blotting, Section 11.7

Blotting onto Chemically Inert Membranes  The easiest way to purify a hydrophobic protein and analyze it is to blot it directly from a detergent containing polyacrylamide gel electrophoresis (SDS-gel) onto a chemically inert membrane. The immobilized protein can then be directly subjected to further analysis, for example, amino acid analysis, sequence analysis, immunological methods, or even mass spectrometry.

1.9  Sample Preparation for Proteome Analysis

The aim of a meaningful proteome analysis is to determine the quantitative relationships of all the proteins in a proteome sample. The ratio of each individual protein in different proteomic states must not change due to the influence of sampling, separations, or analysis procedures. This has the consequence that expensive multistep purification processes, such as those used in classical protein purification applications, can no longer be applied because considerable losses in the individual steps may occur and, even more importantly, they are different and unpredictable for each individual protein. The sample preparation for proteome analysis is very dependent on the biological question and the starting material and therefore cannot generally be treated. But, in principle, all the proteins in a proteome should be brought into solution in a form in which they are directly suitable for separation, for example, for a two-dimensional gel electrophoresis or HPLC-MS analysis.

Further Reading