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Downstream Processing

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For the past twelve years, the series *Advances in Biochemical Engineering* has published high quality scientific information in the areas of both engineering and biology. More recently, these two areas have been successfully integrated into the field known as biotechnology, a subject of growing importance due to the rapid progress being made in applications of molecular genetics and genetic engineering. In order to acknowledge the value of this rapidly-developing technology, the series will now be known as *Advances in Biochemical Engineering/Biotechnology*. In principle, the publishing policy will not change; this change merely reflects our commitment to continue providing the most up-to-date information on recent scientific developments and their practical applications. In the future, too, *Advances in Biochemical Engineering/Biotechnology* will report the latest research progress taking place in the applied fields of biochemistry, molecular biology, microbiology, and engineering, as well as in biotechnology.

Zürich, January 1983 A. Fiechter

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The Formation of Protein Precipitates and Their Centrifugal Recovery

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The methods of precipitating proteins by the addition of reagents are outlined from a physico-chemical viewpoint prior to a more detailed consideration of the molecular and colloidal processes by which precipitation occurs. The mixing necessary for efficient contacting of the reagent and protein and for development of the precipitate also generates forces which can break down precipitate particles. The factors which influence the balance between these opposing processes are analysed as one essential element in reactor design. Other elements, including the influence of reactor configuration, are dealt with and the effects of ageing and other precipitate conditioning methods are described. After outlining the options available for precipitate recovery, centrifugal separation is examined in detail and related to the factors which have been shown to influence precipitate characteristics. Several typical industrial precipitation and recovery operations are described and the key factors influencing process design are summarized.

1 Introduction

Protein precipitation and the subsequent recovery of the precipitate from the mother liquor represents one of the most important operations for the laboratory and industrial scale recovery and purification of proteins. These proteins include vegetable and microbial food proteins, human and animal blood plasma proteins and enzymes for analytical and industrial applications. Proteins produced by genetic engineering are also being recovered and purified by this technique.

The term precipitation will be used in this review to describe an operation in which a reagent is added to a protein solution which causes the formation of insoluble particles of protein. In most applications the intention is to recover the protein in either an unchanged molecular form or one which is readily returned to that form. Therefore, reagents such as urea or some metal ions, which induce major irreversible changes in protein structure and can result in precipitation will not be considered. However methods used in the food industry which precipitate and may alter structure will be mentioned.

There are a number of other ways of forming insoluble protein from protein in solution. Heating may be employed either to coagulate the protein or to bring about drying. Conversely the temperature may be reduced to near 0 °C to induce insolubility in some proteins. These methods are industrially important but they do not share the operation of reagent-protein contacting which connects all the methods to be described.

The recovery of heat coagulated protein and especially that of cryoprecipitates does share common features with those considered here.

This review is particularly concerned with proteins whose solubility properties are determined largely by their polypeptide structure. The formation of such structures from the same pool of twenty amino acids gives a degree of conformity though, for example, histones which are characterized by having many basic amino acid side-chains are soluble at low pH but insoluble under alkaline conditions. Proteins with relatively small non-peptide groups such as haem also conform but lipo-, nucleo- and glycoproteins often exhibit distinctive solubility properties. Thus glycoproteins are very soluble in aqueous solution due to the hydration of the carbohydrate moiety, whereas lipoproteins are relatively insoluble due to the hydrophobic nature of the lipid component. Such changes can also be brought about artificially: the reversible binding of the large heterocyclic apolar molecule, rivanol, (2-exthoxy-6:9-diamino acridine lactate) to a protein reduces its aqueous solubility [1] and the reagent has been used commercially for fractional precipitation of plasma proteins.

Proteins were first classified at the end of the 19th century by their solubility properties and though imperfect the terms adopted are still used. Thus the 'albumins' are characterized by solubility in water and in dilute aqueous salt solutions whereas the 'globulins' though soluble in dilute aqueous salt solutions are insoluble (euglobulins) or sparingly soluble (pseudoglobulins) in water.

The structure of proteins is of central importance in appreciating how precipitating reagents function. The polypeptide chain of water soluble proteins will be folded in

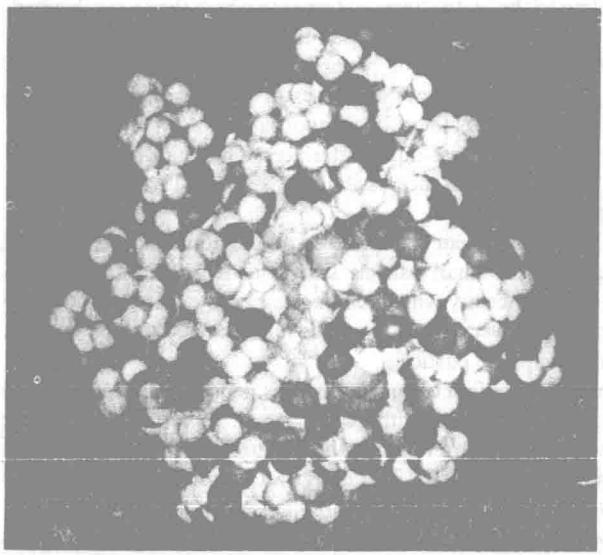

Fig. 1. Model of a globular protein, ferricytochrome c (Tuna). Dark grey spheres are polar atoms, white spheres are hydrogen atoms and pale grey spheres and part spheres are carbon atoms. Thus white and pale grey areas separate from dark grey areas indicate hydrophobic regions. (From computer generated pictures prepared by Dr. R. Feldmann, National Institutes of Health, Bethesda, USA)

such a way that the majority of polar hydrophilic amino acid side-chain groups will be on the exterior and the hydrophobic ones buried (Fig. 1) but this division can never be complete so that changes in the exterior environment brought about by the reagent will affect both types of group as well as the backbone. The overall effect on the protein results from the sum of individual effects which will often be opposed to one another. The situation is complicated by the fact that a protein structure prior to precipitation will not generally be much more stable than a large number of related but undesirable structures. Addition of a reagent can alter the energetic balance in favour of these other structures. Given the complexity of chain folding it will be impractical under industrial conditions to refold the protein to the original structure.

The molecular weights of globular proteins can range from a few thousand to over a million, giving equivalent spherical diameters up to several nanometres. Most globular proteins are ellipsoidal to various degrees. They can often exist as oligomers and this quaternary level of structure may be related to aggregation during precipitation as referred to in this review. Proteins are associated with varying levels of tightly or loosely bound water which forms a hydration shell around the molecule. Up to 0.35 g of water may be tightly bound to 1 g of protein and the amount of loosely bound water is variable but probably stretches up to a few tenths of a nanometre from the molecule surface [2].

A number of biochemical engineering aspects of precipitation are important in establishing efficient operation on an industrial scale. One objective will be to minimize the extent of that part of reagent damage which is due to high localized concentrations. During and after their formation, protein precipitates are subject to particle break-up and further growth according to the hydrodynamic forces to which they are subjected. Such changes may occur during mixing or settling in tanks, passage through pumps and pipelines, and in the equipment used for precipitate recovery. An objective of biochemical engineering studies is to define the causes of these changes in particle size and, where possible, to influence them in a favourable way. During mixing, proteins may be altered irreversibly at gas-liquid interfaces. Their nature is such that foaming readily occurs and this amplifies the possibility of structural damage. Careful equipment design is essential.

The density difference between protein precipitates and the liquid is always small. This, combined with the relatively small particle sizes which can be achieved and the significant enhancement of viscosity which may be caused by proteins and some reagents, leads to problems of recovery. They are made more severe by the hydration and compressibility of the precipitate particles.

Proteins of practical interest are often complex mixtures, many of the components of which have rather similar properties with respect to precipitation. When the objective is to purify a given component protein in the mixture as well as to recover it, precise fractional precipitation is required. The design of a system to achieve this precision is especially demanding.

The biochemical engineering aspects of precipitation will be illustrated by examples from the relatively few systems which have been studied in any detail. They also will be related to engineering design aspects of precipitation reactors, and centrifuges used for precipitate recovery.

Protein precipitation and recovery has some close similarities to the coagulation

and flocculation of biological cells and their separation [3]. The precipitation and recovery of most highly hydrated biological substances also show parallels. Defining protein precipitation in biochemical engineering terms therefore should assist in the transfer of information between these related fields.

2 Physico-chemical Aspects of Protein Precipitation

The methods by which proteins are precipitated can be divided into two groups. In the first, protein solubility is reduced by the addition of high concentrations of reagent which change the nature of the solvent environment in a major way. This category includes organic solvents such as ethanol, acetone and ether and neutral salts such as ammonium sulphate. In the second group, which includes acids, bases and some metal ions, low concentrations of reagent are effective by direct interaction with the protein.

The precipitation methods to be described can be summarized as follows:

a) The addition of high concentrations of neutral salts generally decreases protein solubility, an effect known as 'salting-out'.

b) The ionization of the weakly acidic and basic amino side-chains of proteins is influenced strongly by pH. Solubility is a function of the net charge on these groups and proteins have zero net charge at some pH known as the isoelectric point where they will tend to precipitate from solution.

c) If the dielectric constant of the aqueous medium is reduced, for example by the addition of miscible organic solvents, electrostatic interaction between protein molecules is enhanced and precipitation will result.

d) Proteins are precipitated by non-ionic polymers probably as a result of a reduction in the amount of available water for their solvation.

e) A number of charged polyelectrolytes are capable of precipitating proteins, probably by acting as flocculating agents under appropriate pH conditions.

f) Several polyvalent metal ions which interact directly with proteins have proved valuable in reversibily precipitating proteins.

As would be expected, combinations of reagents produce interactive effects. In addition to the above methods which have been widely applied there are a number of reagents such as rivanol which have narrower though sometimes important applications [4].

It is evident from studies with several precipitants that protein solids probably constitute a protein rich phase rather than a pure solid phase such as occurs with, say, sodium chloride crystals and this complicates the analysis of precipitation [2]. A coherent theory describing the action of all protein precipitants is not available. However, the manner in which the major precipitants function will be described briefly since it bears closely on biochemical engineering aspects of precipitation. For more detailed accounts of the physical chemistry of protein precipitation the reader is referred to several reviews [4, 5].

2.1 Colloidal Stability of Protein Suspensions

The colloidal stability of protein suspensions may best be discussed using the Derjaguin London Verwey Overbeek (DLVO) theory. Forces of attraction and re-

pulsion between lyophobic colloidal spheres of uniform charge or uniform potential may be summed and yield an energy barrier to aggregation. The magnitude of this energy barrier relative to thermal energy of the collisions determines whether or not aggregation will proceed and at what rate. This subject has been extensively reviewed elsewhere [6], and only aspects pertinent to protein precipitation will be discussed here. The feature of protein precipitation not commonly considered in the study of lyophobic colloids is the barrier to aggregation due to a hydration shell [7, 8] which will be discussed in Sect. 2.1.2.

2.1.1 Surface Chemistry of Proteins

A protein may be characterized as a globular, amphoteric polymer with a large number of non-uniformly distributed titratable surface groups [9]. The density of surface charges is often low even for a maximally dissociated protein. For example egg albumin, molecular weight of approximately 40,000, has no more than 28 surface charges [10]. The interaction between these surface groups, the proximity of hydrophobic residues or their areas at the surface and buried within the globular molecule and the presence of hydrogen bonds make the prediction of the surface properties of an individual protein molecule a difficult matter [9]. Proteins may exhibit isoelectric points at pH values ranging from 1 to 12 but, for many proteins, for example soya, casein, some albumins, this range is reduced to pH 4 to 6. Therefore in their normal environment these proteins tend to have an overall negatively charged surface. The picture is complicated by the association and binding of various ions and molecules to the protein surface [9, 11].

An overall negative charge of a protein molecule suspended in an electrolyte will attract positive ions from solution to form a layer (Stern) of counterions close to the molecule surface. The more diffuse layer (Gouy-Chapman) of counter-ions then extends further into the solution developing the overall diffuse electrical double layer surrounding the protein molecule. Characterization of the Stern and Gouy-Chapman layers can be related to the zeta potential [12]. This is the potential at the slip plane between the protein molecule and the solution, the plane occurring somewhere in the diffuse layer surrounding the molecule. For a stable non-aggregating system zeta potentials of the particles of the order of ± 10 to ± 40 mV are commonly reported [12]. One widely studied system is a stable suspension of casein micelles where values of zeta potential ranging from -8 mV [13] to -47.6 mV [14] have been reported. The diffuse and irregular nature of the micelle surface makes experimental analysis difficult [15].

The thickness and nature of the Stern layer is dependent on the size and hydration of the adsorbed ions or surface active substances. It is this layer which controls the ultimate approach of the aggregating species. The Stern layer thus indirectly controls the stability of the colloid by control of the size and magnitude of the diffuse layers. At protein concentrations normally encountered in practice, the proximity of neighbouring protein molecules will also exert considerable influence on the structure of the diffuse layers.

The position and structure of the Stern and diffuse layers can be significantly altered by the presence of adsorbed polymers. Generally if the polymers adsorb to the protein surface, they will do so at several points giving a tightly bound species

which will not desorb on dilution. The adsorbed polymer will extend a considerable distance from the molecular surface due to the presence of loops and trains, so providing a steric barrier to aggregation and for some polyelectrolytes an increased repulsive force due to electrical interactions. Charged or uncharged polymers affect the Stern layer by reducing its charge intensity, the diffuse layer by rearranging the counter-ions, and the zeta potential by altering the slipping plane between the particle and the solution. The method of addition of the polymers and the concentration added will determine whether or not they act as a colloid stabilizing agent or as a flocculation agent (see Sect. 3.1.3).

Despite the many reservations noted above for characterizing protein molecular surfaces, at least a qualitative picture may be built up for the forces controlling the colloid stability or precipitation of protein molecules. As two similarly charged colloidal species are brought together, the electrical diffuse layers will interact giving rise to a repulsive force between the particles. The outer part of the two layers will very rapidly equilibrate so that the rate of approach may be related to repulsive and attractive forces only. The ions in the electrical layer close to the particle surface, including the Stern layer and the inner part of the diffuse layer, will only relax or equilibrate slowly and effective collisions may be dependent on the orientation of the particles and the nature of the species adsorbed to the surface.

Theoretical expressions have been derived for the idealized case of two uniformly charged colloidal spheres approaching each other. The repulsive force is roughly proportional to the square of the zeta potential of the particle, and decreases exponentially with distance between the particles. Attraction between colloidal particles is due to London-van der Waals forces based on induced dipoles between the colliding particles. These are long range forces operating over several tens of nanometres which is also the case for electrostatic repulsion forces at low concentrations of electrolyte. Ionic bonds are very short range and will only come into play when the repulsive forces have been largely overcome.

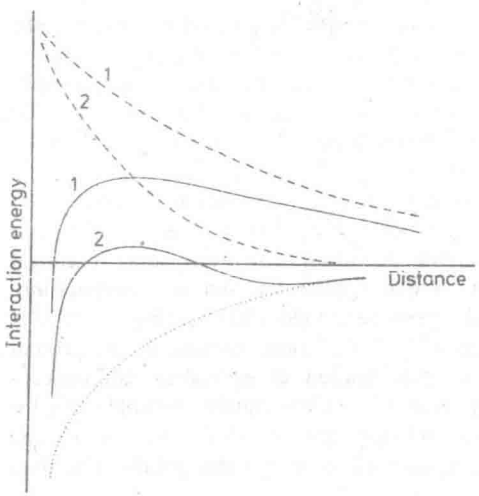

Fig. 2. Combination of attractive and repulsive forces between two charged particles giving overall interaction curves. – – – – electrostatic repulsion forces; · · · · · · London-van der Waals attractive forces; ——— overall interaction energy; (1) low salt concentration; (2) high salt concentration

Considering only the London-van der Waals and the electrostatic forces an overall potential energy barrier to collision may be postulated (Fig. 2). Provided the thermal energy of the particles is sufficient to overcome the overall potential energy barrier then aggregation will take place, the rate of collision being dependent on the relative magnitude of these energy levels. A shallow secondary minimum is noted for the second of the overall energy curves giving rise to loose aggregates which may be easily disrupted on stirring [6].

2.1.2 Hydration of Proteins

Protein molecules cannot be treated simply as hydrophobic colloids. They are very hydrophilic being associated with up to three times their own weight of water. The loosely associated water forms a stabilizing barrier to aggregation [7]. This barrier is not taken into account in the DVLO theory for colloid stability (Sect. 2.1.1).

The loosely held water is not quantifiable due to its similarity to non-associated water. Ten to 100 molecular layers of this water have been estimated to surround a protein molecule probably clustered around the ionic side chains. Some structural ordering would be expected to extend up to a few tenths of a nanometer from the protein molecule surface [2], thereby inhibiting molecules from approaching sufficiently close to promote aggregation.

2.2 Methods of Precipitation of Proteins

2.2.1 Salting-out

The effect of high salt concentration in promoting aggregation and precipitation of proteins is still not well understood. Theoretical approaches have been applied to predict the behaviour but at the high ionic strength involved they cannot be applied strictly and an empirical expression due to Cohn [5] is most widely used:

$$\log S = \beta - KI \qquad (1)$$

where S is the solubility of the protein at ionic strength I and β and K are constants. Figure 3 shows an experimental example of protein precipitation obeying the relation and illustrates that if protein concentration is increased the salt concentration at which the equation applies is shifted in the expected manner. The constant, β, of Eq. (1) varies markedly with the protein but is essentially independent of the salt; β is strongly dependent on pH and temperature, usually passing through a minimum at the isoelectric point. The slope of the salting-out curve, K, is found to be independent of pH and temperature but varies with the salt and protein involved. As has been observed with pectins and other hydrophilic polymers, proteins of similar chemical composition but increasing molecular weight require less salt for precipitation. Myoglobin of low molecular weight, albumins, serum globulins, and high molecular weight fibrinogen represent such a series [16,17]. The more asymmetric the protein molecule the greater is the degree of precipitation at equivalent salt concentrations [7]. For a particular salt, K only varies over approximately a twofold range for different proteins being greatest for large and asymmetric molecules. Salts containing polyvalent anions such as sulphates and phosphates have higher values of K than

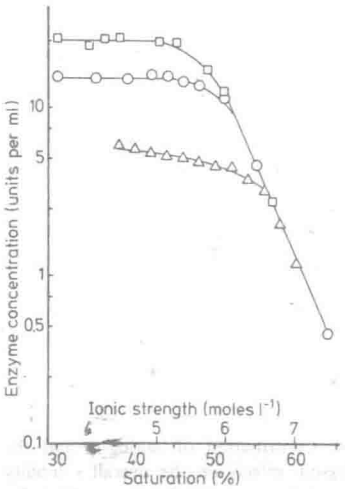

Fig. 3. The effect of enzyme concentration on salting-out of fumarase by ammonium sulphate at 6 °C [112]. Initial enzyme concentrations (units/ml) were: (△) 5.4; (○) 15.2; (□) 25.2

uni-univalent salts but polyvalent cations such as calcium or magnesium depress the value of K. Therefore ammonium and sodium sulphate and potassium and sodium phosphates have been widely used. Sodium sulphate is of low solubility below 40 °C and its use is thus restricted to precipitation of stable proteins such as extracellular enzymes. Although the salting-out effect of phosphates is greater than that of ammonium sulphate the latter is more soluble and its effect is less dependent on temperature, though the variation is still such that temperature must be specified. Ammonium sulphate is also much cheaper. Being the salt of a strong acid and a weak base it does tend to become acid by hydrolysis and the release of ammonia at higher pHs is inconvenient on an industrial scale. Ammonium sulphate is a corrosive material and is difficult to handle and dispose. Residues of it remaining in food products can be tasted at low level, and it is toxic with respect to clinical use so that it must be removed.

The relative effectiveness of neutral salts in salting-out and especially of anions gives rise to a series, the lyotropic or Hofmeister series in which citrate > phosphate > sulphate > acetate ~ chloride > nitrate > thiocyanate. Particularly significant is the observation that the tendency for a salt to cause structural damage to a protein is inversely related to its position in the lyotropic series. Thus sulphate ions are associated with structural stabilization and thiocyanate with destabilization. The specific Hofmeister effects appear to be due to interaction of the ions with hydrophobic groups of the protein [18].

According to a theoretical treatment by Melander and Horvath [19] salting-out of proteins may be described as a balance between a salting-in process due to electrostatic effects of the salt and a salting-out process due to hydrophobic effects. Using a dimensionless form of the Cohn equation (1):

$$\log \frac{S}{S_0} = -KI + \beta' \qquad (2)$$

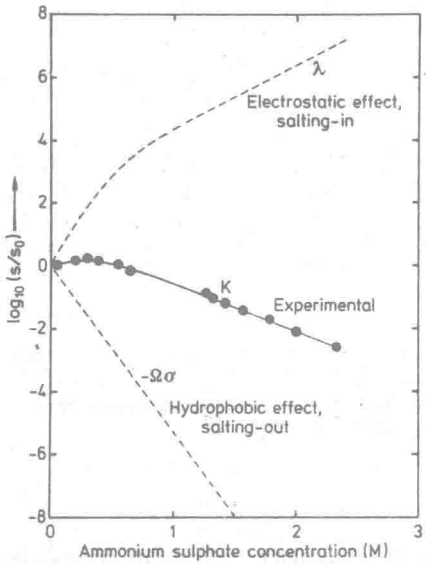

Fig. 4. Contribution of salting-in and the salting-out effects on the overall solubility curve of haemoglobin (see Sect. 2.2.1 for explanation of symbols) [19]

where S_0 is the solubility at zero ionic strength; salting-in, salting-out and overall solubility curves may be summarized as in Fig. 4. The relative surface hydrophobicity determines the contact area between the protein molecules (Ω). At higher salt concentrations the attractive force between the hydrophobic areas is increased due to greater induced dipoles (σ). Concomitant with this increased salting-out effect is the further development of layers of like charges on the molecules, thereby increasing the repulsion between the molecules (λ). The slope of the overall salting-out curve is given by:

$$K = \Omega\sigma - \lambda \tag{3}$$

The type of salt used determines σ; this property is governed by the molal surface tension increment of the salt. The Hofmeister or lyotropic series of salts reflects their ability to precipitate proteins, and in fact lists the salts in the order of their molal surface tension increment. The protein type essentially determines Ω, and generally the magnitude of $\Omega\sigma$ is much greater than λ. Therefore maximum solubility is reached at relatively low salt concentrations, the solubility decreasing very rapidly at higher salt concentrations. For proteins such as tetrameric myoglobin (molecular weight equals 68,000), ovalbumin (46,000), haemoglobin (68,000) and albumin (67,000), the salting-out curve correlates with the relative surface hydrophobicity or the frequency of charged surface groups rather than with the average hydrophobicity of the molecules. The relationship between the theory of Melander and Horvath and the more common one of high salt concentrations simply removing hydration barriers from around the protein molecule is not clear. Probably the salt does tend to attract water from the protein surface as does the lowering of dielectric constant of the protein environment by the addition of organic solvents, or the addition of hydrophilic polymers such as polyethylene glycol.

2.2.2 Isoelectric Precipitation

Adjustment of pH at low ionic strength to the point where a protein has zero net charge will lead to substantially reduced solubility. Figure 5 illustrates this for soya protein total water extract. The effect, which is enhanced for proteins of low hydration constant or high surface hydrophobicity, has been widely used. For example, casein, a protein with the latter characteristic, precipitates at its isoelectric point forming large, strong, aggregates [7]. On the other hand gelatin, an exceptionally hydrophilic protein does not precipitate at its isoelectric point in low ionic strength or high dielectric constant environments.

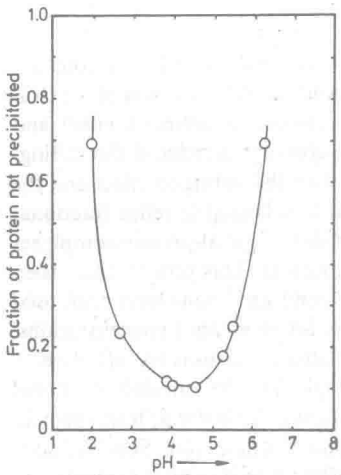

Fig. 5. The effect of pH on the concentration of soya protein remaining in solution, expressed as a fraction of initial concentration of total water extract [99]

The Cohn equation (1) embraces the effect of pH as well as of neutral salts since the constant β for a particular protein is pH dependent, generally reaching a minimum at or near the isoelectric point of the protein. Changes of solubility well in excess of an order of magnitude per pH unit in this region are common. Sometimes β passes through two or more minima, the additional ones evidently being due to formation of specific salts. It is generally observed that as the neutral salt concentration is increased the minimum solubility increases in value and the pH of lowest solubility decreases due to anion binding.

A major advantage of isoelectric precipitation commonly done at acid pHs, is the cheapness of mineral acids and the fact that several such as phosphoric, hydrochloric and sulphuric are acceptable in protein food products. In addition it will usually be possible to proceed directly to another fractionation procedure without the need, faced with salting-out, for removal of the reagent.

The principal disadvantage of using acids is their potential for damaging proteins irreversibly [20]. This comes about because of protein sensitivity to low pH but may be greatly amplified by the action of acid anions from the part of the Hofmeister series (Sect. 2.2.1) associated with protein destabilization.