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Salt-Induced Protein Precipitation in Aqueous Solution: The Effect of Pre-aggregation

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Abstract–A molecular-thermodynamic model is developed for the salt-induced protein precipitation. The protein molecules interact through four intermolecular potentials. An equation of state is derived based on the statistical mechanical perturbation theory with the modified Chiew's equation as the reference system and a perturbation based on the protein-protein interaction potential of mean force. The equation of state provides an expression for the chemical potential of the protein and determines liquid-liquid equilibria. The precipitation behaviors are studied by calculating the partition coefficient with changing conditions such as ionic strength, protein and salt size, and the degree of pre-aggregation effect. The reasonable pre-aggregation effects are determined by comparing the proposed model with experimental data.

Key words: Protein, Interaction Potentials, Precipitation, Phase Equilibria, Pre-aggregation

INTRODUCTION

In the early days of protein chemistry, the only practical way of separating different types of protein was by causing part of a mixture to precipitate through alternation of some property of the solvent.

Protein precipitation is the simplest and the oldest practical way to separate different proteins from a solution mixture. Separation is achieved through the addition of precipitation agents such as inorganic salts, nonionic polymers, polyelectrolytes, and organic solvents [Hong et al., 1997; Park et al., 1994; Foster et al., 1975; Haire et al., 1984; Shih et al., 1992; Niederauer et al., 1992; Rothstein, 1994; Lee et al., 1999, 2000].

A variety of researches have studied the protein precipitation behavior by using various experimental techniques. These experimental results suggest that the protein salting-out may be considered a liquid-liquid phase separation resulting in a supernatant fluid phase with a dense precipitate fluid phase. And the degree of separation is characterized by the partition coefficient, K, which is defined as the ratio of the protein concentration in the dense phase to that in the supernatant phase. Recent theoretical studies have been directed at developing more fundamental models that account for the diverse interactions between the constituents in the protein solution on a molecular level. For example, Mahadevan and Hall [Mahadevan et al., 1990, 1992] present a model, based on Baker-Henderson perturbation theory, for protein precipitation by nonionic polymer. Vlachy et al. [1993] describe a model for a liquid-liquid phase separation for solutions of colloids and globular proteins, based on the random-phase approximation. However, most recent theoretical studies are concerned with aqueous solutions where the electrolyte concentration is less than 0.1 molar. Experimental studies clearly show that the protein precipitation by salts requires an electrolyte concentration in the range 1-10 molar.

Theories that are shown in several works of former researchers have quite deviation from experimental results. Therefore, we modify the previous model to describe the pre-aggregation, which are assumed that protein exist aggregated form to several particles in aqueous solution.

In this study, we present a molecular-thermodynamic frame work for the protein precipitation by highly concentrated inorganic salt. This equilibrium model represents the solution (protein, ions and water) as a pseudo-one-component system containing only a continuous solvent and a globular protein molecule. Equation of state is the sum of a hard-sphere reference contribution and a perturbation. The reference term is derived based on the modified Chiew's model to describe the pre-aggregating effect of protein. We also discuss protein-protein effective two-body potentials. These potentials include coulombic repulsion, dispersion attraction, osmotic attraction, and attractive specific potential to represent specific chemical interactions. The influence of parameters such as protein size, salt size and ionic strength also is studied. Finally, the determination of the degree of pre-aggregation effect is accomplished by comparing with experimental data [Cho et al., 1999; Coen et al., 1995; Yoo et al., 1997; Seo et al., 2000].

THEORETICAL CONSIDERATION

1. The Potential of Mean Force

Protein interactions can be described quantitatively by a two-body potential of mean force; three-body and higher interactions become important at protein concentrations higher than those of reported here.

The overall perturbation potential of mean force between two

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different protein molecules, $W_{pp}(r)$, is given by a sum of four potentials of mean force

$$W_{pp}(r)=W_{elec}(r)+W_{disp}(r)+W_{osmotic}(r)+W_{specific}(r) \quad (1)$$

where r is the center to center separation. $W_{elec}(r)$ is the electric double-layer-repulsion potential, $W_{disp}(r)$ is the dispersion potential of Hamaker, $W_{osmotic}(r)$ is an attractive interaction due to the excluded-volume effect of the salt ions, and $W_{specific}(r)$ is an attractive potential between proteins included to represent any specific chemical effects such as hydrophobic interactions.

The electric double-layer repulsion between two proteins is derived from Debye-Hückel theory [Verwey and Overbeek, 1948]:

$$W_{elec}(r)=\frac{(z_2 e)^2 \exp[-\kappa(r-\sigma_p)]}{4\pi\varepsilon_o\varepsilon_r(1+\kappa\sigma_p/2)^2} \quad \text{for } r>(\sigma_p+2\Delta r) \quad (2)$$

Where z_2 is the valence of the protein, e is the unit of electron charge, $4\pi\varepsilon_o$ is the dielectric permittivity of free space, σ_p is the hard-sphere diameter, ε_r is the relative dielectric permittivity of water, and Δr is the effective-sphere hydration/stern layer. κ is the inverse of the Debye length; given by $K^2=(2e^2N_AI)/(kT\varepsilon_0\varepsilon)$, where N_A is Avogadro's number, and I is the ionic strength of the salt, given by $I=(z_{an}^2\rho_{an}+z_{cat}^2\rho_{cat})/2$, where z_{an} and z_{cat} are the anion and cation valences, respectively, and ρ_{an} and ρ_{cat} are the ionic number densities.

The electric double-layer repulsion is small at high salt concentration due to the screening effect.

The attractive Hamaker dispersion interaction is given by Hamaker[11], Verwey and Overbeek [1948]:

$$W_{disp}(r)=-\frac{H}{6}\left[\frac{\sigma_p^2}{r^2}+\frac{\sigma_p^2}{r^2-\sigma_p^2}+2\ln\left(1-\frac{\sigma_p^2}{r^2}\right)\right] \quad \text{for } r>\sigma_p+2\Delta r \quad (3)$$

where H is the effective Hamaker constant for the protein-protein interaction. Hamaker constants depend on the composition and the density of the protein [Israelachvlili, 1985], and on the chemical nature of the solute [Nir, 1976].

In general, since most proteins have similar densities and compositions, they have similar Hamaker constants.

In concentrated electrolyte solutions, ions occupy a significant fraction of the total solution volume. Protein molecules are so close together that ions are excluded from region between the protein particles. It causes an imbalance in the local osmotic pressure exerted by the ions on the proteins. The osmotic pressure difference is approximated by the ideal osmotic pressure of the bulk solution [$\Pi_{id}=\rho_s k_B T$]. The resulting potential between the proteins, expressed simply by,

$$W_{osmotic}(r)=-\frac{4}{3}\pi\sigma_{ps}^3(\rho_s k_B T)\left[1-\frac{3r}{4\sigma_{ps}}+\frac{r^3}{16\sigma_{ps}^3}\right] \quad \text{for } \sigma_p<r<2\sigma_{ps}$$
$$=0 \quad \text{for } r\geq 2\sigma_{ps} \quad (4)$$

where ρ_s is the total ionic number density, $\sigma_{ps}=(\sigma_p+\sigma_{ion})/2$ and $\sigma_{ion}=(z_{an}\sigma_{cat}+z_{cat}\sigma_{an})/(z_{cat}+z_{an})$ is a valence-weighted ion diameter; here the absolute values of the valences are used.

The specific interaction can be represented by a site-specific square-well potential [Kuehner et al., 1996, 1997]. This interaction includes identity and hydrophobicity of surface amino acid residues, surface roughness, etc.

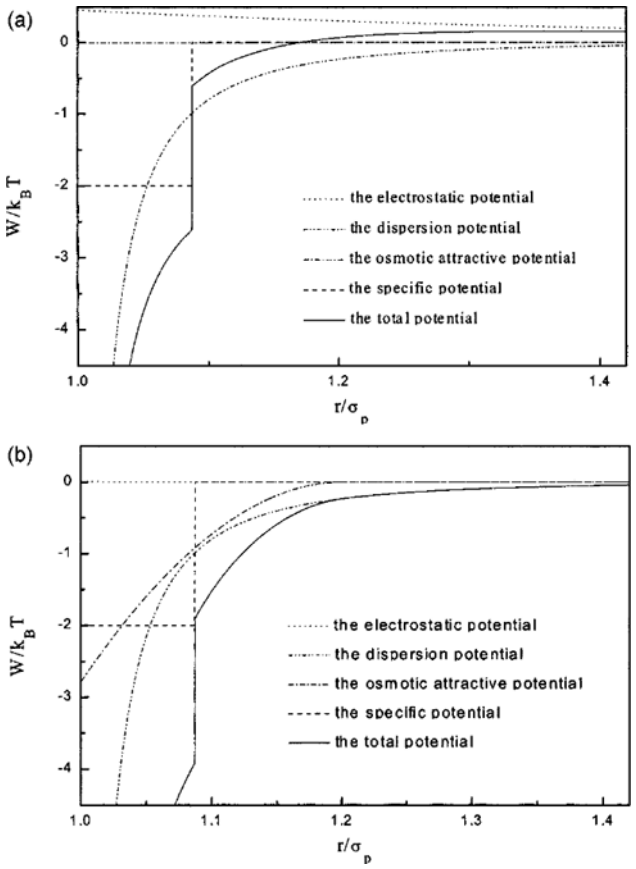

Fig. 1. Contribution to the total potential of mean force as a function of r/σ, in the case of I=0.01 M (a), and I=5 M (b): H/k$_B$T=7, ε$_{sp}$/k$_B$T=2, δ=3 Å, σ$_{ion}$=6.94 Å, pH=4, σ$_p$=34.3 Å, Δr=0.08 Å.

$$W_{specific}(r)=-\varepsilon_{sp} \quad \text{for } \sigma_p<r<(\sigma_p+\delta)$$
$$=0 \quad \text{for } r>(\sigma_p+\delta) \quad (5)$$

where ε_{sp} and δ are model parameters. For example, for hydrophobic interactions between proteins, $\varepsilon_{sp}/kT \sim 5$ provides a reasonable upper bound [Tanford, 1980].

Fig. 1 shows a representative overall protein-protein perturbation potential of mean force, when a) the ionic strength is 0.01 M, b) the electric repulsive potential is significantly larger than that of the ionic strength=5 M. It means that at high ionic strength, the electric double-layer potential is negligible.

2. Equation of State

Perturbation theory is a method, based on statistical mechanics, for predicting thermodynamic properties of the system. In perturbation theory, an assembly of hard spheres is used as the reference system, while the remaining interactions are treated as perturbations;

$$\frac{P}{\rho k_B T}=\left(\frac{P}{\rho k_B T}\right)_{ref}+\left(\frac{P}{\rho k_B T}\right)_{pert}=\left(\frac{P}{\rho k_B T}\right)_{ref}+\frac{\omega_{PA}^2\rho U}{2k_B T} \quad (6)$$

where ρ is the density of protein molecules, P is the pressure, ω_{PA} is the average degree of pre-aggregation, and U is the perturbation energy per unit density, given by,

$$U=4\pi\int W_{pp}(r)r^2 dr \quad (7)$$

where $W_{pp}(r)$ is the sum of the potentials-of-mean-force.

The reference system is given by the modified Chiew's equation [Chiew, 1990; Song et al., 1994]:

$$\left(\frac{P}{\rho k_B T}\right)_{ref} = 1 + 4\omega_{PA}^2\eta\frac{1-\frac{\eta}{2}}{(1-\eta)^3} - (\omega_{PA}-1)\left[\frac{1-\frac{\eta}{2}}{(1-\eta)^3} - 1\right] \quad (8)$$

$$\left(\frac{P}{\rho k_B T}\right) = 1 + 4\omega_{PA}^2\eta\frac{1-\frac{\eta}{2}}{(1-\eta)^3} - (\omega_{PA}-1)\left[\frac{1-\frac{\eta}{2}}{(1-\eta)^3} - 1\right] + \frac{\omega_{PA}^2\rho U}{2k_B T} \quad (9)$$

The general equation for calculating the Helmholtz energy from a pressure-explicit equation of state [Prausnitz, 1986] is

$$A(T,V) = A^{\circ}(T) + \int_V^{\infty}\left(P - \frac{Nk_B T}{V}\right)dV + k_B T N \ln\left(\frac{Nk_B T}{V}\right) \quad (10)$$

$$\frac{A}{N\omega_{PA}k_B T} = \frac{A^{\circ}}{N\omega_{PA}k_B T} + \int_0^{\rho\omega_{PA}}\left(\frac{P}{\rho\omega_{PA}k_B T} - \frac{1}{\omega_{PA}}\right)\frac{d(\rho\omega_{PA})}{\rho\omega_{PA}} + \ln(\rho\omega_{PA}k_B T) \quad (11)$$

The chemical potential is

$$\mu = \left(\frac{\partial A}{\partial N}\right)_{T,V} \quad (12)$$

and

$$\frac{\Delta\mu}{k_B T} = \frac{\mu}{k_B T} - \frac{\mu^{\circ}}{k_B T} = \left(\frac{\Delta\mu}{k_B T}\right)_{ref} + \left(\frac{\Delta\mu}{k_B T}\right)_{pert} = 8\omega_{PA}\eta\frac{4-3\eta}{4(1-\eta)^2} + 4\omega_{PA}\eta^2\frac{5-3\eta}{4(1-\eta)^3} + (\omega_{PA}-1)\ln(1-\eta) - (\omega_{PA}-1)\frac{5-4\eta}{4(1-\eta)^2} - (\omega_{PA}-1)\frac{2\eta^3-6\eta^2+5\eta}{2(1-\eta)^3} + \ln\rho + 1 + \frac{\omega_{PA}^2\rho U}{k_B T} \quad (13)$$

where η is the packing fraction, ω_{PA} represents the average degree of self-aggregation, ρ is the total protein number density, and energy per unit density U given by Eq. (7).

At equilibrium, protein concentrations in the supernatant and dense-fluid phases are calculated from Eqs. (8) and (12) based on the classical equilibrium conditions:

$$\Delta\mu^s = \Delta\mu^d \quad (14)$$

$$P^s = P^d \quad (15)$$

where superscripts "s" and "d" denote the supernatant and dense phases, respectively.

RESULT AND DISCUSSION

For the precipitation of a single protein in an aqueous salt solution, we examine the effect of protein size in phase-separation systems. The partition coefficient, K, of the protein system can be obtained from the equilibrium conditions and is given by the ratio of the equilibrium number density of protein in the dense phase to that in the supernatant phase [$K = \rho_d/\rho_s = \eta_d/\eta_s$].

Fig. 2 shows the predicted partition coefficient K plotted as a function of ionic strength for systems with $H/k_BT=7$, $\varepsilon_{sp}/k_BT=2$, $\delta=3$ Å, $\sigma_{ion}=6.94$ Å, pH=4, $\sigma_p=34.3$ Å, $\Delta r=0.08$ Å, for various values of ω_{PA}. The partition coefficient, K, increases exponentially with the ionic strength. This dependence is commonly observed feature in salting-out not only for proteins but also for other organic substances and dissolved gases [Dixon et al., 1943]. The exponential form has been used extensively in correlating protein salting-out data [Shih et al., 1992; Coen et al., 1995; Cohn et al., 1943].

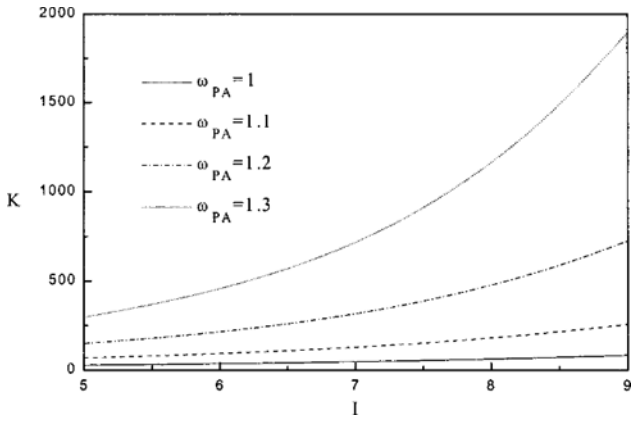

Fig. 2. Effect of ionic strength on protein partitioning: $H/k_BT=7$, $\varepsilon_{sp}/k_BT=2$, $\delta=3$ Å, $\sigma_{ion}=6.94$ Å, pH=4, $\sigma_p=34.3$ Å, $\Delta r=0.08$ Å.

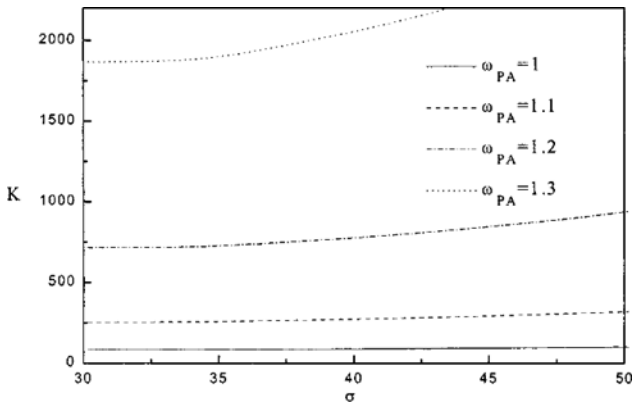

Fig. 3. Effect of protein diameter on partitioning: $H/k_BT=7$, $\varepsilon_{sp}/k_BT=2$, $\delta=3$ Å, $\sigma_{ion}=6.94$ Å, pH=4, $\sigma_p=34.3$ Å, $\Delta r=0.08$ Å.

In the same aqueous solution, large solute molecules partition

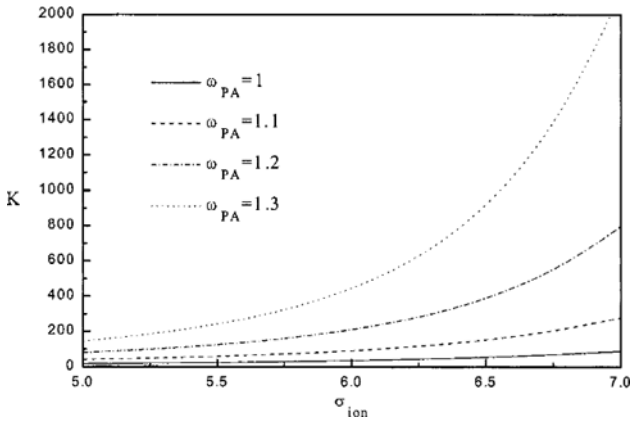

Fig. 4. Effect of ion diameter on partitioning: $H/k_BT=7$, $\varepsilon_{sp}/k_BT=1.5$, $\delta=3$ Å, $\sigma_{ion}=6.94$ Å, pH=4, $\sigma_p=34.3$ Å, $\Delta r=0.08$ Å.

more strongly than those of small molecules [Albertsson, 1986]. Fig. 3, K plotted as a function of the protein diameter, shows that the partition coefficient increases strongly with the size of the protein; large particles separate more efficiently.

Fig. 4, computed partition coefficient, shows a strong dependence on the mean ion diameter, σ_s. For large mean ion diameter, the effective length scale for osmotic attraction increases due to a large region between proteins for ion exclusion. At high ionic strength, the osmotic pressure is sufficiently large so that the osmotic attraction term produces attraction strong enough to yield very high partition coefficients.

Coen et al. [1995] have conducted precipitation experiments for two small globular proteins, hen-egg-white lysozyme and α-chymotrypsin in solutions of ammonium sulfate at various ionic strengths and pH. Fig. 5 shows experimental and calculated values of $C_{p,super}$ - the equilirium protein concentration in the supernatant phase - and K as a function of ionic strength for the hen-egg-white lysozyme (at pH 4). Fig. 6 represents the α-chymotrypsin data (at pH 8.3) for $C_{p,super}$ and K as a function of ionic strength. In those calculation, Hamaker constant and the value of, Δr, the thickness of the hydration/stern layer were 8.9 and 0.8, respectively. These values are coincident with values reported by Kuhner et al. [1997], who indicated that Hamaker constant depends on the value of the thickness

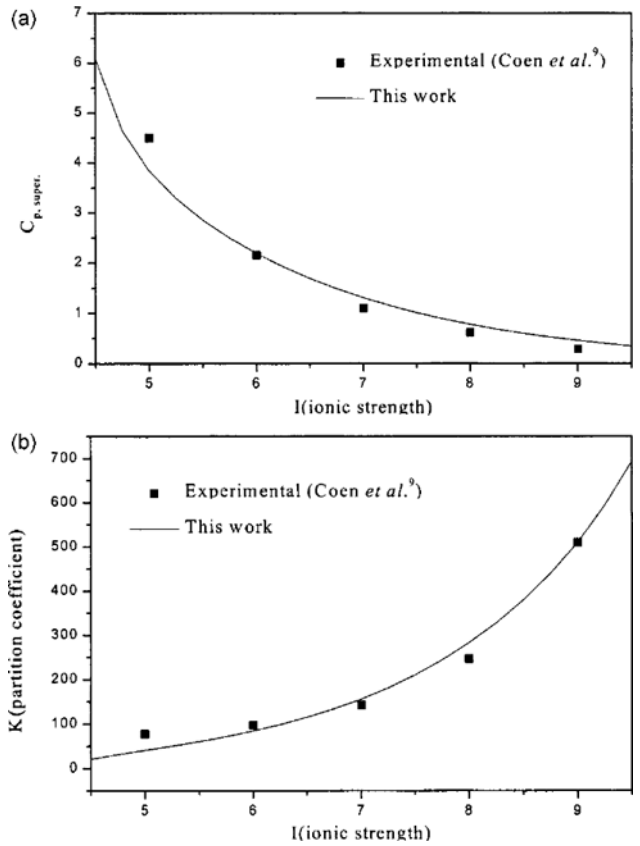

Fig. 5. Experimental and correlated values of $C_{p,super}$ (a) and K (b) in the case of hen-egg-white lysozyme in ammonium sulfate at pH 4: H/k_BT=8.9, Δr=0.8 Å, ε_{sp}/k_BT=3.7, δ=4 Å, σ_{ion}= 6.94 Å, σ_p=34.3 Å, ω_{PA}=1.4. Dark squares are experimental data from Coen et al. and the solid lines are calculated values using the proposed model.

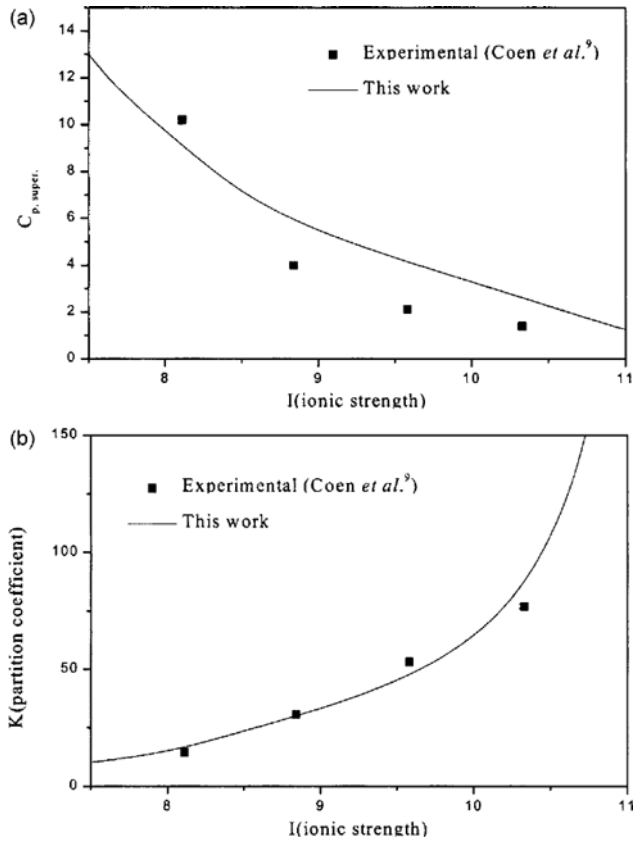

Fig. 6. Experimental and correlated values of $C_{p,super}$ (a) and K (b) in the case of α-chymotrypsin in ammonium sulfate at pH 8.3: H/k_BT=8.9, Δr=0.8 Å, ε_{sp}/k_BT=3.3, δ=3 Å, σ_{ion}=6.94 Å, σ_p=43.3 Å, ω_{PA}=1.25. Dark squares are experimental data from Coen et al. and the solid lines are calculated values using the proposed model.

of the hydration/stern layer. As shown in Fig. 5, calculated equilibrium coefficient and supernatant concentration are in qualitative agreement with experimental results of hen-egg-white lysozyme for ω_{PA}=1.4, ε_{PA}/k_BT=3.7 and δ=4 Å. If the value of ω_{PA}=1.4 is considered, 40% of lysozyme is pre-aggregated before the partition is processed. In Fig. 6, our proposed model also agrees very well with α-chymotrypsin experimental data for ω_{PA}=1.25, ε_{PA}/k_BT=3.3 and δ=3 Å. Considering ω_{PA}=1.25, it implies that 25% of the α-chymotrypsin is pre-aggregated before the partition process. Comparing ω_{PA} values for two model proteins presented in this study indicates that the effect of the specific interaction is more strong in lysozyme solution than in α-chymotrypsin solution.

CONCLUSION

We proposed an approximate equation-of-state model for the salt-induced protein precipitation based on effective potentials of mean force. Thermodynamic properties of the system are developed using a statistical mechanical perturbation theory and the reference term is derived from modified Chiew's equation. Model calculations indicate that the electrolyte concentration plays a primary role in affecting phase separation; the protein partition coefficient, K, increases exponentially with the ionic strength. Partitioning is also

strongly dependent on the protein and ion diameters. Furthermore, our theoretical calculation results show that the pre-aggregation effect of protein plays an important role in the precipitation of proteins. Calculated equilibrium supernatant concentration and partition coefficient are in qualitative agreement with experimental results for both hen-egg-white lysozyme and α-chymotrypsin in solutions of ammonium sulfate when the effect of the pre-aggregation is considered.

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