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Salt-Induced Protein Precipitation: Phase Equilibria from an Equation of State

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Abstract

A molecular-thermodynamic model is developed for salt-induced protein precipitation. The model considers an aqueous solution of a globular protein as a system of interacting hard spheres in a continuum pseudo-solvent (water and salt ions). The protein molecules are considered to interact in a manner described by a set of spherically-symmetric two-body potentials of mean force. These include screened Coulombic repulsion, dispersion (van der Waals) attraction, osmotic attraction, and an attractive square-well potential intended to model specific protein-protein chemical interactions (including the hydrophobic effect and protein self-associations). Following Chiew, *et al.* (1995), an analytical equation of state is derived using the Random Phase Approximation with the hard-sphere fluid as the reference system and a perturbation based on the protein-protein overall potential of mean force. This equation of state provides an expression for the chemical potential of the protein and determines liquid-liquid equilibria. The model is generalized for co-precipitation of several proteins. Experimental single-protein precipitation data are correlated for hen egg-white lysozyme and for α-chymotrypsin in concentrated aqueous solutions of ammonium sulfate.

1. INTRODUCTION

Precipitation has long been used as an early step in the process of purifying proteins from complex solutions [1,2]. Phase separation is achieved through addition of precipitating agents to aqueous protein solutions. Commonly-used precipitating agents include inorganic salts, nonionic polymers, polyelectrolytes, and organic solvents [3,4]. Salting-out of proteins provides one of the simplest precipitation techniques and is used extensively in the laboratory, in biotechnology and in the pharmaceutical industries.

In aqueous solutions, proteins are charged polyions. Their charge depends primarily on solution pH, and they have complex titration curves which depend on the hydrogen-ion dissociation constants of each of the titratable constituent amino acid. Even at the isoelectric point, there may exist a significant dipole [5]. Therefore, in low-ionic-strength solutions, proteins interact through a balance of Coulombic repulsion and weaker attractive charge-dipole and dipole-dipole forces, as well as through attractive dispersion forces, as discussed in DLVO theory [6]. However, in salt solutions where protein precipitation occurs (typically $\geq$ 5M ionic strength), the concentration of ions is so high that there is little free volume for the protein. At such high salt concentrations, where the Debye length is extremely small, Coulombic interactions are essentially completely screened. Also, preferential coordination of water by the ions reduces solvation of the proteins, making precipitation more favorable. Various experimental equilibrium salting-out studies have shown that supernatant-phase protein concentration can depend strongly on protein size, initial concentration, and surface chemistry; on electrolyte identity and ionic strength; and on solution pH [7,8]. Specific interactions (e.g. dimerization or higher oligomerization, hydrophobic interactions) may also play a major role in governing protein-protein interactions in solution.

In most previous studies, salt-induced protein precipitation has been described as equilibrium between a *pure* protein phase (precipitate) and a saturated liquid phase, with the degree of separation quantified by the apparent protein "solubility", i.e. the protein concentration in the supernatant equilibrium liquid phase. However, recent experimental work by Shih, *et al.* [9] with bovine serum albumin and α-chymotrypsin has shown that the precipitated phase contains significant amounts of electrolyte and water, indicating that protein salting-out may be considered a liquid-liquid phase separation resulting in a supernatant fluid phase dilute in protein in equilibrium with a dense precipitate fluid phase rich in protein and containing appreciable amounts of water and

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the principles of fluid-phase equilibria and molecular thermodynamics to construct theoretical working models describing protein salting-out phase separations in the equilibrium limit.

For the single-protein case, this equilibrium model represents the solution (protein, ions, and water) as a system of globular proteins in a continuum pseudosolvent (water and ions). The residual Helmholtz energy of the system is the sum of a hard-sphere reference contribution and a perturbation. In the latter, protein-protein interactions are described by a two-body spherically-symmetric overall potential of mean force which includes a screened Coulombic repulsion term, an attractive dispersion term, an osmotic attraction term to account for ion-excluded-volume effects, and an attractive square-well contribution to represent specific chemical interactions. Because charge-dipole, dipole-dipole and higher-order electrostatic interactions are much weaker than charge-charge interactions, they are ignored here. In this model, the effect of the salt is a strong short-ranged osmotic attraction between proteins and screening of the Coulombic repulsion. Salt-protein or salt-salt interactions are not treated explicitly.

2. EQUATION OF STATE

The Random-Phase Approximation (RPA) has been used previously to model phase transitions and structure factors of colloidal solutions [10], and to describe phase separation of proteins due to addition of polymers or salt [11-13]. In the RPA, an assembly of hard spheres is used as the reference system, while the remaining spherically-symmetric interactions provide perturbations. The RPA equation of state and residual Helmholtz energy are written [14] as a sum of reference and perturbation terms:

$$Z = \frac{P}{\rho kT} = \left(\frac{P}{\rho kT}\right)_{ref} + \frac{\rho U}{2kT} \qquad (2.1)$$

$$\frac{A^{res}}{NkT} = \left(\frac{A^{res}}{NkT}\right)_{ref} + \frac{\rho U}{2kT} \qquad (2.2)$$

where ρ is the total protein number density; and U is the perturbation energy per unit density, given (for a single-protein system) by

$$U = 4\pi \int W_{pp}(r)\, r^2 dr \qquad (2.3)$$

where $W_{pp}(r)$ is the overall perturbation protein-protein potential of mean force defined in §3 below. The reference system equation of state and residual Helmholtz energy are given by the Carnahan-Starling expressions for hard spheres:

$$\left(\frac{P}{\rho kT}\right)_{ref} = \frac{1+\eta+\eta^2-\eta^3}{(1-\eta)^3} \qquad (2.4)$$

$$\left(\frac{A^{res}}{NkT}\right)_{ref} = \frac{4\eta-3\eta^2}{(1-\eta)^2} \qquad (2.5)$$

where the packing fraction $\eta = (\pi\rho/6)\sigma_p^3$ and σ_p is the hard-sphere diameter of the protein. In the RPA, the chemical potential of the protein is given by [13,14]:

$$\frac{\mu-\mu'}{kT} = \frac{\eta(8-9\eta+3\eta^2)}{(1-\eta)^3} + \frac{\rho U}{kT} + ln\rho \qquad (2.6)$$

where $\mu'/kT = ln(\Lambda^3)$; the deBroglie wavelength $\Lambda = h/\sqrt{2\pi mkT}$, where h is Planck's constant and m is the molecular mass. Liquid-liquid equilibria are calculated from Eqs. (2.1) and (2.6) based on the classical equilibrium conditions:

$$\mu^s = \mu^d \qquad (2.7)$$

$$P^s = P^d \qquad (2.8)$$

where superscripts s and d denote the supernatant and dense phases, respectively. In Eq. (2.7), μ' cancels out. Appendix A presents details of phase-equilibrium calculations for multiprotein solutions.

3. PROTEIN-PROTEIN POTENTIAL

The overall perturbation potential of mean force between two different protein molecules i and j, $W_{ij}(r)$, is given by a sum of three spherically symmetric center-to-center potentials of mean force:

$$W_{ij}(r) = W_{elec}(r) + W_{disp}(r) + W_{osmotic}(r) + W_{specific}(r) \tag{3.1}$$

where r is the protein-protein center-to-center distance. Here, W_{elec} is the repulsive screened Coulombic potential due to charge on the protein; W_{disp} is the attractive van der Waals dispersion potential; $W_{osmotic}$ represents an attractive interaction which arises due to the high concentration of ions; and $W_{specific}$ is an attractive potential between proteins included to represent any specific chemical effects, such as hydrophobic interactions.

The screened Coulombic repulsions between proteins are represented by the well-known Debye-Hückel expression:

$$\frac{W_{elec}(r)}{kT} = \frac{z_i z_j e^2 (1/r) \exp\left[-\kappa(r - \sigma_{ij})\right]}{4\pi k T \varepsilon_o \varepsilon_r (1 + \sigma_{ij}/2)^2} \qquad r \geq \sigma_{ij} \tag{3.2}$$

where $z_i e$ is the charge on protein i, σ_{ij} [$= (\sigma_i + \sigma_j)/2$] is the mean hard-sphere diameter of the i-j pair; k is Boltzmann's constant; T is the absolute temperature; $4\pi\varepsilon_0$ is the dielectric permittivity of free space; and ε_r is the relative dielectric permittivity of the solvent. The Debye parameter, κ, is given by $\kappa^2 = (2e^2 N_A I)/(kT\varepsilon_o\varepsilon_r)$, where N_A is Avogadro's number; ionic strength $I = (z_{an}^2\rho_{an} + z_{cat}^2\rho_{cat})/2$; z_{an} and z_{cat} are the anion and cation valences and ρ_{an} and ρ_{cat} are the ionic number densities. Equation (3.2) is derived using a mean-field approximation assuming that the electrolyte ions are point charges. This assumption is poor at ionic strengths greater than 0.1 molar. However, at high ionic strengths, Coulombic repulsion between protein molecules is effectively eliminated by dielectric screening and plays a minor role in phase separation.

The attractive dispersion potential is given by [6]:

$$W_{disp}(r) = -\frac{H_{ij}}{12}\left\{\frac{\sigma_{ij}^2}{r^2 - \sigma_{ij}^2} + \frac{\sigma_{ij}^2}{r^2} + 2\ ln\left(1 - \frac{\sigma_{ij}^2}{r^2}\right)\right\} \qquad r > \sigma_{ij} \tag{3.3}$$

where H_{ij} is the effective Hamaker constant for the i-j interaction. Since H_{ij} is primarily a function of the density of the protein molecules [15], the Hamaker constant is similar for most proteins in solution since most proteins have roughly the same mass density [16]. In a recent experimental study, Coen, *et al.* [17] have reported Hamaker constants regressed from low-angle laser-light-scattering data (at 298K) for bovine α-chymotrypsin and hen egg-white lysozyme in aqueous solutions of potassium sulfate, using a similar potential-of-mean-force model. For both globular proteins in solutions with electrolyte ionic strength greater than 1.0 molar, H_{ij} was constant at approximately 10 kT. Theoretical estimates, based on Lifshitz theory [18], indicate that an appropriate value of H_{ij} for proteins is ~5 kT. The effective dispersion forces are not strongly screened by the presence of added electrolyte; H_{ij} decreases by at most 10% in solutions of high ionic strength.

In concentrated electrolyte solutions, ions occupy a significant fraction of the total solution volume. When two protein molecules approach near the point of surface-surface contact, ions are excluded from the space between proteins, giving rise to a significant imbalance in the local balance of forces exerted by the ions on the protein. The osmotic pressure difference is approximated by the ideal osmotic pressure of the bulk solution ($\Pi_{id} = \rho_s kT$, where ρ_s is the total ionic number density). The result is a strong short-range attractive potential between the proteins, expressed simply by [19]:

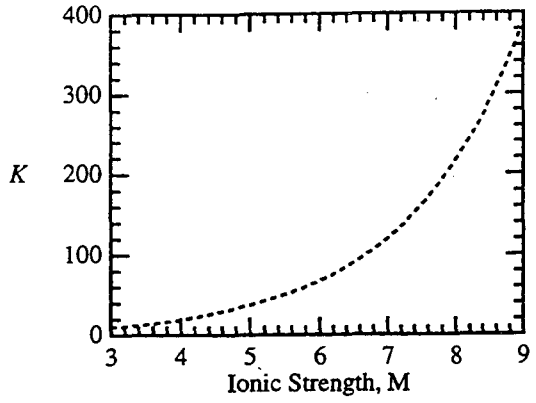

Fig. 1. Effect of ionic strength on protein partitioning. $\sigma_p = 40$Å, $H/kT = 5$, $\delta = 3$Å, $\varepsilon_{sp}/kT = 2$, $\sigma_s = 3.2$Å

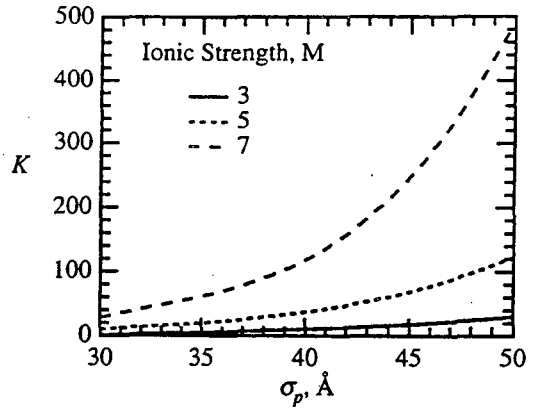

Fig. 2. Effect of protein diameter on partitioning. $H/kT = 5$, $\delta = 3$Å, $\varepsilon_{sp}/kT = 2$, $\sigma_s = 3.2$Å

$$W_{osmotic}(r) = -\frac{4}{3}\pi\sigma_{ij,s}^3(\rho_s kT)\left[1 - \frac{3r}{4\sigma_{ij,s}} + \frac{r^3}{16\sigma_{ij,s}^3}\right] \tag{3.4}$$

where $\sigma_{ij,s} = (\sigma_{ij} + \sigma_s)/2$ and $\sigma_s = (z_{an}\sigma_{cat} + z_{cat}\sigma_{an})/(z_{cat} + z_{an})$ is a valence-weighted ion diameter; here the absolute values of the valences are used. This potential has been used to model phase transitions in colloidal systems induced by the presence of nonadsorbing polymers [11,12] and to describe salting-out phenomena in protein solutions [13].

In this work, we are primarily concerned with precipitation in concentrated electrolyte solutions where ionic effects dominate protein-solution behavior. However, because it is also possible to precipitate proteins in solutions with little added electrolyte, it is likely that specific chemical attractions between proteins such as hydrophobic interactions or self-association leading to aggregation are also important, even at high ionic strength. These interactions can be described by an attractive square-well potential:

$$\begin{aligned} W_{specific}(r) &= -\varepsilon_{sp} \qquad & \sigma_{pp} < r < (\sigma_{pp} + \delta) \\ &= 0 & r > (\sigma_{pp} + \delta) \end{aligned} \tag{3.5}$$

where ε_{sp} and δ are model parameters. Our uncertainty of the molecular details of specific chemical interactions is subsumed in these two parameters; they can only be estimated from characteristic energetics and length scales for such interactions [20]. For example, for hydrophobic interactions between proteins, $\varepsilon_{sp}/kT \sim 3$ provides a reasonable upper bound [21].

IV. RESULTS AND DISCUSSION

For precipitation of a single protein in an aqueous salt solution, we examine first the effect of ionic strength. In phase-separating systems, the partition coefficient, K, is given by the ratio of the equilibrium number density in the dense phase to that in the supernatant phase [$K = \eta_d/\eta_s$]. Figure 1 illustrates the exponential dependence of K on ionic strength. This dependence is a commonly-observed feature in salting-out not only of proteins, but also of other organic substances and dissolved gases [22]. The exponential form has been used extensively in correlating protein salting-out data [9,17,23]. In all calculations in this work, model parameters are chosen to correspond to a small globular protein (taken to have properties similar to bovine α-chymotrypsin) precipitating in ammonium sulfate, one of the most commonly-used inorganic electrolytes for salting-out separations. Experimental precipitation data for small proteins in ammonium sulfate solutions show that partition coefficients range from just above unity (near the critical point for phase separation where typical ionic strengths are ~3-5M) up to several hundred at higher ionic strengths (~10M). These high partition coefficients indicate that substantial purification can be achieved [17].

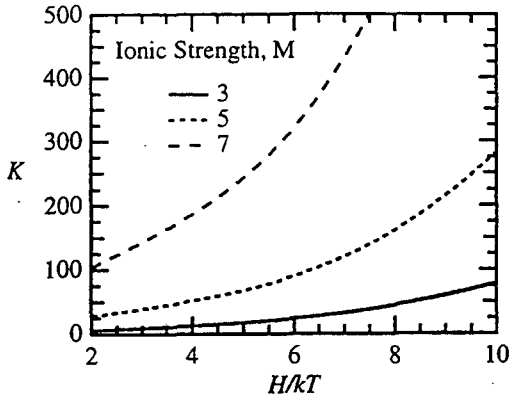

Fig. 3. Effect of Hamaker constant on partitioning. $\sigma_p = 40$Å, $\delta = 3$Å, $\varepsilon_{sp}/kT = 2$, $\sigma_s = 3.2$Å

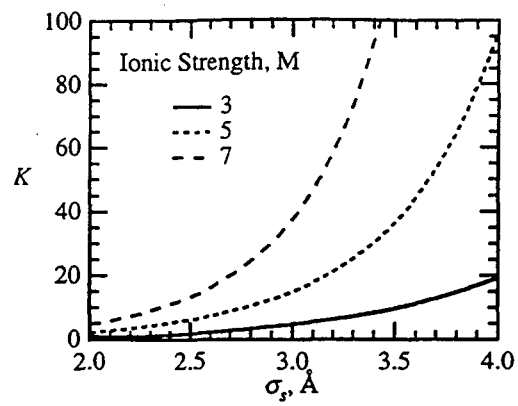

Fig. 4. Effect of mean ion diameter on partitioning $\sigma_p = 40$Å, $H/kT = 5$, $\delta = 3$Å, $\varepsilon_{sp}/kT = 2$

It is well known that, in identical aqueous precipitating solutions, large solute molecules partition more strongly than small molecules [24]. Figure 2 shows a strong dependence of the calculated partition coefficient, K, on protein diameter. As the size of the protein increases in a solution of fixed ionic strength, the length scale over which the osmotic pressure imbalance acts also increases, creating a greater net attractive two-body potential. The effect of protein charge, z_p, on partitioning is negligible, due to strong dielectric screening of Coulombic repulsions at high ionic strengths.

Figure 3 illustrates the dependence of K on the effective Hamaker constant, H_{ij}, for several values of solution ionic strength. For a given ionic strength, the calculated partition coefficients increase by an order of magnitude over the range $2 < H_{ij}/kT < 10$. In Figure 4, the effect of mean ion diameter, σ_s, on partitioning is shown for the same values of ionic strength as those in Figure 3. Calculated partition coefficients show a strong dependence on σ_s, increasing by more than an order of magnitude as σ_s increases from 2 to 4 Å. This range of σ_s includes the mean crystal ionic diameters of most simple electrolytes [25]. For larger mean ion diameters, the effective length scale for osmotic attraction increases, due to a larger region between proteins for ion exclusion. At high ionic strength, the osmotic pressure is sufficiently large that the osmotic attraction term dominates in the total potential of mean force and produces sufficiently strong attraction to yield very high partition coefficients, as shown in Figures 2 and 4.

When considering specific interactions, it is an oversimplification to consider the protein as a smooth hard sphere; details of the surface chemistry (e.g., identity and hydrophobicity of surface amino acid residues, surface roughness, water and ion binding, etc.) become important. However, in our approximate, spherically-symmetric description of specific interactions, we retain the hard-sphere approximation and make the assumption that the above mentioned details are contained within a 3-Å layer on the surface of the protein. In all our calculations, the effect of this assumption is to add an additional 6 Å to the crystal diameter of the protein and to impose a lower limit of 3 Å on square-well width, δ. Figure 5 shows the calculated dependence of partitioning on square-well parameters δ and ε_{sp}/kT. It is apparent that δ has little effect on partitioning for a given ionic strength, while ε_{sp}/kT has a much more significant impact. As the strength of the attractive interaction increases, protein molecules tend to form aggregates. Aggregation increases the protein concentration in the precipitate phase, and lowers the protein concentration in the supernatant phase, resulting in higher partition coefficients at a given solution ionic strength.

Coen, *et al.* [17] have conducted precipitation experiments for two small globular proteins, hen-egg-white lysozyme and bovine α-chymotrypsin in solutions of ammonium sulfate at various ionic strengths and pH. Their data for equilibrium supernatant concentrations are shown in Figure 6, along with model calculations for various values of δ and ε_{sp}/kT. The other model parameters are shown in the figure caption. Effective spherical diameters are known from crystal-structure data for the salt and for the protein [26]; theoretical estimates of the effective Hamaker constant indicate that a reasonable value is $H_{ii}/kT \sim 5$ for these proteins [18]. Agreement of model calculations with experiment is better at higher ionic strengths, where greater precipitation is achieved.

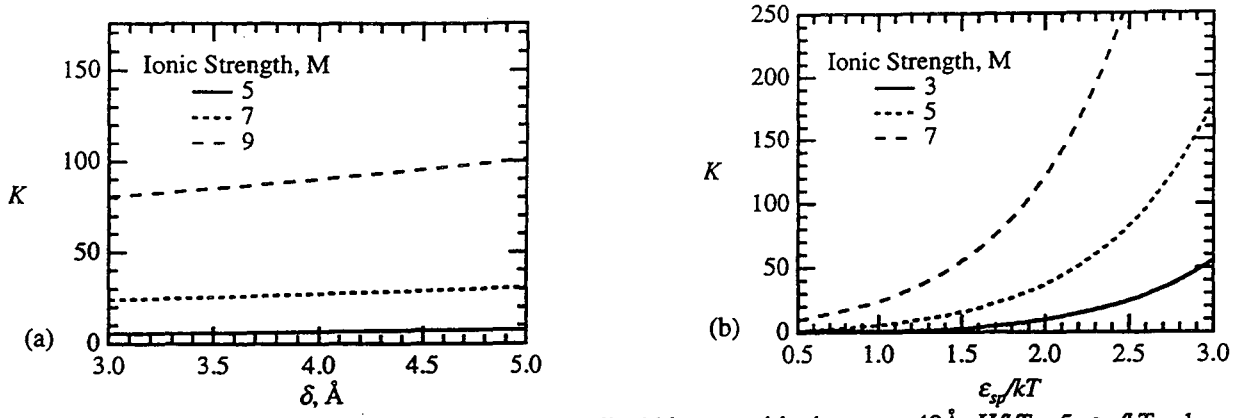

Fig 5. (a) Effect of specific interaction square-well width on partitioning: $\sigma_p = 40$Å, $H/kT = 5$, $\varepsilon_{sp}/kT = 1$, $\sigma_s = 3.2$Å. (b) Effect of square-well depth on partitioning: $\sigma_p = 40$Å, $H/kT = 5$, $\delta = 3$Å, $\sigma_s = 3.2$Å.

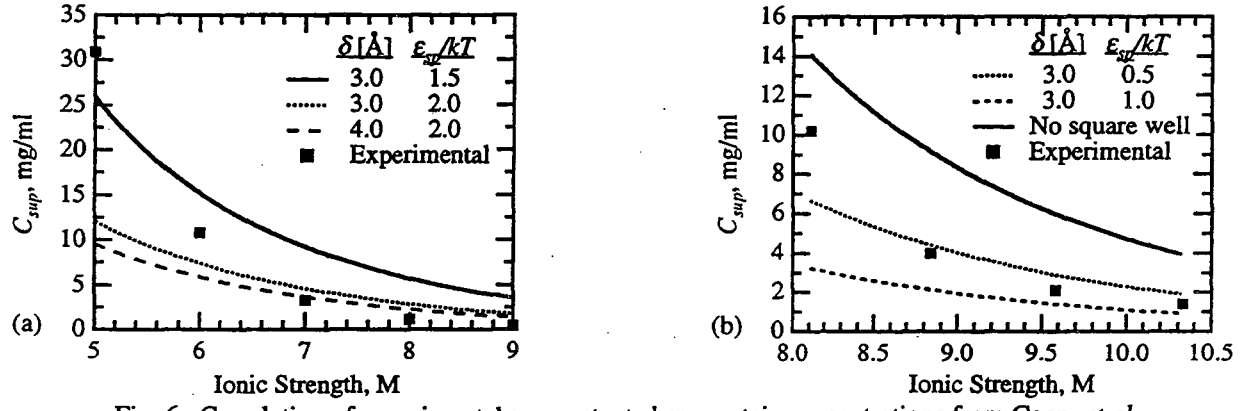

Fig. 6. Correlation of experimental supernatant phase protein concentrations from Coen, *et al.*
(a) Hen-egg-white lysozyme in ammonium sulfate at pH 8: $\sigma_p = 34.3$Å, $H/kT = 5$, $\sigma_s = 3.2$Å
(b) α-chymotrypsin in ammonium sulfate at pH 8.3 (pI): $\sigma_p = 43.4$Å, $H/kT = 5$, $\sigma_s = 3.2$Å

However, the model underpredicts equilibrium supernatant concentrations at the lowest ionic strength for each protein. These ionic strengths were near the respective critical points for lysozyme and α-chymotrypsin phase separation, where the RPA equation of state is less accurate. Figure 6(a) again illustrates the relatively small influence of δ on calculated partitioning.

Although lysozyme is the smaller of the two proteins, it partitions more strongly in ammonium sulfate (pH 8) at a given ionic strength than α-chymotrypsin, as indicated by higher values of ε_{sp}/kT for lysozyme. Lysozyme has been reported to undergo an isodesmic aggregation in low-ionic-strength solutions [27,28], while α-chymotrypsin apparently does not aggregate significantly. These correlated values for ε_{sp}/kT are approximate estimates of characteristic interaction energies, within the limits of the approximations described above.

V. CONCLUSION

We have derived an approximate equation-of-state model for salt-induced protein precipitation based on effective potentials of mean force. Thermodynamic properties of the system are derived using a statistical mechanical perturbation theory. Model calculations indicate that electrolyte concentration plays the primary role in affecting phase separation; protein partition coefficient K increases exponentially with ionic strength. Partitioning is also strongly dependent on protein and ion diameters. Furthermore, aggregation of protein due to specific interactions may play an important role in the precipitation of proteins, even at high salt concentrations. The extent of aggregation is a strong function of ε_{sp}/kT, the depth of the specific interaction square-well potential. Calculated equilibrium supernatant concentrations are in semi-quantitative agreement with experimental results for bovine α-chymotrypsin and hen-egg-white lysozyme in solutions of ammonium sulfate at 25°C.

Appendix A

For co-precipitation of two or more proteins, the reference system compressibility and residual Helmholtz energy are given by the Boublík-Mansoori-Carnahan-Starling expressions for hard-sphere mixtures:

$$\left(\frac{P}{\rho kT}\right)_{ref} = \left[\frac{1}{1-Q_3} + \frac{3Q_1Q_2}{Q_0(1-Q_3)^2} + \frac{Q_2^3(3-Q_3)}{Q_0(1-Q_3)^3}\right] \tag{A.1}$$

$$\left(\frac{A^{res}}{NkT}\right)_{ref} = \left[\frac{Q_2^3 + 3Q_1Q_2Q_3(1-Q_3)}{Q_0Q_3(1-Q_3)^2} - \left(1 - \frac{Q_2^3}{Q_0Q_3}\right) ln(1-Q_3)\right] \tag{A.2}$$

where $Q_k = (\pi/6)\sum_i \rho_i \sigma_i^k$, ρ_i is the number density of protein species i, and σ_i is the hard-sphere diameter of protein species i. Liquid-liquid equilibria are calculated from Eq. (2.8) and a generalization of Eq. (2.7): $\mu_i^s = \mu_i^d$ for every i. Equilibrium phase densities and compositions are obtained by a flash calculation. In an aqueous solution of m protein species, perturbation energy per unit density is given by:

$$U = 4\pi \sum_{i,j=1}^{m} x_i x_j \int W_{ij}(r)\, r^2 dr \tag{A.3}$$

where $x_i = \rho_i/\rho$ and $W_{ij}(r)$ is the protein-protein perturbation potential of mean force for the i-j pair

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