MS detection, the increasing use of a combined CE-MS technique can be expected.

To be more widely accepted in the area of biomedical research, CE-based protein separations must demonstrate a number of features that match the success of conventional (gel) electrophoretic systems. Besides profiling complex protein samples, these systems allow for immunological and enzymatic assaying of separated proteins as well as for simultaneous transfer of sample components into another separation dimension without altering the separation in the first one. All of these are achieved with minimal disturbance of zone integrity. Thus, the major efforts will probably be made in developing both multidimensional separation systems involving CE and CE-based separation systems permitting post- or on-column enzymatic and immunological analysis of the separated components of complex biological samples. Incorporating immobilized enzymes or antibodies into CE-MS systems will revolutionize the analysis of protein structure and, especially, glycoprotein analysis.

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Centrifugation

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Introduction

Modern technological developments have made centrifugation one of the most important and widely applied techniques in experimental research. In biological studies centrifugation is used for the extraction and isolation of biological materials and for the measurement of physical properties of macromolecules. Indeed, biological materials have been extracted and isolated for more than a thousand years using centrifugal forces. In the 1920s, Svedberg and other researchers developed motor-driven centrifuges which had an optical system to observe sedimentation of macromolecules during centrifugation, and used these instruments for the measurement of physical properties of macromolecules, especially proteins. The molecular mass of most proteins was determined using these analytical centrifuges until 1970, but they have not been recently used for that purpose because much easier methods for the measurement of molecular mass, such as size exclusion chromatography and sodium dodecyl sulfate (SDS)-gel electrophoresis, have been developed. More recently, centrifugation has become an indispensable tool for the isolation of proteins, nucleic acids and subcellular particles. The use of centrifuges has also been revived for the measurement of physical properties of proteins, especially for the characterization of protein associations and protein–protein interactions. In this section, important points of theory and practice for the separation and isolation of proteins by centrifugation are summarized.

Theoretical Basis of Centrifugation

Although a rigorous understanding of sedimentation theory is not required for the separation and isolation of proteins, a review of some basic principles will be helpful for understanding the establishment of conditions and the interpretation of experimental results obtained. Because of their random thermal motion,

macromolecular particles in a solution do not show any perceptible sedimentation in a uniform gravitational field. However, these macromolecular particles do sediment under a centrifugal force. If the effect of diffusion is neglected, in a solution (density ρ) the motion of a particle (mass m and volume V_p) that is located a distance r from the axis revolving with angular velocity ω can be expressed by the following equation:

$$\upsilon f = m\omega^2 r - \rho\omega^2 r V_p \quad [1]$$

where υ is the velocity of the sedimenting particle, f its frictional coefficient; $m\omega^2 r$ the centrifugal force and $\rho\omega^2 r V_p$ the buoyant force. This equation may be rearranged to give:

$$\upsilon = dr/dt = s\omega^2 r \quad [2]$$

where:

$$s = \frac{m - \rho V_p}{f}$$

This is the well-known sedimentation equation in which s is the sedimenting coefficient and has dimensions of time. For most biological macromolecules, the magnitude of s is about 10^{-13} s. Therefore, the unit of sedimentation, the Svedberg (S), has been defined as being equal to 10^{-13} s. The standard sedimentation coefficient ($S_{20,w}$) is defined as that equivalent to sedimentation in water at 20°C. The sedimentation coefficients ($S_{20,w}$) of some proteins are shown in **Table 1**.

The sedimentation coefficient s may be transformed to a more practical form. The mass of 1 mole of particles, M, is

$$M = mN_o \quad [3]$$

Table 1 Sedimentation coefficients of some proteins

Protein	$S_{20,w}$
Cytochrome *C* (bovine heart)	1.71
Egg-white lysozyme	1.9
Insulin	1.95
Ribonuclease A (bovine pancreases)	2.00
Myoglobin (horse heart)	2.04
α-Chymotrypsin (bovine pancreases)	2.40
Pepsin	2.8
g-Actin	3.7
Lactate dehydrogenase (pig heart)	6.93
Catalase (horse liver)	11.2
Glutamate dehydrogenase (bovine liver)	26.6
Fibrinogen (human)	7.63
Haemocyanine (octopus)	58.7
Haemocyanine (snail)	100

where N_o is Avogadro's number. Thus, a particle's volume, V_p, may be expressed in terms of its molar mass:

$$V_p = m\upsilon = \upsilon M/N_o \quad [4]$$

where υ is the particle's partial specific volume.

Eqns [1], [3] and [4] may be combined to give:

$$\upsilon f = \frac{M(1 - \upsilon\rho)\omega^2 r}{N_o} \quad [5]$$

When eqns [2] and [5] are combined, s may be expressed as:

$$s = \frac{\upsilon}{\omega^2 r} = \frac{M(1 - \upsilon\rho)}{N_o f} \quad [6]$$

Since the particle's partial volume, υ, may be expressed by the reciprocal of the buoyant density of the particles, ρ_p, as $\upsilon = 1/\rho_p$, s may also be expressed as:

$$s = \frac{\upsilon}{\omega^2 r} = \frac{M(1 - \rho/\rho_p)}{N_o f} \quad [7]$$

Since $f = 6\pi\eta r_p$, where η is the viscosity of the liquid medium and r_p is the radius of unsolvated spherical particle, these equations indicate that the sedimentation velocity, υ, is related to the sedimentation coefficient s, which is mostly a function of particle size, density of the particle ρ_p, density of the medium ρ and the viscosity of the liquid medium, η. In other words, for a given particle, sedimentation is directly related to particle size, particle density and the centrifugal field, and inversely to the viscosity and density of the liquid medium.

Centrifugation for Protein Separation

Centrifuges may be classified on the basis of the maximum speed, namely, low speed, high speed and ultracentrifuges. Each of these can be used in the various steps of protein separation and isolation from biological materials. Low speed centrifuges are used routinely for the initial processing of biological samples. This type of centrifuge can be mainly used to isolate cells and organelles that contain target proteins by pelleting these materials. High speed centrifuges, with maximum speeds of 8000–25 000 rpm, are mainly used for the preparation of subcellular fractions. These centrifuges can generate about 60 000 **g**, which is enough to separate proteins from debris of cells and organelles. In order to isolate proteins from other proteins, ultracentrifuges are

required. Modern ultracentrifuges can generate approaching 1 000 000 **g**, which is sufficient to pellet even small proteins. Ultracentrifuges can be divided into two types: analytical and preparative. Analytical ultracentrifuges have a device by which the sedimentation rate of molecules can be optically measured during centrifugation and can be used to obtain data on the sedimentation properties of particles. The masses of most proteins were determined by these ultracentrifuges before development of simpler molecular mass determination methods. Eqn [4] indicates that the particle's mass $m = M/N_o$ can be determined from its sedimentation coefficient s, if its frictional coefficient f, is known, as indicated in eqn [6].

Preparative ultracentrifuges are designed for sample preparation. This kind of ultracentrifuge is also commonly used for quantitative estimations of sedimentation coefficients of particles in a density gradient, although the data obtained are not as accurate as those obtained using analytical ultracentrifuges. Preparative ultracentrifugation can be divided into two methods, namely differential ultracentrifugation and density gradient centrifugation. Differential centrifugation is based on the differences in the sedimentation rates of particles in samples. If a suspension of particles is centrifuged in a tube without a density gradient, each particle will move toward the bottom of a tube. In this case, the rate of sedimentation, v, is dependent upon s (eqn [2]). Since s is mostly a function of particle size, the rate of sedimentation is proportional to particle size. In the course of the ultracentrifugation, two fractions can be obtained from a solution of particles: a pellet containing sedimented particles and a supernatant solution of the unsedimented fraction. A given particle in the solution may sediment to the pellet or near the bottom, as illustrated in **Figure 1**. As might be expected, this centrifugation will first sediment the largest particles in the sample solution to the bottom of the tube. The only particle that is in purified form is the most slowly sedimenting one, but the yield is very low. The major problem with differential centrifugation is that the centrifugal force necessary to pellet the larger particles is also often sufficient to pellet the smaller particles initially near the bottom of the tube (Figure 1). To separate one particle from another effectively, a 10-fold difference in mass is usually required. Thus, this centrifugation is recommended for the separation of proteins from large particles such as cells or organelles. However, it cannot be used for the isolation of one protein from another because the partial specific volume, v, of most proteins (in eqn [6]) is not sufficiently different.

Eqn [6] assumes that centrifugation is performed in a homogeneous medium. However, centrifugation

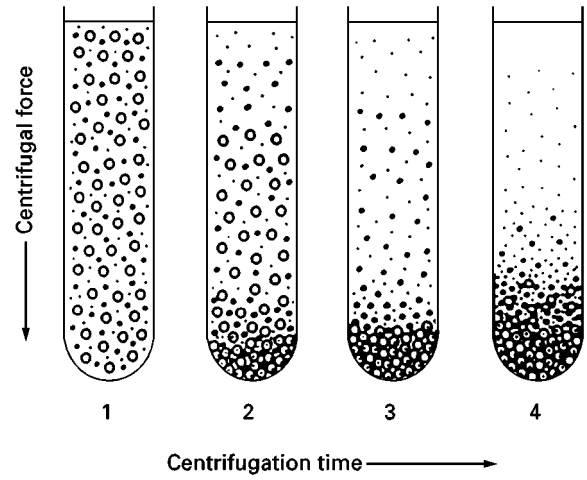

Figure 1 Fractionation of particles by differential centrifugation. Reproduced with permission from Griffith (1979).

can be carried out in a solution of an inert substance in which the concentration increases from the top to the bottom of the centrifuge tube, i.e. density increases from top to bottom. In such density gradient centrifugation of a mixture of particles with different sizes or buoyant densities, a particle will become stationary when $(1 - v\rho)$ in eqn [6] is zero. Thus, various components will separate according to size or buoyant densities, and form bands or zones of particles with similar densities. Thus, the use of such density gradients greatly enhances the resolving power.

There are two types of density gradient ultracentrifugation: isopycnic and rate-zonal ultracentrifugation. In isopycnic centrifugation, separation is based on the centrifugation of particles in a density gradient through which the particles move until their densities are the same as those of the surrounding medium, i.e. in eqn [7], $\rho_p = \rho$ (**Figure 2**). The sample is mixed with a relatively concentrated solution of a low molecular mass substance, such as CsCl, and is centrifuged until the solution achieves equilibrium under the high centrifugal field. The low molecular mass substance forms a steep density gradient. It is not obligatory to load the sample on top of the gradient. In the centrifugation, particle size only affects the rate at which particles reach their isopycnic position. Since variations in amino acid composition give proteins with only slightly different densities, isopycnic centrifugation can be used only when proteins are associated with nonprotein components such as lipids or polysaccharides, and their density differences are sufficient for the separation. Various gradient media can be used for the separation of these proteins because proteins form a band at low density in most gradient media. For example, in addition to CsCl,

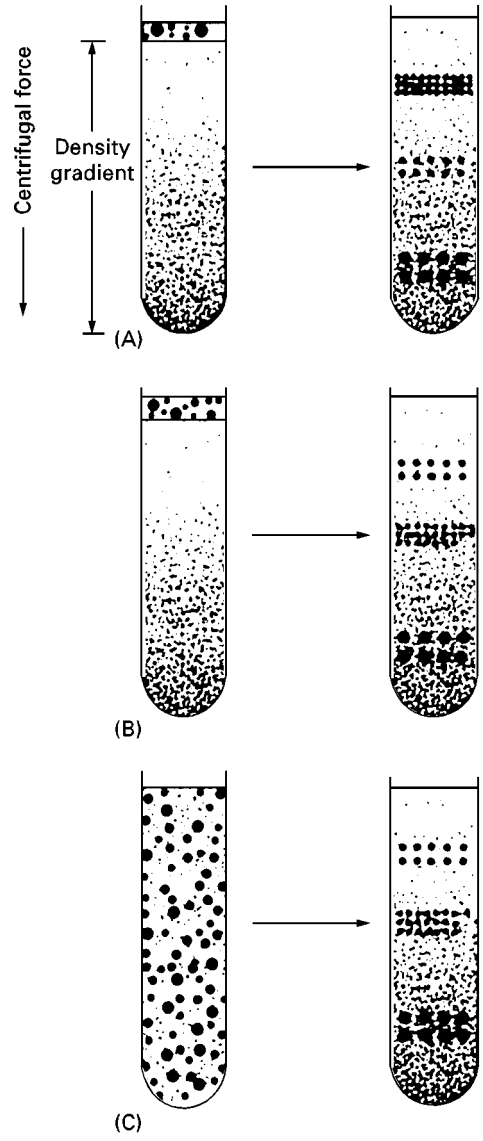

Figure 2 Types of density gradient centrifugation. (A) Rate-zonal centrifugation. (B) Isopycnic centrifugation using a preformed density gradient. (C) Isopycnic centrifugation using a self-forming gradient. Reproduced with permission from Rickwood (1992) by permission of Oxford University Press.

RbCl, NaBr or KBr can also be used to form shallower gradients for better resolution of these proteins.

Rate-zonal centrifugation is ideal for particles of defined size such as protein and RNA. In the rate-zonal ultracentrifugation, a mixture containing particles is layered on top of a density gradient. Loading the concentrated samples to the top of the gradient increases the eventual resolution of recovered particles. Sucrose is commonly used to form a density gradient. During centrifugation, particles move through the gradient at their characteristic sedimentation rates, forming zones that can be recovered at the end of the run (Figure 2). Because the sedimentation rate is more affected by molecular size, the rate-zonal ultracentrifugation separates similarly shaped macromolecules largely on the basis of their molecular masses. It should be noted that particles separated by the rate-zonal centrifugation may not be homogeneous because particles with similar mass, even proteins, are sometimes heterogeneous.

Practical Aspects for Protein Separation by Centrifugation

Since rate-zonal centrifugation is commonly used for the separation of proteins, the following discussion will focus on a practical approach for this technique.

Types of Rotor

Preparative centrifuge rotors can be classified into four types: fixed angle, swinging-bucket, vertical and zonal. In fixed-angle rotors, the tubes are positioned at a fixed angle. These rotors are often used for differential ultracentrifugation and are very efficient for the separation of proteins from cells and organelles. Typically, a sample is loaded atop a gradient which reorients as the rotor is spun (**Figure 3**). During centrifugation, the larger particles are first sedimented across the tube, hit the wall of the tube, and slide down to form a pellet at the bottom.

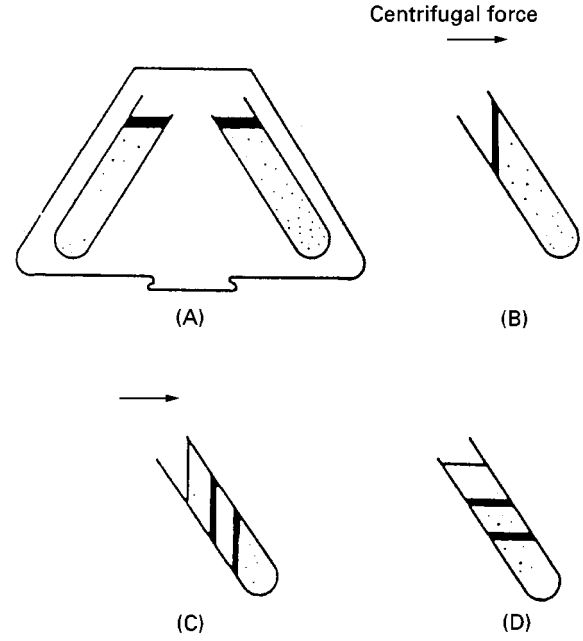

Figure 3 Operation of fixed-angle rotors. (A) The gradient is prepared, the sample is loaded and the centrifuge tubes are placed into the rotor. (B) Both sample and gradient reorient during acceleration. (C) Bands form as particles sediment. (D) Bands and gradient are both reoriented when the rotor is at rest. Reproduced with permission from Rickwood (1992) by permission of Oxford University Press.

Efficiency for the pelleting of particles is high due to the short sedimentation path. However, fixed-angle rotors are not common for protein separation because the pelleting process also disrupts sample zones as particles sediment through the gradient. Thus, fixed-angle rotors are mainly used for the pelleting of materials.

For the separation of proteins from other proteins, especially for small scale separation, the swinging-bucket rotor is widely used for rate-zonal centrifugation. This type of rotor is also used for the estimation of sedimentation coefficients of proteins. As shown in **Figure 4**, in the swinging-bucket rotor, the sample tubes are loaded into individual buckets which hang vertically while the rotor is at rest. When the rotor begins to rotate, the buckets swing out perpendicular to the axis of rotation. In these rotors, resolution of particles is high because particles sediment with a relatively long path length. For the same reason, run times are generally longer. Many types of swinging-bucket rotors are commercially available. The centrifuge tube should be as long as possible if high resolution is the objective. For large volume samples, swinging-bucket rotors with wider tubes should be used because the sample can be loaded in a narrow zone while still reducing particle interactions during sedimentation.

Vertical rotors are suitable for isopycnic as well as for rate-zonal separations. However, this type of rotor is not practical for the separation of proteins. As a result of diffusion and reorientation during centrifugation, sample bands will be significantly broader than analogous bands in swinging-bucket rotors. In addition, if the sample contains pellets or floats, these materials will distribute along the length of the tube and can subsequently contaminate the supernatant during reorientation at the end of run.

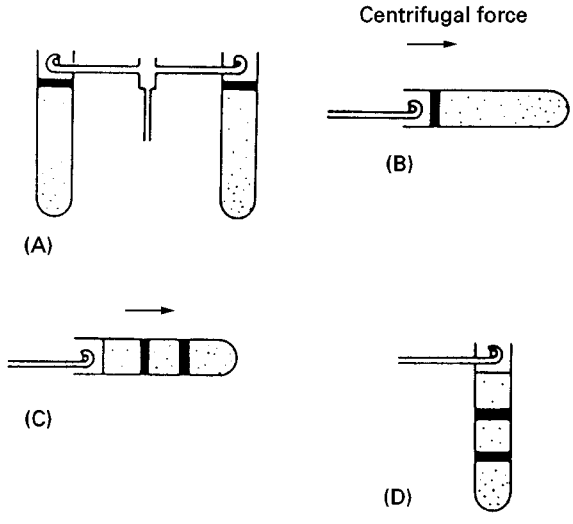

Figure 4 Operation of swinging-bucket rotors. (A) The gradient is performed and the sample is loaded on the top of the gradient. (B) Centrifuge bucket reorients as rotor accelerates to lie perpendicular to the axis of rotation. (C) Bands form as the particle sediment. (D) Rotor decelerates. Centrifuge bucket comes to rest in its original vertical position. Reproduced with permission from Rickwood (1992) by permission of Oxford University Press.

Choice of Density Gradient

A density gradient is essential for rate-zonal centrifugation to support the zones of particles as they sediment. In addition, the sample can be loaded on to the top of the gradient as a narrow zone and the increasing density from the top to the bottom of the density gradient suppresses mechanical disturbances. Moreover, the presence of a gradient of increasing viscosity serves to sharpen the sample zones during centrifugation. The density gradient material for protein separation requires the following properties.

1. The materials should be sufficiently soluble in water to produce the range of densities needed.
2. Solutions of the gradient materials should be adjustable to a pH and ionic strength that are not harmful to proteins in the sample.
3. The materials should not interfere with methods of analysis of the target protein.

Sucrose has most often been used as a gradient material. Sucrose is inexpensive and extremely soluble in aqueous media and can be used to produce density gradients ranging up to 1.35 g mL^{-1}. Thus, it is suitable for separation of almost all proteins in cells. Although concentrated solutions of sucrose have high osmotic potential that cause shrinkage of certain cells and organelles, the high osmotic pressure has relatively less effect on the biological properties of proteins. Generally, sucrose is relatively inert to proteins, although contaminants in many commercial sources of sucrose may interact with proteins. Such impurities can be removed by treatment with activated charcoal. However, it is best to purchase specially purified sucrose for density gradient work. To sterilize sucrose solutions, autoclaving (100°C or above) of the solution should be avoided and treatment with 0.1% diethylpyrocarbonate is recommended. As described above, isopycnic centrifugation can be used for the separation of different types of proteins. However, it should be noted that the density of sucrose, even of a saturated solution, is too low for the separation. For this purpose, RbCl, NaBr or KBr can be used to form shallower gradients for better resolution.

Glycerol is used to stabilize some proteins, especially membrane-bound proteins, and provides gradient densities ranging up to 1.26 g mL^{-1}. Thus, glycerol gradients are widely used for the separation

of proteins by rate-zonal separations. However, it should be noted that the high viscosity of glycerol reduces the effective density range and glycerol appears to inhibit some enzyme activities.

Preparation of Gradients

Density gradients can be divided into two types: continuous and discontinuous. For protein separation, continuous gradients are usually used in rate-zonal centrifugation. The most common continuous gradient for protein separation is a linear gradient in swinging-bucket tubes. A linear gradient is a gradient in which the density increases linearly in a tube of constant cross-sectional area with increasing distance from the centre of rotation. Thus, in this configuration the linear gradient can be defined as one where the density increases linearly with volume.

When designing a linear gradient in swinging-bucket rotors, several points should be emphasized. The density at the top of the gradient must be sufficient to support the sample while the density of the bottom of the gradient must not exceed the density of proteins to be separated. In general, the greater the slope of the gradient, the better the resolution obtained because the viscous drag rises rapidly as the sucrose concentration increases. Usually, as a first attempt, a 5–30% or 10–40% sucrose gradient should be used. It should be emphasized that the sample volume is related to the slope of the gradient because a given slope of gradient can only tolerate a limited amount of sample before gradient inversion occurs. Poor resolution during rate-zonal centrifugation almost always results from overloading.

Linear gradients are prepared using gradient makers. Many configurations of gradient maker are available. The simplest gradient makers consist of two vessels of equal cross-sectional area joined by a connecting channel with a stopcock. One chamber is a reservoir and the other chamber has a mixing device and an exit connected to the centrifuge tube. There are two methods for preparing linear gradients:

1. The reservoir contains the less dense solution, the mixing chamber contains the denser solution, and the gradient is routed to the wall of the centrifuge tube at the top. This method is readily applicable to centrifuge tubes made of hydrophilic materials such as cellulose nitrate and cellulose acetate butyrate.
2. The reservoir contains the denser solution, the mixing chamber contains the less dense solution, and the gradient is routed to the bottom of the centrifuge tube. This method can be applied to any type of centrifuge tube and it is much easier to prepare the gradient without disturbance.

The gradient should be prepared and maintained at 4°C.

Preparation of Sample

The sample should be ready for loading before the gradient is prepared and should be kept cold for many preparations. The sample is usually prepared in the same buffer as the gradient. In addition, several points are important in sample preparation:

1. The sample solution must have a density less than that of the gradient.
2. Gradients should be centrifuged as soon as possible after the sample has been loaded to prevent so-called droplet sedimentation.
3. For optimal resolution in rate-zonal centrifugation, the sample must be loaded on to the top of gradient and the sample volume should not exceed 2–3% of the gradient volume.

Loading of the sample on to the density gradient is one of the most crucial steps in rate-zonal centrifugation. The simplest method is to use a pipette to load the sample directly to the meniscus at the tube wall.

Conditions During Centrifugation

Smooth acceleration and deceleration are important for all gradient work. In addition, control of the temperature of the sample and gradient are important for reliable and reproducible sedimentation. Fortunately, most modern ultracentrifuges are equipped with programmed acceleration and deceleration modes which minimize the disturbance of gradient and temperature control. It should be emphasized that, during the gradient reorientation phase of a run using a swinging-bucket rotor, the rotor should be accelerated as slowly as possible up to 1000 rpm, and the brake switch should be off below 1000 rpm during deceleration.

Recovery of Fractions from the Gradient

After centrifugation, gradients are fractionated to recover protein bands. Great care must be taken at this stage to avoid loss of resolution. Several points should be emphasized.

1. All operations should be designed to minimize disturbance of the gradient.
2. The volume of the tubing from the gradient to the fraction collector should be minimized.
3. Care must be taken to avoid contamination of the recovered fractions by pelleted materials.
4. The gradient should be fractionated at a slow flow rate, particularly if viscous materials are used for the gradient.

In order to collect the entire gradient in a series of fractions, several methods may be applied. The simplest is to pierce the bottom of the tube with a needle, and collect the gradient as it drops out. Another method is to pump the gradient from the bottom of the tube with a narrow capillary tube. However, this method is not recommended because of the potential to disturb the gradient and resulting loss of resolution.

See also: **III/Proteins:** Capillary Electrophoresis; Crystallization; Electrophoresis; Field Flow Fractionation; High-Speed Countercurrent Chromatography; Ion Exchange.

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Crystallization

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Introduction

The first protein crystals described in the literature were obtained by Hunefeld in 1840. Hunefeld observed hemoglobin crystals after slow drying of blood pressed between two slides of glass. It is remarkable that this first result demonstrated the basic principle used today, that protein crystals similar to inorganic crystals may be produced by concentration of a protein in solution through slow dehydration. Throughout the history of protein crystal growth, the rationale for protein crystallization has been, firstly, separation of proteins from complex extracts, and then, starting in the 1930s, as purification as determination of the three-dimensional structure of protein molecules.

Knowledge about the three-dimensional structure is necessary to better understand the functions of protein molecules in living systems and plants. Three-dimensional structure can be determined by X-ray diffraction. For X-ray diffraction, good quality protein crystals of appropriate sizes are required. Crystal sizes in each direction should be at least 0.1 mm, if using a strong beam of synchrotron radiation, or at least 0.3 mm for conventional sources of X-rays.

Protein molecules in the crystalline state are more stable than in solution. Therefore, crystallized proteins are more stable against denaturation and may be preserved for a significantly longer period of time than in solution. That is the reason that protein crystallization is often directed as much on preservation as on separation and purification.

This article comprises a brief description of general principles of protein crystal growth and a description of various techniques of protein crystallization with the emphasis on methods using a small amount of a crystallizing solution, from about 1 to 20 μL. The consumption of small amounts of protein is of value, since screening and optimization tests of determination of crystallization conditions typically require many portions of protein solution.

General Principles of Protein Crystallization

Intermolecular Interaction

To crystallize a protein it should be first of all dissolved to give a solution where the protein molecules become close one to another to create a nucleus that grows into a crystal.

An essential feature of protein solutions, associated with the complex structure and large size of protein molecules, is that the molecules may be charged by electric charges, of the same polarity, resulting in long-range electrostatic repulsion. This peculiarity is mediated by the ability of macromolecules to acquire protons from a solution or give up protons into the solution depending on the pH. The charge value of