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Abstract

Protein salting-out phase equilibria are reported for lysozyme and α-chymotrypsin in concentrated ammonium sulfate solutions. Supernatant and dense-phase protein concentrations and the resulting protein partition coefficients are reported as a function of solution pH and ionic strength. Phase equilibria with a trivalent salt (sodium citrate) suggest that ionic strength is the appropriate primary variable to describe phase equilibria. For an aqueous mixture containing both lysozyme and α-chymotrypsin, selective precipitation of one protein exhibits phase equilibria similar to those obtained from single-protein measurements.

Osmotic second-virial coefficients from low-angle laser-light scattering (LALLS) are reported over a range of pH for dilute chymotrypsin concentrations in aqueous electrolyte solutions containing potassium sulfate or sodium phosphate at ionic strengths 0.01 M and 1.0 M. Hamaker constants regressed from experimental osmotic second-virial coefficients are used to obtain protein-protein potential-of-mean-force models. In addition to DLVO potentials, protein-protein interactions are modeled using attractive protein-dipole potentials and an osmotic attraction potential that becomes important at high salt concentrations.

Introduction

Separation and recovery of a protein from aqueous solution is an increasingly important concern in biotechnology as proteins are produced on larger scales. The purification of therapeutic proteins is crucial to the approval and performance of a drug. Noteworthy reviews of protein purification are by Scopes (1994) and by Wheelwright (1991). Protein salting-out remains one of the simplest methods for crude protein separations since it was employed to separate blood proteins into distinct fractions in the mid 1850's (Green, 1931).

To date, most studies have focused on simple systems containing only one protein, water and salt. A commonly-used correlation for protein salting-out data (Cohn and Edsall, 1943) is

$$\log S = \beta - K_s I \qquad (1)$$

where S is the protein solubility, I is the ionic strength, and β and K_S are empirical parameters fit to experimental supernatant-phase protein-concentration data as a function of ionic strength. Melander and Horváth (1977) modeled a protein in solution using electrostatic repulsion and the energy of forming a cavity in the solvent for the protein. While this model includes effects of salt type (e.g. the lyotropic series) and the hydrophobic surface area of the protein, it has not been satisfactory for predicting experimental phase equilibria due to the presence of additional intermolecular forces not included in the model (Przybycien and Bailey, 1989). These and other studies, both experimental and theoretical (e.g. Bell et al., 1983; Arakawa and Timasheff, 1985), have represented precipitation phase equilibrium as a saturated protein solution in equilibrium with a pure protein solid phase. Recent experiments have shown that this representation is not realistic; significant amounts of water, protein and salt are present in both phases (Taratuta et al., 1990; Shih et al., 1992). Thus, it may be preferable to represent salting-out of proteins as a liquid-liquid phase transition represented by the protein partition coefficient (Shih et al., 1992):

$$K_2 = \frac{c_{2,dp}}{c_{2,sp}} \qquad (2)$$

where $c_{2,dp}$ is the concentration of the protein in the dense phase and $c_{2,sp}$ is the concentration of the protein in the supernatant phase.

For the purification of proteins it is useful to predict the selectivity of a salting-out process for a target protein. Richardson et al., (1990) developed an empirical method to salt out selectively alcohol dehydrogenase from yeast extract. They generalized this procedure, showing how to estimate the maximum achievable selectivity by salting-out. An excellent overall review of selective precipitation is given by Niederauer and Glatz (1992). Both of these studies indicate the empirical nature of selective salting-out separations, requiring extensive salting-out experiments to optimize the selective precipitation of a target protein.

A comprehensive description of protein salting-out thermodynamics requires an understanding of intermolecular forces in solution. Colloid solution theory forms the basis for several models of protein solution thermodynamics (Vilker et al., 1981; Mahadevan and Hall, 1990; Haynes et al., 1992; Mahadevan and Hall, 1992a; Mahadevan and Hall, 1992b; Vlachy and Prausnitz, 1992; Vlachy et al., 1993). The DLVO model (Verwey and Overbeek, 1948) describes the interactions of spherical colloids with uniform surface properties, with attractive van der Waals forces and repulsive coulombic forces in a continuum solvent where salt ions are considered solely through electrostatic screening. The DLVO model assumes the pairwise additivity of the potentials of mean force:

$$W_{DLVO}(r) = W_{disp}(r) + W_{q\text{-}q}(r) \qquad r > d_2 \qquad (3)$$

where r is the center-to-center distance between spherical molecules, d_2 is the hard-sphere diameter, $W_{disp}(r)$ is the dispersion potential of Hamaker and $W_{q\text{-}q}(r)$ is the repulsive charge-charge potential of mean force. The DLVO potential can predict protein-solution osmotic pressures up to high protein concentrations at low salt concentrations (Vlachy and Prausnitz, 1992). However, following Grimson (1983), Vlachy et al. (1993) observed that the DLVO model does not adequately describe protein phase equilibrium at high salt concentrations.

Vlachy et al. (1993) suggested use of the osmotic-attraction potential of Asakura and Oosawa (1954, 1958) to describe the effect of concentrated electrolytes on protein phase separation. The Asakura and Oosawa potential has been used with good success to model the phase separation of colloids and proteins resulting from the addition of non-adsorbing polymers (De Hek and Vrij, 1981; Gast et al., 1983; Mahadevan and Hall, 1990; Mahadevan and Hall, 1992a; Vlachy and Prausnitz, 1992; Vlachy et al., 1993). Here, the osmotic attraction of colloids is due to depletion of the polymer in the region between two colloidal particles, resulting in an osmotic attraction. Salting-out phase equilibria calculations using the osmotic attraction potential (Vlachy et al., 1993) yield calculated protein partition coefficients similar to those measured earlier (Shih et al., 1992).

Protein dipole moments, often on the order of several hundred Debye (Tanford, 1961), also contribute significant attractive protein-protein potentials (Vilker et al., 1981; Haynes et al., 1992). While such electrostatic interactions may be necessary to describe protein-protein interactions at low ionic strengths, they are less important at high salt concentrations where they are electrostatically screened (Phillies, 1974). These potentials are briefly discussed later.

The literature describes several other protein solution-interactions such as hydrophobic and specific protein-protein interactions (e.g. lysozyme: Bruzzesi et al., 1965; Banerjee et al., 1975; Wills et al., 1980; chymotrypsin: Egan et al., 1957; Aune and Timasheff, 1971). However, it is difficult to incorporate these interactions into a general model of protein-protein potentials. Pratt and Chandler (1977) and Lazaridis and Paulaitis (1992) have shown the complexity of modeling water interactions at hydrophobic surfaces by regarding water as a discrete component.

Experimental protein-protein osmotic second-virial coefficients measured by low-angle laser-light scattering (LALLS) and by membrane osmometry have been useful for investigating protein intermolecular potentials (Haynes et al., 1992; Haynes et al., 1993). Both experiments yield osmotic second-virial coefficients.

This work reports experimental salting-out phase equilibria for lysozyme and chymotrypsin single-protein solutions. Results are presented for protein partition coefficients as functions of ionic strength and pH. Salting-out phase equilibria for aqueous lysozyme-chymotrypsin mixtures are similar to those for the single-protein solutions. Progress toward a protein-interaction potential model requires parameters from low-angle laser-light scattering data. Such data are reported for chymotrypsin solutions over a range of pH at low and high ionic strengths.

Experimental

Materials

Bovine α-chymotrypsin (C-4129), hen-egg-white lysozyme (L-6876) and phenyl-methyl-sulfonyl fluoride (PMSF) were purchased from Sigma, St. Louis, MO. A.C.S. grade salts were used. Distilled water was de-ionized and filtered (0.20 μm) by a NANOpure system prior to use.

Methods

The following experiments were performed: 1) single and two-protein salting-out phase equilibria, 2) low-angle laser-light scattering (LALLS) and 3) UV spectrophotometer measurements of protein concentration. All experiments were carried out at 25±0.1°C.

Protein salting-out phase equilibria

The procedure of Shih et al. (1992) was followed for salting-out phase separations. Protein solutions were prepared by dissolution in pure water. To prevent autolysis, α-chymotrypsin solutions were inhibited according to the method of Fahrney and Gold (1963) with a 10% molar excess PMSF from a stock solution of 0.11 M PMSF in 2-propanol. Concentrated salt solution was added dropwise and pH was adjusted using dilute solutions of the appropriate conjugate acid or base (e.g. NH_4OH and H_2SO_4 for $(NH_4)_2SO_4$ salt experiments). Samples were equilibrated for 4 hours under mild agitation and then centrifuged in a Jouan CT422 temperature-controlled centrifuge for 2 hours at 5000g to separate the supernatant phase from the dense phase.

The two equilibrated phases were then separated and analyzed. Samples of the supernatant and dense phases were diluted and analyzed by UV absorbance (280 nm) to determine protein concentration. For ammonium sulfate, the salt concentration of each phase was measured by a titration method (Fritz and Schenk, 1974). Based on observed partitioning of ammonium sulfate and other salts, sodium citrate was assumed to partition uniformly between both phases. The water content of the dense phase was measured by weight loss after freeze drying for 40 hours at 40 mtorr. Error in protein concentration measurements was ± 5%.

Phase separation for mixtures of lysozyme and chymotrypsin was accomplished by the procedure stated above. Protein concentrations were determined by ion-exchange HPLC with UV detection at 280 nm. A Hewlett Packard Series II 1090 HPLC was employed with an HRLC MA7S, 1 mL capacity, cation exchange column (Bio-Rad, Richmond, CA). All elution buffers contained 0.02 M bis-tris propane buffer at pH 7.3 and 0.05% (w/v) sodium azide as a preservative. 0.108 M NaCl was employed to elute chymotrypsin and 0.5 M NaCl to elute lysozyme. To achieve linear detector response, dense-phase samples were diluted up to 100 fold. To remove excess salt which interferes with protein binding to the ion-exchange column, supernatant samples were passed through Bio-Spin 6 size-exclusion columns which contain Bio-Gel P polyacrylamide with a 6000 Dalton molecular weight cutoff (Bio-Rad, Richmond, CA) . Bio-Spin columns were centrifuged at 1100g for 3.5 min. Salt and water determinations were made as above.

Low-angle laser-light scattering (LALLS)

Static light-scattering experiments were conducted with a KMX-6 LALLS photometer from Chromatix/Milton Roy with a 2 mW helium-neon laser at 633 nm. Measurements were made using the 6-7° annulus and an average of Rayleigh ratios was taken from three field stops (0.3, 0.2 and 0.15 mm). Refractive indices were measured with a KMX-16 Laser Differential Refractometer (LDR) with a 0.5 mW helium-neon laser at 633 nm.

Stock solutions of α-chymotrypsin at 5 g/L were prepared and dialyzed overnight against the desired salt solution to remove the 2-propanol and excess PMSF and to maintain constant ionic strength and pH among all samples after diluting to the desired protein concentration with the dialysate. Dialysis tubing was from Spectropor (#132660) with a molecular-weight cutoff of 8000. pH was adjusted with the conjugate acid or base as necessary. No buffer was used with K_2SO_4 solutions, limiting the pH range. Ionic strengths of phosphate solutions were kept constant at different pH's by adjusting the salt concentration to account for differing amounts of $H_2PO_4^-$ and HPO_4^{-2} species. Ionic strengths were calculated using dilute solution pK_a's. Samples were filtered inline using a Millipore 0.22μm syringe-tip filter. Solutions were pumped through the light-scattering cell at 0.2 mL/min.

To determine osmotic second-virial coefficients using LALLS, measurements were made of the reduced Rayleigh ratio, $\overline{R}_\theta = (R_{\theta,solution} - R_{\theta,solvent})$ at several protein concentrations. The protein osmotic second-virial coefficient, B_2, and weight-average molecular weight $M_{w,2}$ were determined from

$$\frac{Kc_2}{\overline{R}_\theta} = \frac{1}{M_{w,2}} + 2B_2c_2 \qquad (4)$$

where c_2 is the protein concentration (g/mL) and K is the optical constant which depends on the refractive index of the solvent, n_0; the refractive-index increment with respect to protein concentration, dn/dc_2; and the wavelength of light, λ:

$$K = \frac{2\,\pi\, n_0^2\,(dn/dc_2)^2}{N_A\lambda^4} \qquad (5)$$

Since the solvent is taken to be the pseudosolvent of water and salt, the regressed second-virial coefficient is only valid for the particular solvent in which the experiment was conducted.

Analytical Methods

Lysozyme and chymotrypsin concentrations were determined by absorbance at 280 nm using a Milton-Roy 1201 Spectrophotometer. Extinction coefficients used were: 2.04 L/(g cm) for chymotrypsin (Fasman, 1989) and 2.635 L/(g cm) for lysozyme (Sophianopoulos et al., 1962).

pH was measured using a Corning General-Purpose Combination electrode with a Sargent-Welch model LSX pH meter.

Results and Discussion

Salting-out phase equilibria

Reversible phase equilibrium has been demonstrated by addition of either water or concentrated salt solution to solutions containing protein, salt and water which were previously phase separated. The resulting phase equilibria at the new salt concentrations were equivalent to those obtained previously. Because observed protein supernatant and dense-phase concentrations are independent of path, reversible phase equilibrium is achieved in our salting-out experiments; these results are similar to those obtained in our earlier studies (Shih et al., 1992).

Tables 1 and 2 show phase equilibria for lysozyme and chymotrypsin in aqueous ammonium-sulfate solutions over a range of pH's and ionic strengths. Experiments with ammonium sulfate were limited to pH's below 9 to prevent evolution of gaseous ammonia. Partitioning of salt between the dense and supernatant phases was insignificant for all experiments reported here.

Figure 1 shows protein partition coefficients (K_2) for lysozyme in ammonium sulfate as a function of pH and ionic strength. Initial protein concentrations (i.e. protein concentrations before phase separation) varied from 20-50 mg/g with no effect on lysozyme partitioning (Shih et

al., 1992). As ionic strength rises, larger partition coefficients are observed, as expected. The trend with respect to pH is less clear. From pH 6-8, K_2 is independent of ionic strength. However, at ionic strengths 5-7 m, K_2 increases steadily as pH approaches 4 whereas, at ionic strengths 8-9 m, K_2 decreases at pH 5. Shih et al. (1992) observed similar behavior for lysozyme supernatant concentrations for NaCl salting-out equilibria at low pH. In Shih's work, the supernatant concentrations are higher at pH 5 confirming the lower partition coefficients at pH 5 observed here. Shih suggested that binding of anions to the highly-charged proteins at low pH causes an increase of partitioning to the dense phase. This increased partitioning may occur due to both a decrease in effective protein charge and an increase in apparent protein size. It is well known that, under the same conditions, protein solubility is roughly inversely proportional to protein size. The cause of the drop in K_2 at pH 5 is not clear.

Figure 2 shows protein partition coefficients for chymotrypsin in aqueous ammonium sulfate as a function of pH and ionic strength. The initial protein concentration was 30 mg/g water. Data at pH 8.3 in Figure 2 were interpolated from data in Table 2. As ionic strength rises, larger partition coefficients are observed, as expected. Chymotrypsin partitioning behavior is similar to lysozyme partitioning as a function of pH. Increased partitioning at low pH may also be explained through anion binding as for lysozyme. At its isoelectric point (pI=8.3), the net protein charge is zero; therefore, a minimum in partitioning might be expected as the coulombic repulsions are reduced. However, chymotrypsin's minimum supernatant concentration (often referred to as solubility) is not a minimum at pH 8.3. Protein partition coefficients further indicate that chymotrypsin is more likely to partition to the dense phase at low pH than at its isoelectric point.

To explore the effect of a trivalent anion in salting out of proteins, chymotrypsin was also salted out with sodium citrate. Experiments were carried out at pH 9 where citrate is trivalent. Figure 3 shows that the partition coefficients for chymotrypsin with sodium citrate (pH 9) are similar to those with ammonium sulfate (pH 8.3, limited by ammonia evolution) when plotted on

an ionic strength basis (Figure 3A) but they differ significantly when plotted as a function of salt concentration (Figure 3B). Figure 3 suggests that ionic strength (rather than salt concentration) may be the appropriate primary variable even when the salt concentration is large.

Two-Protein Phase Equilibrium Measurements

Initial experiments were performed to measure phase equilibria in concentrated aqueous salt systems with two proteins. Figure 4 shows partition coefficients for lysozyme and for chymotrypsin with ammonium sulfate at pH 7 as a function of ionic strength. Partition coefficients are also shown for single-protein phase equilibria obtained under the same solution conditions (filled symbols). Initial protein concentrations were 20 mg/g water for both proteins. The curvature observed in the protein-mixture partition coefficients (empty symbols) at high ionic strength can be attributed to the inability of the Bio-Spin columns to remove all of the salt from the supernatant solutions, causing the protein concentration analysis to be less accurate at the highest ionic strengths.

Figure 4 shows that the two-protein equilibria are almost identical to the single-protein equilibria. At pH 7, both lysozyme and chymotrypsin are positively charged and therefore little lysozyme-chymotrypsin interaction is expected. However, in an aqueous two-protein mixture, partition coefficients for hemoglobin and for lysozyme at pH 7 show the same correspondence to the single-protein data (data not shown), although, in this mixture the protein charges are of opposite sign.

LALLS Measurements to Determine Intermolecular Potentials of Mean Force

LALLS measurements were performed for dilute chymotrypsin solutions. Measurements were made in aqueous sodium phosphate and potassium sulfate solutions at I=0.01 M and I=1.0 M over a range of pH's. Results are shown in Table 3. Figure 5 shows experimental osmotic second-virial coefficients, determined from Eq. 4, plotted as a function of pH with ionic

strength as a parameter. Since no buffer was used, the accessible pH range for K_2SO_4 solutions was limited by carbon dioxide dissolution.

Second virial coefficients for 1.0 M ionic-strength solutions, represented by triangles, are relatively constant with respect to protein charge, probably because of effective screening of electrostatic forces. As expected, these virial coefficients are negative, indicating attractive forces that are not pH-dependent, e.g. dispersion forces which are independent of pH.

On the other hand, virial coefficients for the 0.01 M ionic-strength solutions, represented by circles, fall with increasing pH. The net charge on α-chymotrypsin is positive at low pH and approaches zero at pH 8.3. Consequently, the coulombic repulsion, which is not well screened at I=0.01 M, decreases with rising pH. At pH 3, coulombic forces are strongest, producing a positive (repulsive) experimental second-virial coefficient. Attractive forces dominate at higher pH's, yielding negative second-virial coefficients. The negative (attractive) second-virial coefficients at higher pH's suggest the presence of electrostatically-screened attractive forces since they are not observed at I=1.0 M. As suggested in previous studies (Vilker et al., 1981; Haynes et al., 1993), these forces are likely to be dipole forces which are both attractive and electrostatically screened (Phillies, 1974). The contribution of these dipole potentials to the osmotic second-virial coefficients is discussed in the following section.

Determination of the Effective Hamaker Constant

Experimental osmotic second-virial coefficients may be used to study the potentials of mean force for aqueous protein-protein interactions. The Hamaker constant (H), considered here to be an adjustable parameter, can be determined using Eq. 6 and a potential-of-mean-force expression.

Osmotic second-virial coefficients obtained by LALLS are in the Lewis-Randall framework but Eq. 6 is in the McMillan-Mayer framework (Hill, 1959; Cabezas and O'Connell, 1993). However, calculations based on the method of Hill and also those based on the method by

Cabezas, show that the conversion of LALLS data to the McMillan-Mayer framework changes the osmotic second-virial coefficients by less than 2% which is within experimental error.

The following equation relates the osmotic second-virial coefficient, B_2, to the potential of mean force, W:

$$B_2\left(a_1^0, T\right) = B_2^{hs} - \frac{N_A}{2}\int_{d_2+3\,\text{Å}}^{\infty} \left[\exp\left(-W(r, a_1^0, T)/kT\right) - 1\right] 4\pi r^2\, dr \qquad (6)$$

where a_1^0 is the activity of pure solvent, $B_2^{hs} = \frac{2\pi d_2^3}{3}$ is the hard-sphere contribution to the second-virial coefficient and d_2 is the protein diameter. As suggested by Vilker et al. (1981), the lower integration limit is taken as $d_2 + 3$ Å. For Eq. 6, the potential-of-mean-force expressions are first given by Eq. 3 (DLVO); second, by an expression including charge-dipole, $W_{q\text{-}\mu}$, and dipole-dipole, $W_{\mu\text{-}\mu}$, interactions

$$W(r) = W_{disp}(r) + W_{q\text{-}q}(r) + W_{q\text{-}\mu}(r) + W_{\mu\text{-}\mu}(r); \qquad (7)$$

and a third by an expression (Eq. 7 plus $W_{OA}(r)$) including osmotic attraction. Table 4 gives expressions for the various potentials of mean force.

Figure 6 shows Hamaker constants regressed for the three potential-of-mean-force models using LALLS experimental osmotic second-virial coefficients for chymotrypsin in I=1.0 M K_2SO_4. The following parameters were used in the calculations: T=298 K, d_2=43.4 Å (Stryer, 1988), mean ionic diameter d_3=5 Å, z_2(pH) and μ(pH) from Haynes et al. (1992). Results using the DLVO potential (Eq. 3) show that the Hamaker constants are pH dependent. Hamaker constants should be constant with respect to solution conditions provided the conformation of chymotrypsin does not change significantly. Hence, it appears that additional electrostatic forces are present. Further, these regressed Hamaker constants are an order of magnitude larger than those calculated for bovine serum albumin (1-2 kT) (Nir, 1976). These larger Hamaker constants

compensate for the observed attractive behavior that is otherwise not accounted for in the DLVO model.

Dipole-dipole and charge-dipole potentials are introduced (Eq. 7) to compensate for the limitations of the DLVO model at low ionic strength. These dipole forces are attractive and pH-dependent because chymotrypsin's dipole moment is a strong function of pH. Figure 6 shows that the addition of dipole forces significantly reduces the observed Hamaker constants but does not alter their pH dependence. Values of the Hamaker constant at pH 3 and 4 are greater than those calculated by Nir while values of the Hamaker constant at higher pH are smaller than the calculated values.

Inclusion of the osmotic attraction potential (Eq. 7 plus $W_{OA}(r)$) does not significantly affect the regressed Hamaker constants because the salt concentration is only 0.33 M, not large enough to observe significant osmotic attraction. Osmotic second-virial coefficients at significantly higher salt concentrations are needed to study the osmotic-attraction potential.

The larger regressed Hamaker constants at low pH indicate the presence of pH-dependent attractive forces not accounted for in any of the potential-of-mean-force expressions. These attractive forces observed at low pH with LALLS, may also be responsible for the greater partitioning observed at low pH in the experimental phase equilibria presented in Figures 1 and 2. Regression of model parameters, such as the Hamaker constant, from experimental osmotic second-virial coefficients permit empirical modeling of intermolecular forces that are not explicitly identified.

Conclusions

Salting-out phase equilibrium measurements are reported for lysozyme and for chymotrypsin in concentrated ammonium-sulfate solutions. Protein partition coefficients indicate that pH-dependent interactions are present at high ionic strength. For the systems studied here, two-protein partition coefficients show behavior similar to that for one-protein

partition coefficients. Osmotic second-virial coefficients measured by LALLS for chymotrypsin solutions are reported and used to examine expressions for the intermolecular potential of mean force. Results indicate that pH-dependent interactions are present beyond those accounted for with charge and dipole protein-protein potentials. Effective Hamaker constants regressed from osmotic second-virial coefficients obtained from LALLS provide a method for empirically incorporating potentials not included in the model.

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Notation

B_2	osmotic second-virial coefficient, mL mol/g^2
c_2	protein concentration, g/mL
$c_{2,dp}$	protein concentration in dense phase, mg/g water
$c_{2,sp}$	protein concentration in supernatant phase, mg/g water
d_i	hard-sphere diameter of species i, Å
dn/dc_2	protein specific refractive-index increment, mL/g
e	electron charge, 1.602×10^{-19} C
H	Hamaker's constant, J
I	Ionic strength, m (moles/kg water) or M (moles/L)
k	Boltzmann's constant, J/K
K	optical constant, cm^2 mol/g^2
K_2	protein partition coefficient

K_s	salting-out parameter
$M_{w,2}$	protein molecular weight, g/mol
N_A	Avagadro's number, mol^{-1}
n_0	refractive index of solvent
r	radial distance, Å
$\overline{R}_\theta$	reduced Rayleigh ratio, cm^2/mL
S	solubility
T	temperature, K
W_{disp}	dispersion potential of mean force, J
W_{DLVO}	DLVO potential of mean force, J
W_{OA}	osmotic attraction potential of mean force, J
$W_{q\text{-}q}$	charge-charge potential of mean force, J
$W_{q\text{-}\mu}$	charge-dipole potential of mean force, J
$W_{\mu\text{-}\mu}$	dipole-dipole potential of mean force, J
z_2	net protein valence

Greek Symbols

β	salting-out parameter
ε	absolute permittivity, $C^2\ J^{-1}\ m^{-1}$
ε_0	vacuum permittivity, $C^2\ J^{-1}\ m^{-1}$
ε_r	relative permittivity
ε_s	permittivity at protein surface $C^2\ J^{-1}\ m^{-1}$
κ^{-1}	Debye screening length, Å
λ	wavelength of light, cm
μ	protein dipole moment, C Å (C Å = 3.336×10^{-20} Debye)

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Table Captions

Table 1 - Lysozyme phase equilibria measurements in ammonium-sulfate solutions.

Table 2 - Chymotrypsin phase equilibria measurements in ammonium-sulfate solutions.

Table 3 - LALLS osmotic second virial coefficients for chymotrypsin in salt solutions.

Table 4 - Contributions to the potential of mean force for proteins in aqueous electrolyte solutions, $W_{p\text{-}p}(r)^{\dagger}$.

†Definition of symbols: disp = dispersion, q-q = charge-charge, q-μ = charge-dipole, μ-μ = dipole-dipole and OA = osmotic attraction potentials. H = Hamaker constant, r = radial distance, d_2 = protein diameter, d_3 is the mean ionic salt diameter, $d_{23}=(d_2+d_3)/2$, ρ_3 = ion number density, z_2 = net protein charge, μ = protein dipole moment, $\zeta_{i\text{-}j}(r)$ = screening factor, κ^{-1} = Debye screening length, e = electron charge, $\varepsilon = 4\,\pi\,\varepsilon_0\,\varepsilon_r$ where ε_0 = vacuum permittivity and ε_r = relative permittivity, ε_s = permittivity at protein surface ($\varepsilon_s/\varepsilon = 4$ (Phillies, 1974)), k = Boltzmann's constant, and T = temperature. An earlier version of this table was presented by Vilker et al. (1981). A similar table (Haynes et al., 1992) had a misprint in the $W_{disp}(r)$ equation. References: (1) Verwey and Overbeek, 1948 (2) Haynes et al., 1992 (3) Vlachy et al., 1993.

Table 1 - Lysozyme phase equilibrium measurements in ammonium-sulfate solutions.

I (m)	$c_{2,sp}$ (mg/g water)	$c_{2,dp}$ (mg/g water)	K_2
pH 4			
5	4.50	352	78.2
6	2.39	234	98.0
7	1.25	179	143
8	0.67	97.1	145
9	0.29	69.1	238
pH 5			
5	24.0	147	6.13
6	7.90	141	17.8
7	3.30	178	53.9
8	1.27	89.4	70.4
9	0.42	53.9	128
pH 6			
5	44.9	284	6.33
6	12.9	154	11.9
7	4.23	146	34.5
8	1.52	132	86.8
9	0.63	134	213
pH 7			
5	38.4	254	6.61
6	12.1	159	13.1
7	4.04	140	34.7
8	1.43	121	84.6
9	0.50	104	208
pH 8			
5	34.5	218	6.32
6	11.9	161	13.5
7	3.63	127	35.0
8	1.27	109	85.8
9	0.46	107	233

Table 2 - Chymotrypsin phase equilibrium measurements in ammonium-sulfate solutions.

I (m)	$c_{2,sp}$ (mg/g water)	$c_{2,dp}$ (mg/g water)	K_2
pH 4			
7.5	14.1	202	14.3
8.1	5.15	183	35.5
8.7	2.35	155	66.0
9.3	1.16	147	127
9.9	0.67	156	233
pH 5.5			
7.5	26.0	219	8.42
8.1	10.4	133	12.8
8.7	4.12	142	34.5
9.3	2.18	148	67.9
9.9	1.21	151	125
pH 7			
7.5	23.3	281	12.1
8.1	9.01	185	20.5
8.7	4.05	159	39.3
9.3	2.09	154	73.7
9.9	1.28	144	112
pH 8.3			
8.11	10.2	148	14.5
8.84	3.98	123	30.9
9.58	2.11	113	53.6
10.33	1.39	108	77.7

Table 3 - Osmotic second-virial coefficients obtained by LALLS for chymotrypsin in salt solutions.

pH	$B_2 \times 10^4$ (mL mol/g^2)	M_w
I=0.01 M Sodium Phosphates		
4.2	3.64	25100
4.9	-1.45	30000
6	-6.67	40700
7	-15.1	44100
8.3	-34.3	55700
I=1.0 M Sodium Phosphates		
5	-3.08	28700
7	-1.43	24900
8.3	-1.10	31300
I=0.01 M Potassium Sulfate		
3	3.27	29500
4	0.692	26400
5	-5.50	28700
5.8	-10.6	33700
I=1.0 M Potassium Sulfate		
3	-1.21	30600
4	-1.23	28400
5.2	-3.37	28300
6.6	-1.66	27100

Table 4 - Contributions to the potential of mean force for proteins in aqueous electrolyte solutions, $W_{p\text{-}p}(r)^{\dagger}$.

Potential, W(r)	Screening Parameter, $\zeta(r)$	Ref.
$W_{disp}(r) = -\frac{H}{12}\left[\frac{d_2^2}{r^2} + \frac{d_2^2}{r^2 - d_2^2} + 2\ln\left(1 - \frac{d_2^2}{r^2}\right)\right]$		(1)
$W_{q\text{-}q}(r) = \frac{(z_2 e)^2 \zeta_{q\text{-}q}(r)}{\varepsilon r}$	$\zeta_{q\text{-}q}(r) = \frac{\exp[-\kappa(r - d_2)]}{(1 + \kappa d_2/2)^2}$	(1)
$W_{q\text{-}\mu}(r) = -\frac{2}{3}\frac{(z_2 e)^2 \mu^2 \zeta_{q\text{-}\mu}(r)}{\varepsilon^2 kT r^4}$	$\zeta_{q\text{-}\mu}(r) = \left\{\frac{3(1 + \kappa r)\exp[-\kappa(r - d_2)]}{(1 + \kappa d_2/2)[2 + \kappa d_2 + (\kappa d_2/2)^2 + (1 + \kappa d_2/2)\varepsilon_s/\varepsilon]}\right\}^2$	(2)
$W_{\mu\text{-}\mu}(r) = -\frac{2}{3}\frac{\mu^4 \zeta_{\mu\text{-}\mu}(r)}{\varepsilon^2 kT r^6}$	$\zeta_{\mu\text{-}\mu}(r) = \frac{3^4[2 + 2\kappa r + (\kappa r)^2]^2 \exp[-2\kappa(r - d_2)]}{(2 + \kappa d_2 + (\kappa d_2/2)^2 + (1 + \kappa d_2/2)\varepsilon_s/\varepsilon)^4}$	(2)
$W_{OA} = -\frac{4}{3}\pi d_{23}^3 \rho_3 kT\left[1 - \frac{3r}{4d_{23}} + \frac{r^3}{16d_{23}^3}\right]$	for $d_2 < r < d_{23}$	(3)
$W_{OA} = 0$	for $r > 2d_{23}$	

†Definition of symbols: disp = dispersion, q-q = charge-charge, q-μ = charge-dipole, μ-μ = dipole-dipole and OA = osmotic attraction potentials. H = Hamaker constant, r = radial distance, d_2 = protein diameter, d_3 is the mean ionic salt diameter, $d_{23}=(d_2+d_3)/2$, ρ_3 = ion number density, z_2 = net protein charge, μ = protein dipole moment, $\zeta_{i\text{-}j}(r)$ = screening factor, κ^{-1} = Debye screening length, e = electron charge, $\varepsilon = 4\pi\varepsilon_0\varepsilon_r$ where ε_0 = vacuum permittivity and ε_r = relative permittivity, ε_s = permittivity at protein surface ($\varepsilon_s/\varepsilon = 4$ (Phillies, 1974)), k = Boltzmann's constant, and T = temperature. An earlier version of this table was presented by Vilker et al. (1981). A similar table (Haynes et al., 1992) had a misprint in the $W_{disp}(r)$ equation. References: (1) Verwey and Overbeek, 1948 (2) Haynes et al., 1992 (3) Vlachy et al., 1993.

Figure Captions

Figure 1 - Phase partitioning of lysozyme in ammonium-sulfate solutions.

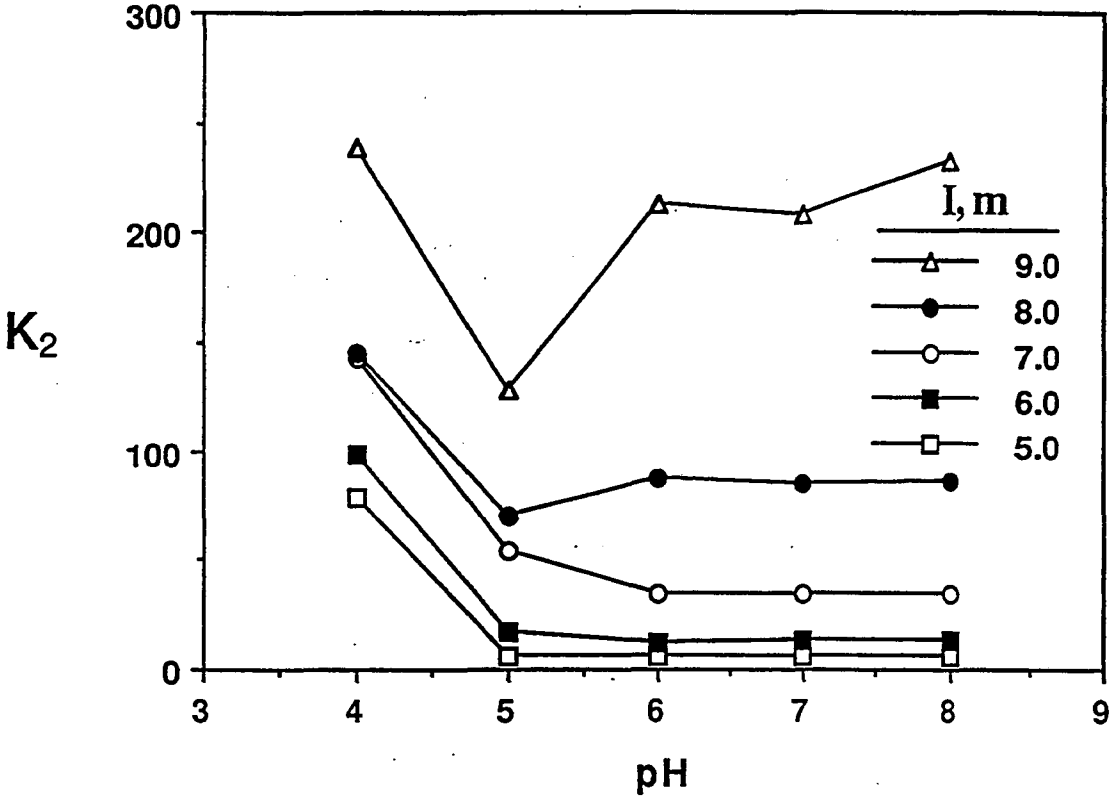
Figure 2 - Phase partitioning of chymotrypsin in ammonium-sulfate solutions.

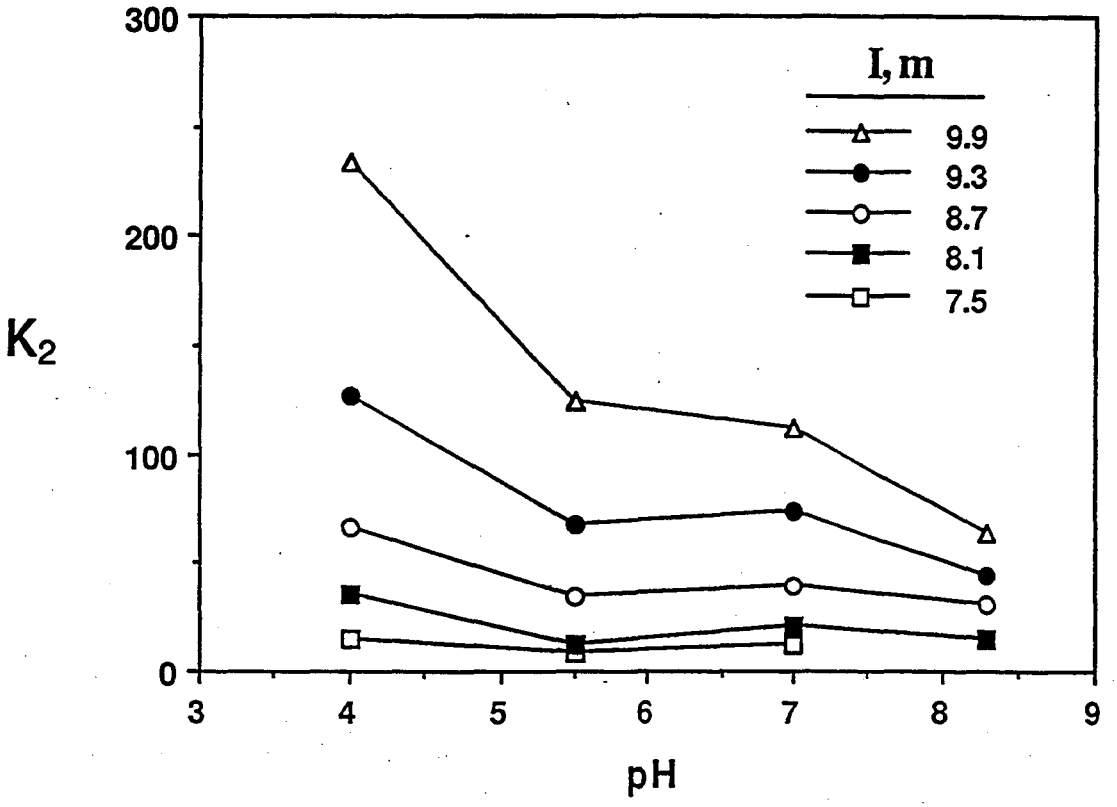
Figure 3 - Phase partitioning of chymotrypsin in sodium citrate (pH 9) and in ammonium sulfate (pH 8.3) as functions of salt ionic strength (A) and salt concentration (B).

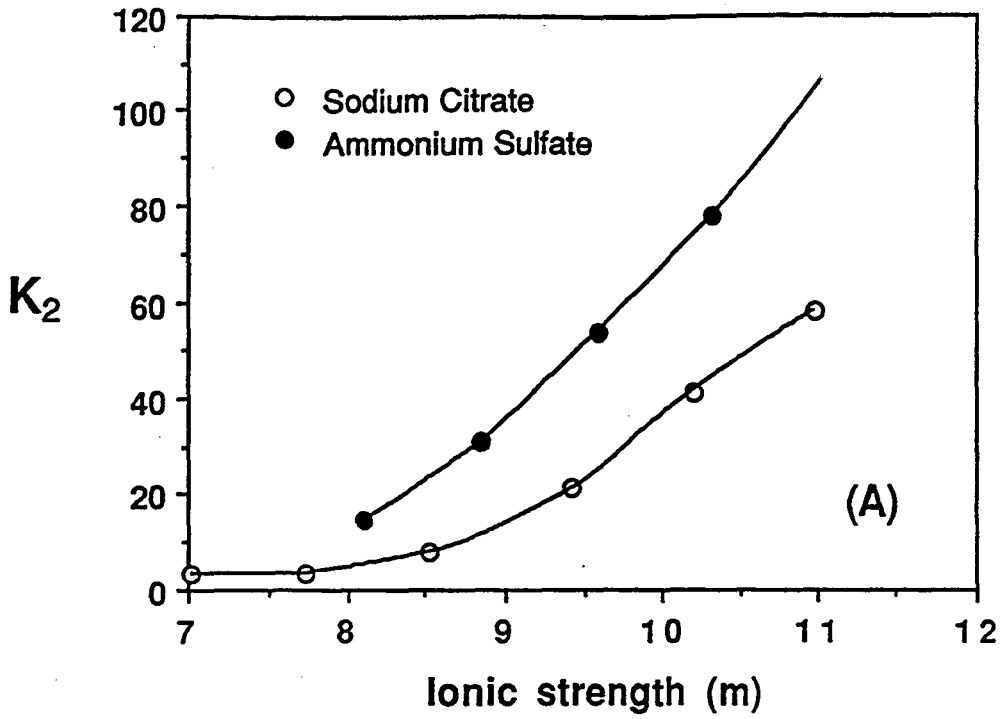
Figure 4 - Partition coefficients for lysozyme and for chymotrypsin in aqueous two-protein (open symbols) and one-protein (filled symbols) systems at pH 7 using ammonium sulfate.

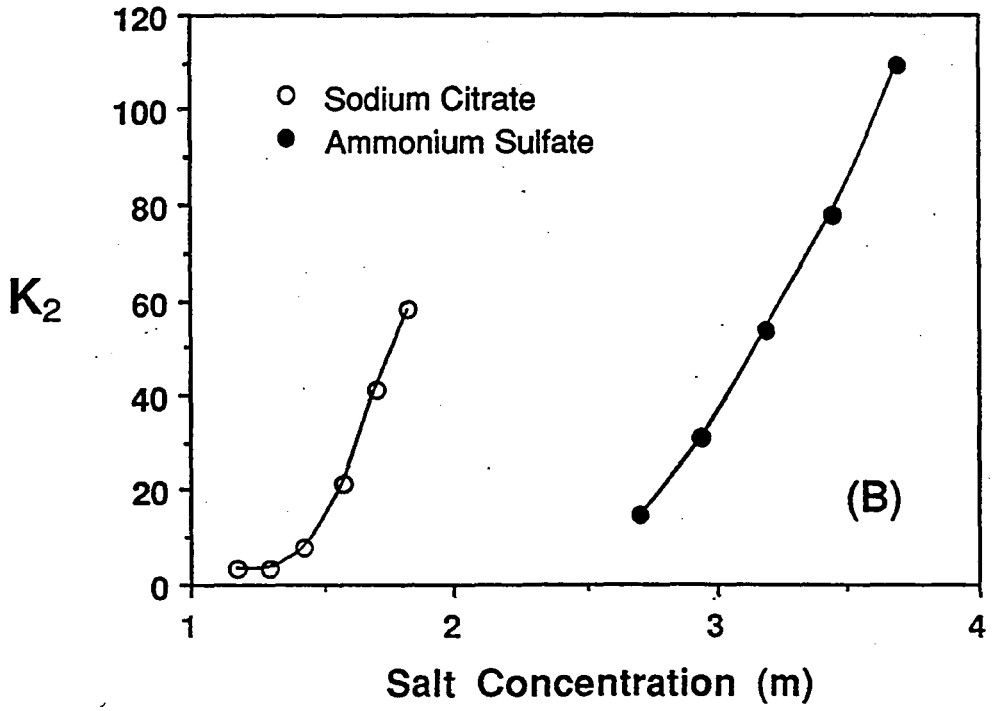
Figure 5 - Osmotic second-virial coefficients obtained by LALLS for chymotrypsin. Lines drawn to indicate individual ionic strength trends.

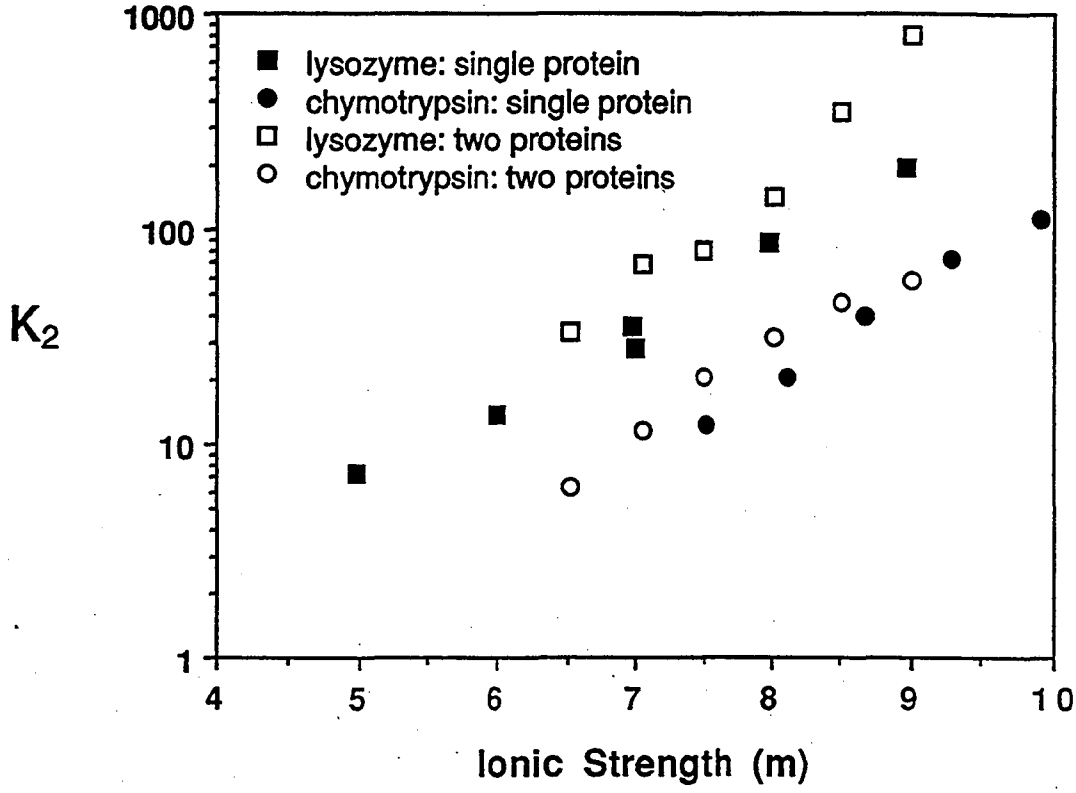
Figure 6 - Reduced Hamaker constants regressed from osmotic second-virial coefficients obtained by LALLS for three potential-of-mean-force expressions: Eq. 3, DLVO potential; Eq. 7, DLVO and dipole potentials; and Eq. 7 plus $W_{OA}(r)$, DLVO, dipole and osmotic attraction potentials.

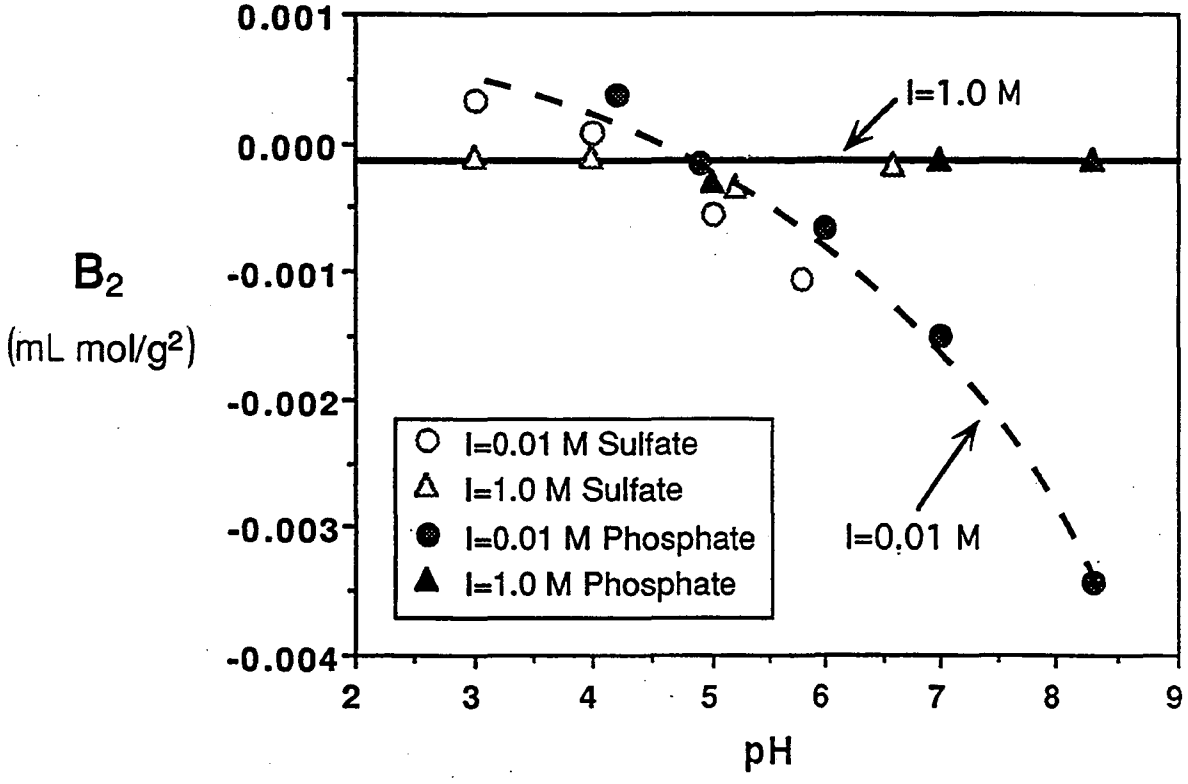

300
200
100
0
K2
3
4
5
6
7
8
9
pH
I, m
9.0
8.0
7.0
6.0
5.0

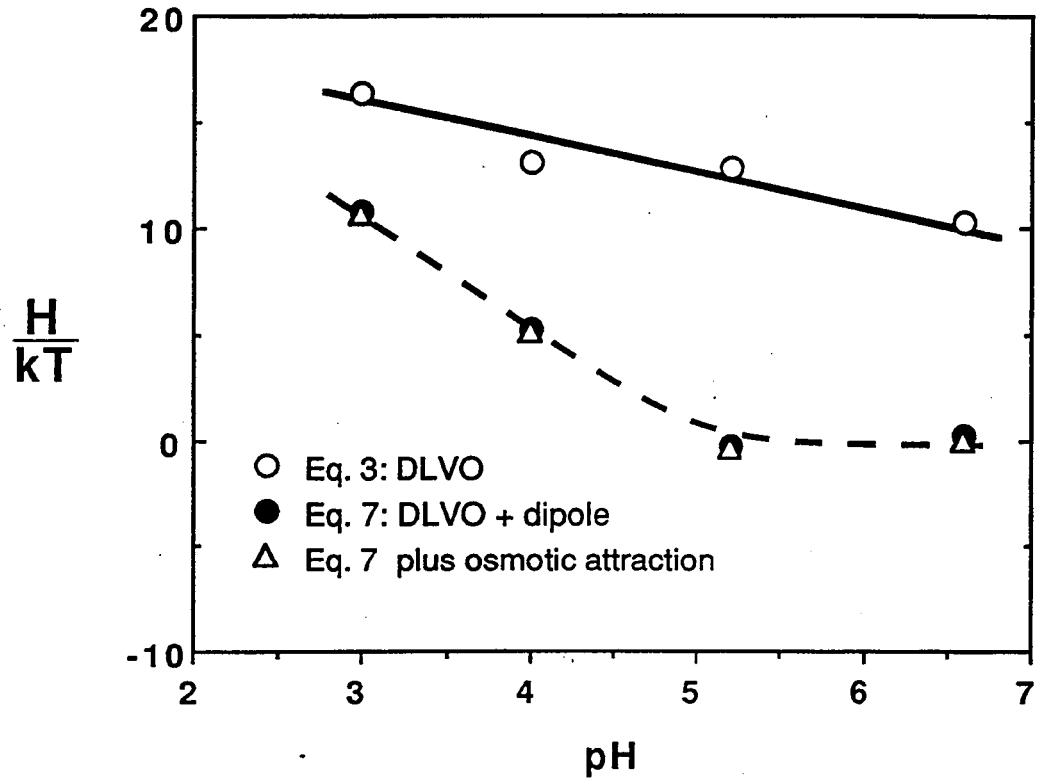

300
200
100
0
K2
3
4
5
6
7
8
9
pH
I, m
9.9
9.3
8.7
8.1
7.5


120
100
80
60
40
20
0
K_2
Sodium Citrate
Ammonium Sulfate
(A)
7
8
9
10
11
12
Ionic strength (m)


120
100
80
60
40
20
0
K_2
Sodium Citrate
Ammonium Sulfate
(B)
1
2
3
4
Salt Concentration (m)


1000
100
10
1
K_2
lysozyme: single protein
chymotrypsin: single protein
lysozyme: two proteins
chymotrypsin: two proteins
4
5
6
7
8
9
10
Ionic Strength (m)


0.001
0.000
-0.001
-0.002
-0.003
-0.004
B2
(mL mol/g2)
2
3
4
5
6
7
8
9
pH
I=1.0 M
I=0.01 M
I=0.01 M Sulfate
I=1.0 M Sulfate
I=0.01 M Phosphate
I=1.0 M Phosphate


20
10
0
-10
H
kT
2
3
4
5
6
7
pH
Eq. 3: DLVO
Eq. 7: DLVO + dipole
Eq. 7 plus osmotic attraction

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