LBL-32145
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## CHEMICAL SCIENCES DIVISION

Submitted to Biotechnology & Bioengineering

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March 1992

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Prepared for the U.S. Department of Energy under Contract Number DE-AC03-76SF00098

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LBL-32145

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# Some Characteristics of Protein Precipitation by Salts

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This work was supported by the Director, Office of Energy Research, Office of Basic Energy Sciences, Chemical Sciences Division of the U.S. Department of Energy under Contract Number DE-AC03-76SF00098.

## Summary

The solubilities of lysozyme, α-chymotrypsin and bovine serum albumin were studied in aqueous electrolyte solution as a function of ionic strength, pH , chemical nature of salt and initial protein concentration. Compositions were measured for both the supernatant phase and the precipitate phase at 25°C.

Salts studied were sodium chloride, sodium sulfate and sodium phosphate. For lysozyme, protein concentrations in supernatant and precipitate phases are independent of the initial protein concentration; solubility can be represented by the Cohn salting-out equation. Lysozyme has a minimum solubility around pH 10, close to its isoelectric point (pH 10.5). The effectiveness of the three salts studied for precipitation were in a sequence: sulfate> phosphate> chloride, consistent with the Hofmeister series.

However, for α-chymotrypsin and bovine serum albumin, initial protein concentration affects the apparent equilibrium solubility. For these proteins, experimental results show that the compositions of the precipitate phase are also affected by the initial protein concentration. We define a distribution coefficient $\mathcal{K}_e$ to represent the equilibrium ratio of the protein concentration in the supernatant phase to that in the precipitate phase. When the salt concentration is constant, the results show that, for lysozyme, the protein concentrations in both phases are independent of the initial protein concentrations, and thus $\mathcal{K}_e$ is a constant. For α-chymotrypsin and BSA, their concentrations in both phases are nearly proportional to the initial protein concentrations, and therefore, for each protein, at constant salt concentration, the distribution coefficient $\mathcal{K}_e$ is independent of the initial protein concentration. However, for both lysozyme and α-chymotrypsin, the distribution coefficient falls with increasing salt concentration. These results indicate that care must be used in the definition of solubility. Solubility is appropriate when the precipitate phase is pure, but when it is not, distribution coefficient better describes the phase behavior.

**Keywords**: Protein Solubility, Precipitation, Salting-out

# Introduction

The inorganic salting-out process of proteins has widespread application as an isolation procedure in protein recovery on both laboratory and industrial scales. Examples include recovery of proteins such as diagnostic enzymes, insulin, human growth hormone and interferon (27) and food proteins. For example, salting-out by ammonium sulfate provides a typical method for initial protein separation. In this process, a concentrated salt is added to a protein solution producing a protein-rich precipitate.

In this work we report fundamental experimental precipitation data for lysozyme, α-chymotrypsin and bovine serum albumin. The effect of concentrated salt for promoting aggregation and precipitation of proteins is not well understood. In the salting-out region, salt exerts a specific effect on the protein which depends on the nature of the salt and its concentration. Cohn (14) found a simple relation between the solubility of protein and the ionic strength of the solution:

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

where S is the solubility of the protein of interest, I is the ionic strength; $K_s$ is a protein- and salt-specific constant while $\beta$ is a constant that depends on temperature and the protein but not on the salt (19, 20). In Figure 1, constant $K_s$, which is known as the salting-out constant, is the slope and $\beta$ is the intercept with the vertical axis which represents the hypothetical logarithm of the solubility of protein at zero ionic strength. This salting-out equation is not only suitable for proteins, but also for most organic substances and dissolved gases which may also be salted-out by electrolytes (15). The theoretical basis of this equation is not clear.

Experimental results show that polyvalent anions in salts such as sodium sulfate and sodium phosphate have higher $K_s$ values than those for 1-1 electrolytes, but polyvalent cations such as magnesium or calcium depress the $K_s$ value. Therefore, salts with single valent cations and polyvalent anions such as ammonium and sodium sulfate have been widely used as protein precipitants. Ammonium sulfate is more widely used than sodium sulfate because the solubility of sodium sulfate is low at room temperature.

The relative effectiveness of salts in protein salting-out has been of interest since its discovery by Hofmeister (22). In order of decreasing effectiveness, this series is : citrate > sulfate > phosphate > chloride > nitrate > thiocyanate. When this series is compared with the empirical salting-out equation ( Eq. 1 ), it is apparent that salts with large Hofmeister effects tend to have high salting-out constants, $K_s$. The tendency for a salt to cause denaturation of a protein is inversely related to its position in Hofmeister series (10).

Von Hippel (39) suggested that the Hofmeister series is related to the interaction between ions and the hydrophobic groups of the protein. It is the hydrophobic part of a protein that governs the salting-out of protein in salt solution. Generally, protein molecules fold in aqueous solution and the number of exposed hydrophobic amino acid residues is minimized. However, close examination of the three-dimensional structure of a number of proteins (13, 24, 35) shows that a substantial fraction of the hydrophobic amino-acid residues is indeed located at the surface of the protein molecules. Therefore, the influence of these hydrophobic residues on protein-protein interaction cannot be ignored. Some of the hydrophobic amino acid residues are buried in the protein-protein contact area during the association of protein molecules in salt solution. This association of protein molecules is often accompanied by a decrease in the Gibbs energy of the system.

Melander and Horvath (28) related the hydrophobic effect to protein solubility. They assumed that, *in the absence of conformational change*, the energetics of the salting-out of protein is only affected by two contributions: (1) the formation of a cavity in the solvent to accommodate a protein molecule, and (2) the electrostatic interactions between protein and electrolyte. This assumption leads to a relationship between protein solubility and ionic strength (28)

$$\ln S = x_0 + \beta_0 - [\,\Omega\,\sigma - \Lambda\,]\,I \qquad (2)$$

where $\Omega$ is a constant which depends on the molar hydrophobic surface area, $\sigma$ is the molal surface tension increment, $\beta_0$ represents the Debye-Hückel contribution, which considers the protein as a spherical macromolecule with a uniform charge, valid only at low ionic strength. $\Lambda$ represents the Kirkwood contribution, which considers the protein as a neutral dipole. This contribution is proportional to the ionic strength (16). The constant $x_0$ represents the contributions other than those of cavity formation and electrostatic interactions between protein and electrolyte. Comparing Eq. (2) with Cohn's salting-out equation (Eq. 1), the constants in the empirical Eq. (1) are given by

$$K_s = \Omega\,\sigma - \Lambda \qquad (3)$$

and

$$\beta = x_0 + \beta_0 \qquad (4)$$

Combining the concept of the Hofmeister series and Cohn's salting-out equation, a salt with high effectiveness to precipitate protein tends to have a high salting-out constant $K_s$. Melander and Horvath's estimate (Eq. 3) provides a quantitative basis for the Hofmeister series. According to Eq. (3), the salt with a high molal surface tension increment $\sigma$ also has a high $K_s$, and hence has a high Hofmeister effect. Melander

and Horvath (28) used a number of salts to evaluate the constant $\sigma$. The results show that the sequence of $\sigma$ can serve as a quantitative measure for the qualitative Hofmeister series.

Melander and Horvath's hydrophobic theory is valid for conformally inert proteins. However, Przybycien and Bailey (33) found that the conformation of $\alpha$-chymotrypsin is perturbed to different extents by the salt used. Przybycien and Bailey (32) employed Melander and Horvath's hydrophobic theory to predict the salting-out constants of $\alpha$-chymotrypsin in various salt solutions. They found that the experimental $K_s$ have significant discrepancies from the theoretical values obtained from Eq. (3). Przybycien and Bailey (32) suggested that these discrepancies result from the conformational change of $\alpha$-chymotrypsin in salt solution since hydrophobic and electrostatic interaction terms $\Omega$ and $\Lambda$ depend on the hydrophobic surface area and on the dipole moment of protein, which may be altered by the conformation change due to the specific protein-salt interaction (9). Przybycien and Bailey's experimental results (32) showed the importance of the protein-salt interaction in the study of protein precipitation.

Extensive studies of protein-salt interactions in aqueous solution have been reported by Timasheff and co-workers (1, 2, 3, 4, 5, 6, 7, 8, 18, 30, 31, 36, 37). They showed that conformational stability and solubility of proteins in salt solutions are strong functions of the interactions between the salt and the protein. They expressed the interactions in terms of a *preferential interaction parameter* defined by

$$\xi_3 = \left(\frac{\partial m_3}{\partial m_2}\right)_{T,\,\mu_1,\mu_3} \qquad (5)$$

where $m_3$ is the concentration of component 3 (salt) in molality, T is the Kelvin temperature, $\mu_i$ is the chemical potential of species i, where 1 stands for water, 2 for

protein and 3 for salt. Interaction parameter $\xi_3$ provides a measure of the excess salt present in the immediate domain of the protein molecule beyond its concentration in the bulk solution; it may be determined by high-precision densimetry or differential refractometry (1, 3, 6, 31). A positive value of $\xi_3$ means an excess of salt in the domain of protein (30); a negative value means a deficiency of salt, or an excess of water, in the immediate domain of a protein molecule.

This protein-salt interaction may be considered as a combination of two contributions (3): (a) the non-specific preferential exclusion of salt caused by the surface tension effect stated in Melander and Horvath's hydrophobic theory, and (b) the specific binding of salt to a protein which depends on the chemical nature of both the protein surface and the salt:

$$\xi_3 = \xi_3^{excl} + \xi_3^{bind} \tag{6}$$

where the sign of the first term on the right-hand side is negative, and that of the second term is positive. Note that binding does not imply the formation of any stable stoichiometric complexes.

KSCN has a positive molal surface tension increment (28). According to Melander and Horvath's hydrophobic theory (28), KSCN is a salting-out reagent for proteins. However, Arakawa and Timasheff (1) examined the solubility of bovine serum albumin (BSA) in KSCN solution, and observed a salting-in effect of KSCN on BSA rather than salting-out. They explained this result by the observation of considerable binding of salt to protein molecules which compensates for salt exclusion due to the hydrophobic effect caused by salt.

Salting-in reagents have a tendency to denature proteins. Denaturation is attributed to the effect of binding of the salt to the protein (1). The increase of the net charge of the protein due to the binding of the ions increases the electrostatic Gibbs energy of the protein. The resulting intramolecular repulsive forces cause a decrease in the conformational stability of the protein. The intermolecular electrostatic repulsive force also prevents protein aggregation and thus increases the solubility of protein in solutions of these salts.

The protein-salt interaction parameter is also a reflection of the perturbation of the chemical potential of protein and salt due to their mixing. The relation is given by Arakawa and Timasheff (1, 2)

$$\left(\frac{\partial \mu_2}{\partial m_3}\right)_{T,P,m_2} = -\frac{RT}{m_3}\xi_3 \quad . \qquad (7)$$

Abundant experimental results for $\xi_3$ for various salts and proteins have been reported ( 1, 2, 3, 4, 6, 7, 8, 18,30, 31, 32, 37 ). The change of the chemical potential of a protein due to the addition of salt can be calculated by Eq. (7). The values of $\xi_3$ for salting-out electrolytes, such as sodium chloride and sodium sulfate, are negative (32, 36), i.e., proteins are preferentially hydrated. According to Eq. (7), a negative $\xi_3$ produces a positive change in the chemical potential of the protein due to the addition of salt, and eventually leads to protein precipitation. Some salting-in electrolytes, such as KSCN, have positive $\xi_3$ due to the strong interaction of salts to protein molecules (32). The positive $\xi_3$ causes a decrease of the chemical potential of protein due to the addition of salt, and thus increases the solubility of protein. Generally, the protein-salt interaction parameter $\xi_3$ depends on the particular protein and salt, pH, and salt

concentration. The magnitude of $\xi_3$ increases with increasing salt concentration (8, 32).

However, in spite of widespread application, few theoretical or detailed experimental studies on protein precipitation have been reported. To improve our understanding of protein precipitation, this research describes experimental studies on aqueous one-protein systems. Several important parameters (pH, type of salt, ionic strength and initial protein concentration ) were varied to identify some characteristics of protein precipitation in aqueous electrolyte solutions.

# Materials and Methods

## Materials

Reagent grade bovine serum albumin (BSA), egg-white lysozyme, bovine pancreatic α-chymotrypsin and phenyl-methane sulfonyl fluoride (PMSF) were purchased from Sigma. Reagent grade NaCl, $Na_2SO_4$, $H_3PO_4$, $NaH_2PO_4$ and $Na_2HPO_4$ were purchased from Fisher Chemical Inc. Water was purified by passage through a Barnstead NANOpure II system and a 0.22 μm filter, all maintained at 25 ± 0.5 °C.

The pH of a solutions was measured by Corning Electrode purchased from Fisher Chemical Inc. The pH of NaCl and $Na_2SO_4$ solutions was adjusted by appropriate acid and base (e. g., NaOH and HCl for NaCl ). The pH of phosphate solutions was adjusted by mixing appropriate ratios of $Na_2HPO_4$ , $NaH_2PO_4$ and $H_3PO_4$ solutions.

## Methods

***Inhibition of α-chymotrypsin by Phenyl-Methane Sulfonyl Fluoride***

α-chymotrypsin may self-digest in aqueous solution, except at pH 3 or less (26). Inhibition of α-chymotrypsin was accomplished by alkylating histidine-57 with PMSF (17). 0.1 M PMSF stock solution was prepared by dissolving PMSF in isopropanol. Immediately after α-chymotrypsin was dissolved in water, PMSF stock solution was added with 10 mole percent excess. The mixture was then gently shaken for one hour to complete binding of PMSF to histidine-57.

***Protein Precipitation***

Protein solutions were prepared by dissolution in 6 ml NANOpure water in a 20-ml vial. For α-chymotrypsin, inhibition followed the dissolution step. A concentrated salt solution was then added dropwise while stirring the protein solution to reduce any local concentration gradients. Stirring speed was adjusted to eliminate foaming. The mixture was then gently shaken for 4 hours, during which precipitation occured. The precipitate-containing mixture was centrifuged in a Jouan CT4 22 temperature-controlled centrifuge at 5000 G and 25°C for 2 hours to separate the supernatant phase from the precipitate phase. 2 hours of centrifugation at 5000G was chosen since no increased amount of precipitate resulted from higher gravity force or longer centrifugation.

After centrifugation, the clear supernatant phase was withdrawn and diluted 30- to 50-fold with water to eliminate the influence of salt on the UV absorbance of protein. Protein concentration was determined by UV absorbance in a Milton Roy 1201 UV Spectrophotometer. The extinction coefficients used were 23.7 dL / (g cm) at 281.5 nm for lysozyme, 6.58 dL / (g cm) at 278 nm for BSA and 18.4 dL / (g cm) at 281 nm for α-chymotrypsin (3).

The precipitate phase was freeze-dried at 40 mtorr for 40 hours. The water content in the precipitate phase was determined by the weight difference before and after freeze drying. The dry protein-salt mixture was redissolved, and the protein

content was determined by UV absorbance. The salt content in the precipitate phase was determined by a Perkin-Elmer 2280 Atomic Absorbance Spectrophotometer based on the concentration of sodium ions, which has a characteristic absorbance at 589 nm.

# Results and Discussion

## *Effect of Ionic Strength*

The Cohn equation relates the logarithm of protein solubility S to the salt concentration $C_{salt}$

$$\log S = \beta - K_s' C_{salt} \tag{8}$$

If the salt concentration is given in terms of ionic strength I, we obtain Eq. (1). We use mg/g water for S and molality for $C_{salt}$ and I. Figure 2 shows the solubilities of lysozyme as a function of ionic strength at various pH in sodium chloride solutions. The straight lines in Figure 2 were fitted to Eq. (1) by the method of least squares. Table I summarizes the effect of ionic strength on the solubility of lysozyme in various salt solutions. The values of $\beta$, $K_s'$ and $K_s$ are computed from the intercepts and the slopes of the two forms of the Cohn equation (Eqs. 1, 8).

Green (19, 20) suggested that $\beta$, the hypothetical logarithm of the solubility of protein at zero ionic strength, depends on temperature, pH and the type of protein but not on the salt. As indicated in Table I, $\beta$ is a function of pH. There is no significant difference in $\beta$ for lysozyme in sodium sulfate solutions or in sodium phosphate solutions. However, the $\beta$'s for lysozyme in sodium chloride solutions are about one third of those in sodium sulfate and sodium phosphate solutions.

Lysozyme is often used in studies of protein crystallization. Howard et al (23) reported experimental studies on the solubility of crystalline lysozyme in salt solution. Figure 3 compares the solubilities of lysozyme in equilibrium with its amorphous and crystalline forms. The solubility of lysozyme is higher when in equilibrium with amorphous than crystalline lysozyme.

If the concentration of lysozyme is high, precipitation does not occur as salt is added to the solution due to the formation of a gel. A similar observation has been reported by Ries-Kautt et al (34). Generally, gelation occurs if the lysozyme concentration is higher than 60 mg/ml and if the ionic strength is higher than 0.8 m. Once the gel forms, lysozyme cannot be recovered by centrifugation.

## *Effect of pH*

Figure 4 shows the effect of pH on the solubilities of lysozyme in sodium chloride solutions. In Fig. 4, a solubility minimum is observed around pH 10, which is close to the isoelectric point of lysozyme ( pH 10.5 ). It is well known that a protein has a minimum solubility at its isoelectric point where the protein has a zero net charge. A decrease of protein solubility was observed at pH values less than pH 5. Carr (12) and Bull and Breese (11) have shown that at acidic pH, the anions of salts bind to proteins, reduce the net positive charge on the protein and thus decrease its solubility.

Protein molecules in salt solution can be considered as a mixture of differently charged protein ions in chemical equilibrium. Protein solubility, S, is the sum of the concentration of neutral protein molecules, $S_n$, and the concentrations of protein cations, $P^{+1}$, $P^{+2}$, $P^{+3}$, $P^{+n}$, and the concentrations of protein anions, $P^{-1}$, $P^{-2}$, $P^{-3}$, $P^{-m}$, where the positive and negative charges are the net charge of protein molecules. Following the description by Cohn and Edsall (14),

$$P^n \xleftrightarrow{K_n} P^{n-1} + H^+ \tag{9}$$

$$[P^n] = \frac{[P^{n-1}][H^+]}{K_n} \tag{10}$$

$$S = S_n + (P^{+1} + P^{-1}) + (P^{+2} + P^{-2}) + (P^{+3} + P^{-3}) + \cdots + (P^{+n} + P^{-m}) \ . \tag{11}$$

Since the concentrations of protein cations and anions depend on the pH of the solution, combining Eq (10) and (11),

$$S = S_n\left[1 + \left(\frac{[H^+]}{K_n} + \frac{K_{n+1}}{[H^+]}\right) + \left(\frac{[H^+]^2}{K_nK_{n-1}} + \frac{K_{n+1}K_{n+2}}{[H^+]^2}\right) + \left(\frac{[H^+]^3}{K_nK_{n-1}K_{n-2}} + \frac{K_{n+1}K_{n+2}K_{n-3}}{[H^+]^3}\right) + \cdots\right] \tag{12}$$

Eq. (12) relates the solubility of protein to the pH value of the solution. Each term in Eq. (12) corresponds to a specific net charge of protein. The sum of the first terms in each parenthesis measures the dissociation into cations; the sum of the second terms in each parenthesis gives the dissociation into anions. In sufficiently acid solutions, the second term in each parenthesis vanishes; whereas at highly basic solutions, the first term in each parenthesis is negligible. Generally, at constant pH, only one term in Eq. (12) dominates, and the corresponding charge is considered the most likely charge of protein at this pH.

As indicated in Table I, the salting-out constant based on the ionic strength ($K_s$) has no significant variation with the change of the pH value of the solution, but strongly depends on the nature of salt. The salting-out constant based on salt concentration ($K_s'$) has a significant dependence on pH when phosphate is used as

the anion because phosphate buffer solution contains several types of anions, and the composition is a function of pH.

### *Effect of the Type of Salt*

The nature of the salt has a large influence on protein solubility. Experimental results (28) show that the salting-out effect of salts mainly depends on the type of anions. Salts exert their effect by dehydrating proteins through competition for water molecules. Their ability to dehydrate depends primarily on the square of the valence of the anion of the salt (22). Thus, salts with polyvalent anions are more effective at salting-out than those containing univalent anions. Since the cation was common to the three salts studied in the present work, solubility difference can be attributed to the anions.

Comparison of salting-out ability of salts should be made on the basis of salt concentration rather than ionic strength since the Hofmeister classification is based on salt concentration (38). As illustrated in Table I, for lysozyme, divalent sulfate and phosphate have higher $K_s'$ than univalent chloride indicating that, *at the same salt concentration*, the precipitation potentials of the three salts studied here have the sequence:

*sulfate > phosphate > chloride*

which is consistent with the Hofmeister series.

For lysozyme solutions, at high pH, phosphate has a high $K_s'$ value, close to that of sulfate. The $K_s'$ of phosphate decreases at lower pH, as phosphate buffer solutions contain a higher mole fraction of the univalent anion $H_2PO_4^-$. For sulfate, $K_s'$ falls with decreasing pH. Comparing the $K_s'$ of sulfate with that of phosphate, the value of $K_s'$

of sulfate is less sensitive to pH since sodium sulfate is a strong electrolyte where the majority of anions is $SO_4^{-2}$, even at low pH.

Table I provides a compilation of $\beta$, $K_s'$ and $K_s$ values for lysozyme and three salts, and also gives a comparison of $K_s$ based on ionic strength. The data show that $K_s$ is less sensitive to pH than $K_s'$. According to Table I, *at the same ionic strength*, the precipitation potentials of the three salts studied here have the sequence:

*phosphate ≈ sulfate > chloride*

Phosphate and sulfate have a similar effect in precipitating lysozyme at the same ionic strength, and both are better precipitants than chloride.

## *Effect of Centrifugation*

The magnitude and duration of centrifugal force have an important influence in protein precipitation. As indicated in Figure 5, the protein concentration in the supernatant phase depends on the time of centrifugation. The time required for precipitation recovery depends mainly on the particle-size distribution of the protein aggregate, the density difference between salt solution and protein particle, the viscosity of the salt solution and the external gravity force applied.

Generally, the time required for centrifugation is longer at low gravity force than that at high gravity force. Different gravity forces (2000G, 5000G, 8000G and 9000G) were applied to identify their effect on protein solubility. For these different gravity forces, no significant dependence of protein solubility on gravity force was observed after 2 hours of centrifugation. Therefore, 2 hours of centrifugation at 5000G was chosen to precipitate protein aggregates. No significant variations in protein solubility resulted from longer centrifugation.

At some particular salt concentration, the density of the salt solution may equal that of the protein aggregate, making centrifugal recovery of protein precipitate impossible. Fig. 6 illustrates an example of this phenomenon. In 2.63 m $Na_2SO_4$ solution at pH 9, the density of lysozyme and that of salt solution are so close that precipitation of lysozyme by centrifugation is not possible. This critical density should be avoided in practical applications.

## *Effect of Initial Protein Concentration*

The solubility of a protein is typically determined by increasing salt concentration in an aqueous protein solution to a desired level. For lysozyme, the observed solubility is independent of the initial protein concentration as illustrated in Figure 7, where solubilities at various salt concentrations of sodium phosphate buffer solution are shown as a function of initial lysozyme concentration. Each solubility curve is a straight line with a unit slope, up to a point where the lysozyme in the solution is saturated. Beyond this point, the precipitate phase forms, the slope of the solubility curve is zero and the lysozyme concentration in the supernatant phase is a constant.

However, other proteins, such as α-chymotrypsin and bovine serum albumin (BSA), show different behavior. At a constant salt concentration and pH, the protein concentration in the supernatant phase depends on the initial amount of protein in the system (Figs. 8, 9). This influence of the initial protein concentration is often neglected in studies of protein solubility; few experimental data on this effect are available.

For these types of proteins, the protein concentration in the supernatant phase is almost directly proportional to the amount of protein initially added. Figures 8 and 9 show the solubilities of α-chymotrypsin and BSA, respectively, in sodium phosphate buffer solutions at pH 5. The concentrations of α-chymotrypsin and BSA in the supernatant phases are approximately linear functions of the initial protein

concentrations, and the slopes fall with increasing salt concentrations. At high salt concentration, the slope approaches zero, similar to that found with lysozyme.

Hardy (21) and Mellanby (29) reported similar observations with serum globulin in salt solutions. Edsall (14) proposed that this result was to be expected because serum globulin is a mixture of several components, However, Edsall's explanation cannot explain our observations with α-chymotrypsin. It appears that the result is general. It has been observed that α-chymotrypsin coexists with its dimer in dilute salt solution at pH 4.3 without buffer, but the dimer represents only 5% of the total protein (25).

Based on their solubility behavior in salt solutions, we can consider two classes of proteins:

**Type I**: At a constant salt concentration and pH, the protein concentration in the supernatant phase is a constant. Lysozyme is representative.

**Type II**: At a constant salt concentration and pH, the protein concentration in the supernatant phase is not a constant but is approximately proportional to the initial protein concentration. α-Chymotrypsin and bovine serum albumin are representative.

Experimental results ( Figure 10 ) strongly support the argument that the dependence of protein concentration in the supernatant on the initial protein concentration is controlled by thermodynamics rather than kinetics. The concentration of α-chymotrypsin in the supernatant phase depends uniquely on the salt concentration, pH of the solution and the initial protein concentration, but is independent of the experimental path, as illustrated in Figure 10. Pure water was added to a two-phase mixture of protein in 2.92 m phosphate buffer at pH 5 to dilute it to 2.60 m. The new 2.60 m solubility curve obtained from this dilution experiment corresponds to that obtained with phosphate solution without dilution. Similarly, the 2.92 m solubility curve can also be obtained by adding the appropriate amount of concentrated salt solution into a two-phase protein-salt solution to raise the salt

concentration to 2.92 m. These results suggest that the dependence of protein solubility on the initial protein concentration is thermodynamically controlled.

To explain the results, it is necessary to define carefully what we mean by solubility. Normally, solubility of a solute is the concentration of that solute in a solvent when the dissolved solute is in equilibrium with *pure* solid solute. But in the experiments reported here, the condensed ("solid") phase is not pure. To understand two-phase equilibria where both phases are mixtures, it is necessary to determine the compositions of *both* phases. For α-chymotrypsin, the precipitate phase contains approximately 70 wt.% water, 20 wt. % salt and 10 wt. % protein. By comparing this composition to that of the supernatant phase (approximately 75% water, 24% salt and 1% protein ), the precipitate phase is a dense liquid phase.

We define the distribution coefficient $\mathcal{K}_e$ of protein between the two phases as:

$$\mathcal{K}_e = \frac{c^s}{c^p} \tag{13}$$

where $c^s$ and $c^p$ are the protein concentrations in the supernatant phase and the precipitate phase, respectively.

Lysozyme and α-chymotrypsin are chosen as the model proteins of type I and type II, respectively. Their $c^s$, $c^p$ and $\mathcal{K}_e$ in sodium phosphate buffer solution at pH 5 were measured.

**Type I:** At a fixed salt concentration and pH, the lysozyme concentration in the supernatant phase ($c^s$) is independent of the initial protein concentration (Fig.11.**I**). Experimental results (Fig.11.**II**) show that the lysozyme concentration in the precipitate phase ($c^p$ ) is also independent of the initial protein concentration. Constant values of $c^s$ and $c^p$ give a constant equilibrium distribution coefficient $\mathcal{K}_e$ (Fig. 11.**III**). However, $\mathcal{K}_e$ falls with increasing salt concentration.

**Type II**: At a constant salt concentration and pH, the concentration of α-chymotrypsin in the supernatant phase ($c^s$) is nearly proportional to the initial protein concentration (Fig.12.I). The concentration of α-chymotrypsin in the precipitate ($c^p$ ) is *also* proportional to the initial protein concentration (Fig. 12.II). Because both $c^s$ and $c^p$ are proportional to the initial protein concentration, the equilibrium distribution coefficient $\mathcal{K}_e$ is a constant at a fixed salt concentration and pH, and is *independent* of the initial protein concentration (Fig. 12.III). However, $\mathcal{K}_e$ falls with increasing salt concentration.

In other experiments, sodium sulfate solution was used and the concentrations of protein in both phases are measured. These results (Figs. 13, 14) show that the effect of the initial protein concentration on protein solubility depends primarily on the type of protein, but is independent of the type of salt. At constant salt concentration and pH, the solubility of lysozyme is independent of the initial concentration of lysozyme in both sodium phosphate and sodium sulfate solutions (Figs. 11, 13). However, the solubilities of α-chymotrypsin show similar dependence on initial concentration of α-chymotrypsin in both sulfate and phosphate solutions( Figs. 12, 14).

These results indicate that the initial protein concentration has an effect on the compositions of *both* phases at equilibrium. When the protein precipitate is not pure, the concept of protein solubility is not applicable. A meaningful term is the protein distribution coefficient.

# Conclusions

Salting-out of proteins has widespread application as an isolation procedure in protein recovery on both laboratory and industrial scales. However, few fundamental studies on protein precipitation have been reported. In this work, several system parameters (pH, type of salt, ionic strength and initial protein concentration) were varied to identify some characteristics of protein precipitation in aqueous salt solutions.

Three salts (sodium chloride, sodium sulfate and sodium phosphate) were used as precipitants in this work. At constant salt concentration and pH, the solubility of lysozyme is a constant. The results show that the dependence of lysozyme solubility on ionic strength fit the Cohn equation (Eq. 1). By comparing our solubilities of amorphous lysozyme with that of crystalline lysozyme reported by Howard et al (23), we found that the solubilities of lysozyme are higher when in equilibrium with amorphous as opposed to crystalline lysozyme.

If the concentration of lysozyme is high, precipitation does not occur due to the formation of a gel.

The solubility of lysozyme depends on pH of the solution. A minimum is observed around pH 10, which is close to the isoelectric point of lysozyme (pH 10.5). A decrease of lysozyme solubility was observed at pH value less than 5. This decrease is related to binding of salts to proteins.

Salting-out constants $K_s$ have no significant variations with pH, consistent with the observations of Green (19, 20). However, salting-out constants depend on the type of salt. For the three salts used in this work, their sequence of ability to precipitate proteins is consistent with the Hofmeister series.

Solubilities of α-chymotrypsin and BSA in aqueous salt solutions are approximately proportional to the initial protein concentration. Based on solubility behavior in salt solutions, we considered two types of proteins: (1) at constant ionic strength and pH, the protein solubility is a constant, and (2) at constant ionic strength and pH, the protein solubility is approximately proportional to the initial protein concentration. Among the proteins studied, lysozyme belongs to the first type, whereas α-chymotrypsin and BSA belong to the second type.

To understand the effect of the initial protein concentration on the two-phase equilibria in protein-salt-water systems, the compositions of the precipitate phases were examined. Precipitates contain water and salt in addition to protein. We define a

distribution coefficient $\mathcal{K}_e$ to represent the equilibrium ratio of the protein concentration in the supernatant phase to that in the precipitate phase. The results show that, for lysozyme, the protein concentrations in both phases are independent of the initial protein concentrations, and thus $\mathcal{K}_e$ is a constant. For α-chymotrypsin and BSA, the concentrations in both phases are proportional to the initial protein concentrations; therefore, for each protein, the distribution coefficient $\mathcal{K}_e$ is also independent of the initial protein concentration. However, for both lysozyme and α-chymotrypsin, $\mathcal{K}_e$ falls with increasing salt concentration. An important conclusion of this work is that care must be used in the definition of solubility. That word is useful when the precipitate phase is pure but when it is not, solubility must be replaced by distribution coefficient.

## Acknowledgements

The authors would like to thank Charles Haynes and Christopher Coen for their valuable assistance. This work was supported by the Director, Office of Energy Research, Office of Basic Energy Sciences, Chemical Sciences Division of the U.S. Department of Energy under Contract Number DE-AC03-76SF00098. The support provided by the National Science Foundation is also gratefully acknowledged.

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| Salt | pH | $\beta$ | $K'_s(m^{-1})$ | $K_s(m^{-1})$ |
|---|---|---|---|---|
| Sodium Chloride | 4 | 1.48 | 0.526 | 0.526 |
| | 5 | 1.70 | 0.426 | 0.426 |
| | 6 | 1.47 | 0.481 | 0.481 |
| | 8 | 1.18 | 0.480 | 0.480 |
| | 9 | 0.903 | 0.447 | 0.447 |
| | 10 | 0.848 | 0.453 | 0.453 |
| | 11 | 0.839 | 0.297 | 0.297 |
| Sodium Sulfate | 4 | 2.93 | 1.949 | 0.683 |
| | 6 | 3.47 | 1.946 | 0.682 |
| | 7 | 3.51 | 2.085 | 0.695 |
| | 8 | 3.55 | 2.136 | 0.712 |
| | 9 | 3.71 | 2.380 | 0.780 |
| | 11 | 3.45 | 2.295 | 0.765 |
| Sodium Phosphate | 5 | 3.70 | 1.303 | 0.914 |
| | 6 | 3.91 | 1.659 | 0.870 |
| | 7 | 3.84 | 1.890 | 0.799 |
| | 8 | 3.77 | 2.095 | 0.720 |

Table I: Cohn-Equation Parameters $\beta$, $K'_s$ and $K_s$ of Lysozyme in Aqueous Solutions of Various Salts at Several pH. Salting-out constants $K'_s$ and $K_s$ are calculated based on salt concentration and ionic strength, respectively, defined by Eqs. 8 and 1.

## FIGURE CAPTIONS

| Figure | Captions |
|---|---|
| 1 | A Typical Solubility Curve with Salting-in and Salting-out Region |
| 2 | Solubilities of Lysozyme in Sodium Chloride Solutions (ionic strength in molality). pH 5 ( ○ ); pH 6 ( ● ); pH 8 ( □ ); pH 10 ( ■ ). |
| 3 | Solubilities of Lysozyme in Equilibrium with Crystalline ( ○ )and Amorphous ( ● ) Lysozyme in Sodium Chloride Solutions at pH 5 at 25°C. The solubilities of lysozyme in equilibrium with crystalline lysozyme are reported by Howard et al (1988). |
| 4 | Effect of pH on the Solubilities of Lysozyme in Sodium Chloride Solutions. I = 1.0 m ( —■— ); I = 1.5 m ( —○— ); I = 2.0 m ( —●— ); I = 2.5 m ( —□— ); I = 3.0 m ( —■— ). |
| 5 | Effect of Centrifuge Time on the Concentration of Protein in the Supernatant Phase (2.60 m pH 4 Sodium Phosphate; centrifugation at 1400G) |
| 6 | Densities of pH 9 Sodium Sulfate Solutions ( —○— )and Estimation of the Density of Lysozyme Precipitate. The density of lysozyme precipitate is equal to that of 2.63 m Sodium Sulfate Solution, which is approximately 1.27 g / ml . |
| 7 | Solubility Curves for Lysozyme in Sodium Phosphate Solutions at pH 5. At constant salt concentration and pH, the solubility of lysozyme is a constant, independent of the initial concentration of lysozyme. I = 1.90 m ( ■ ); I = 2.09 m ( ○ ); I = 2.25 m ( ● ); I = 2.53 m ( □ ); I = 2.76 m ( ■ ). |

8 Solubility Curves for α-Chymotrypsin in Sodium Phosphate Solutions at pH 5. At constant salt concentration and pH, the solubility of α-Chymotrypsin is nearly proportional to the initial protein concentration. I = 2.60 m (—△—); I = 2.92 m (—○—); I = 3.23 m (—●—); I = 3.50 m (—□—); I = 3.81 m (—■—).

9 Solubility Curves for BSA in Sodium Phosphate Solutions at pH 5. At constant salt concentration and pH, the solubility of BSA is nearly proportional to the initial protein concentration. I = 3.30 m ( ○ ); I = 3.61 m ( ● ); I = 3.75 m ( □ ).

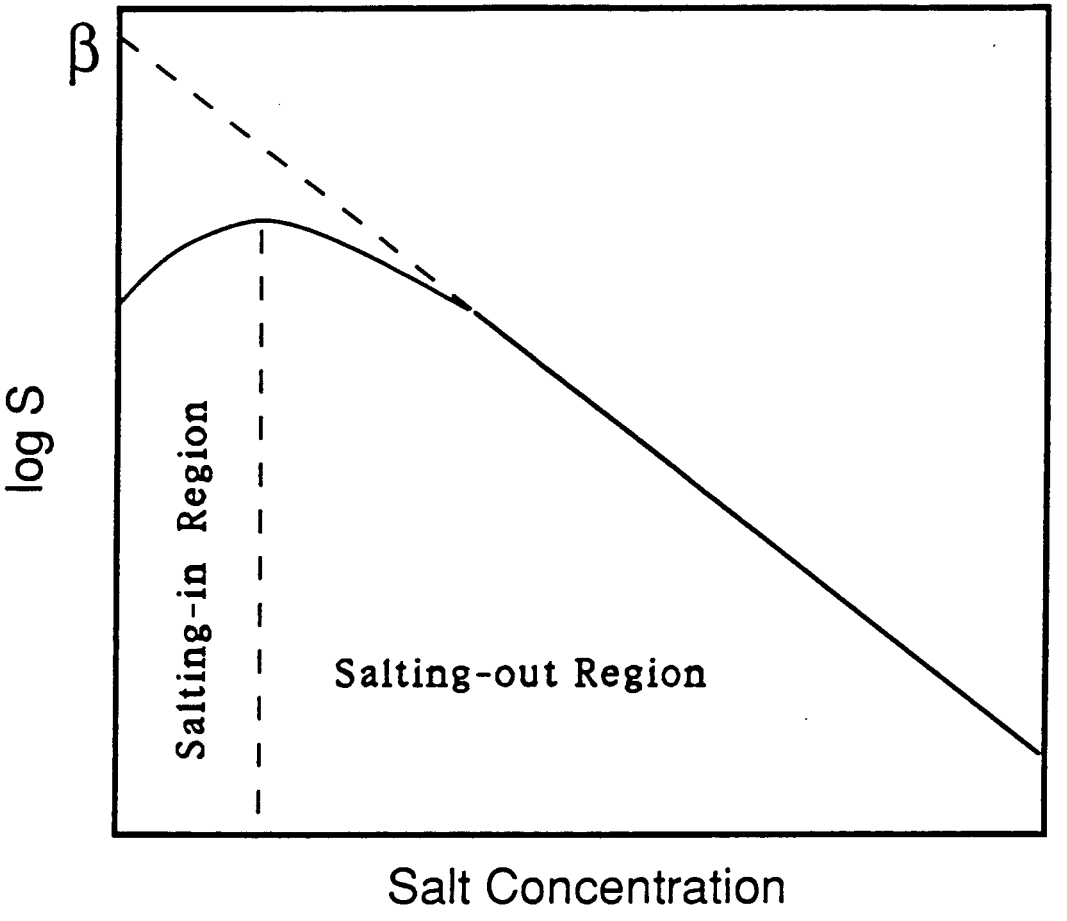
10 The 2.60 m solubility curve B ( —■— ) is obtained by diluting a 2.92 m two-phase salt-protein-water mixture to 2.60 m. After equilibrium was reached, curve B is identical with the original 2.60 m solubility curve A obtained without dilution ( —△— ). Curve C ( —□— ) is the 2.92 m solubility curve. (Salt is pH 5 sodium phosphate buffer solution)

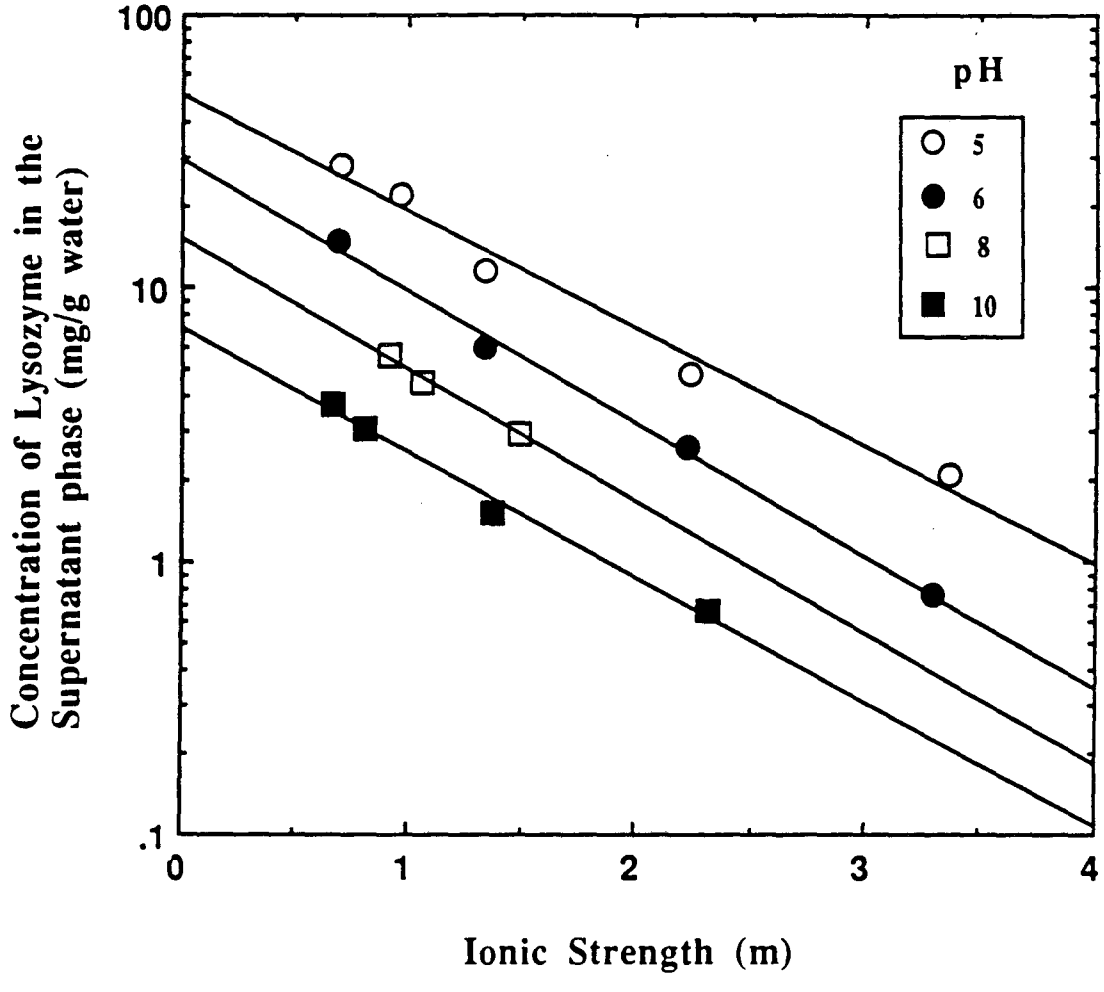
11 In pH 5 sodium phosphate buffer solution, both the concentration of lysozyme in the supernatant (I) and precipitate (II) phases are independent of the initial protein concentration. The distribution coefficient (III) is a constant at fixed salt concentration and pH. I = 2.09 m ( ○ ); I = 2.53 m ( ● ).

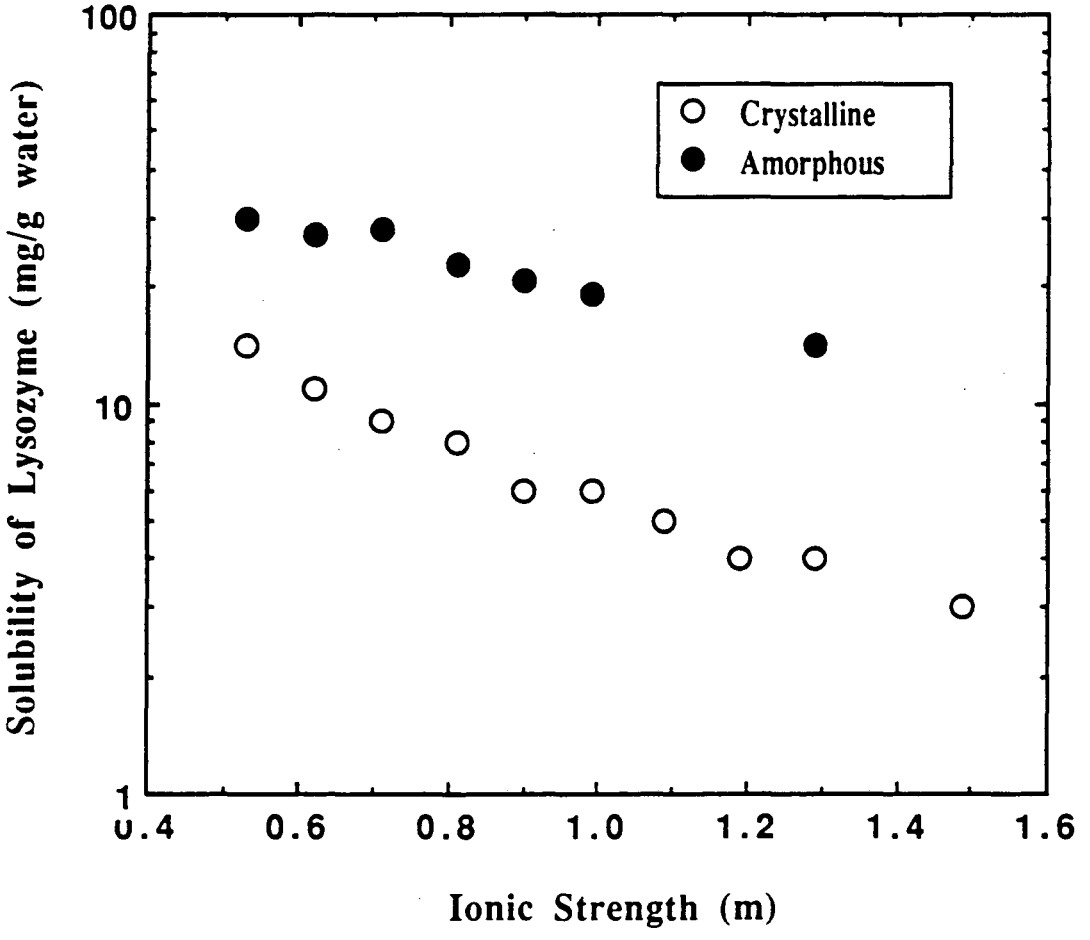
12 In pH 5 sodium phosphate buffer solution, both the concentrations of α-chymotrypsin in the supernatant (I) and precipitate (II) phases are proportional to the initial protein concentration. Therefore, the distribution coefficient (III) is a constant at fixed salt concentration and pH. I = 2.82 m ( ○ ); I = 3.05 m ( ● ).

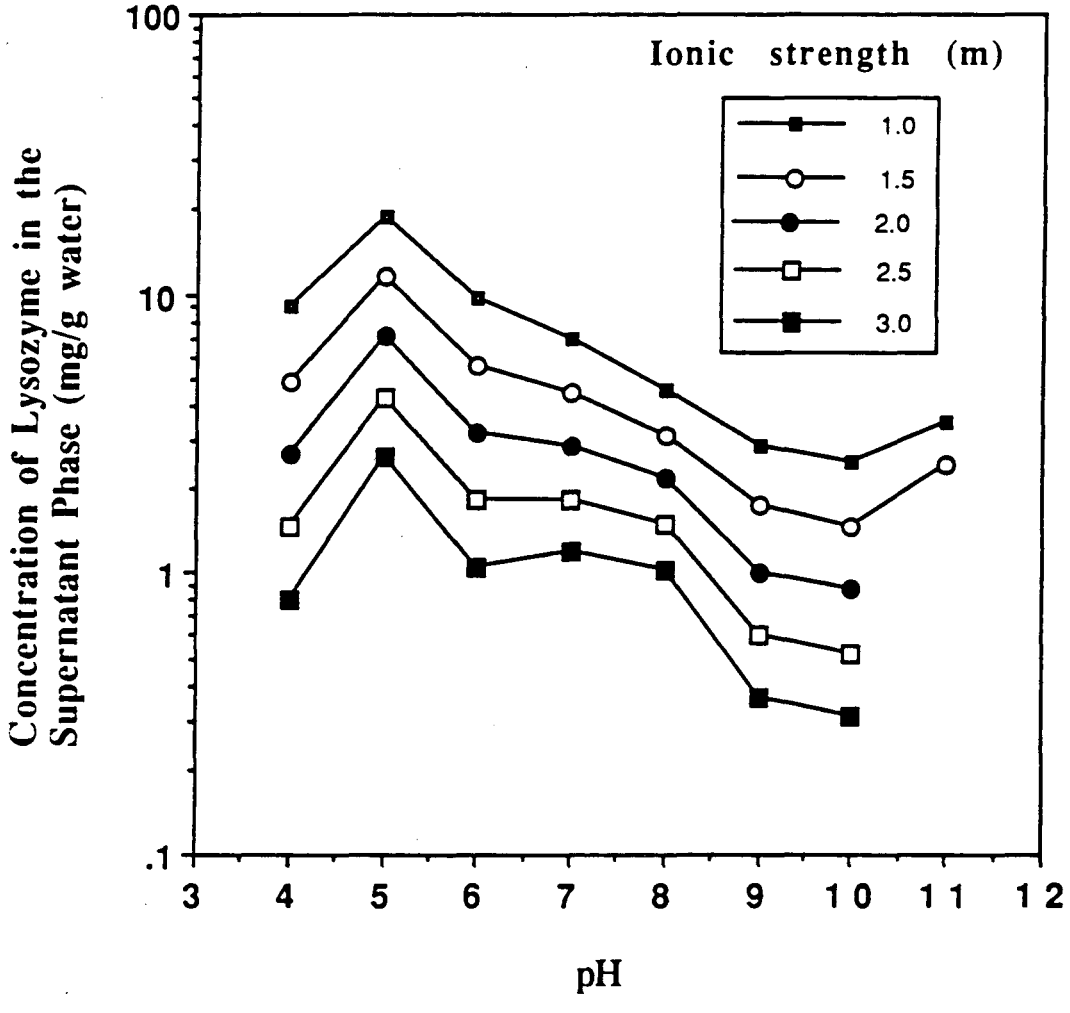
13 In 1.56 m pH 5 sodium sulfate buffer solution, both the concentration of lysozyme in the supernatant (I) and precipitate (II) phases are independent of the initial protein concentration. The distribution coefficient (III) is a constant at fixed salt concentration and pH.

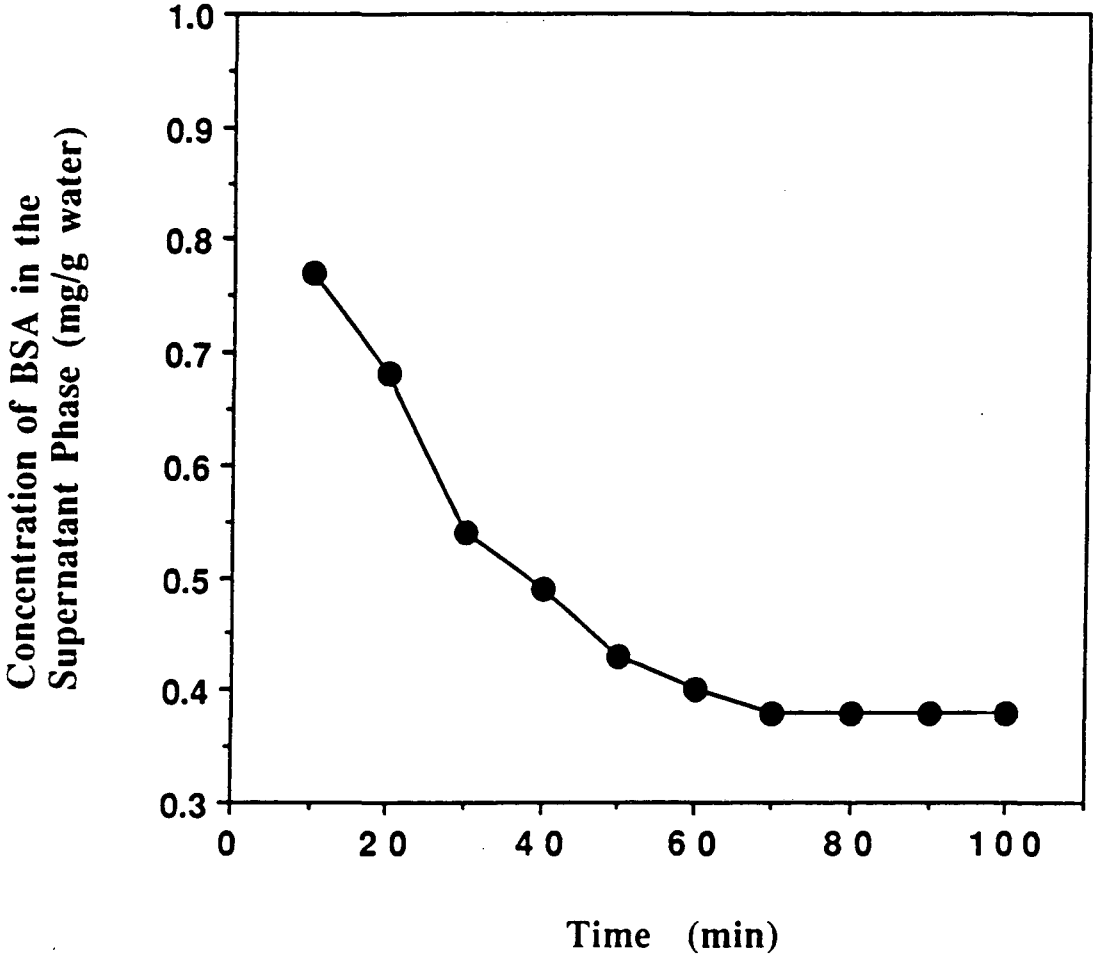
14 In 1.56 m pH 5 sodium sulfate buffer solution, both the concentrations of α-chymotrypsin in the supernatant (**I**) and precipitate (**II**) phases are proportional of the initial protein concentration. Therefore, the distribution coefficient (**III**) is a constant at fixed salt concentration and pH.

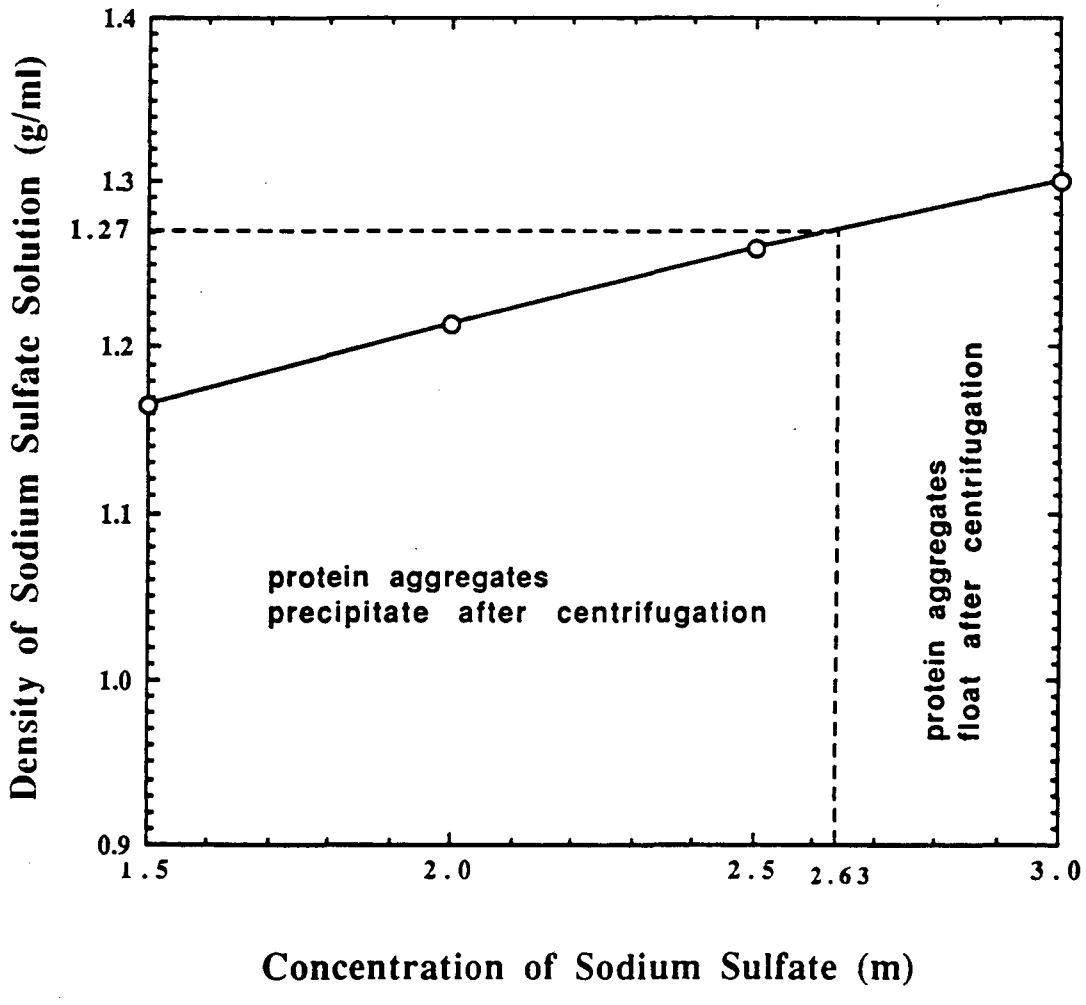

β
log S
Salting-in Region
Salting-out Region
Salt Concentration

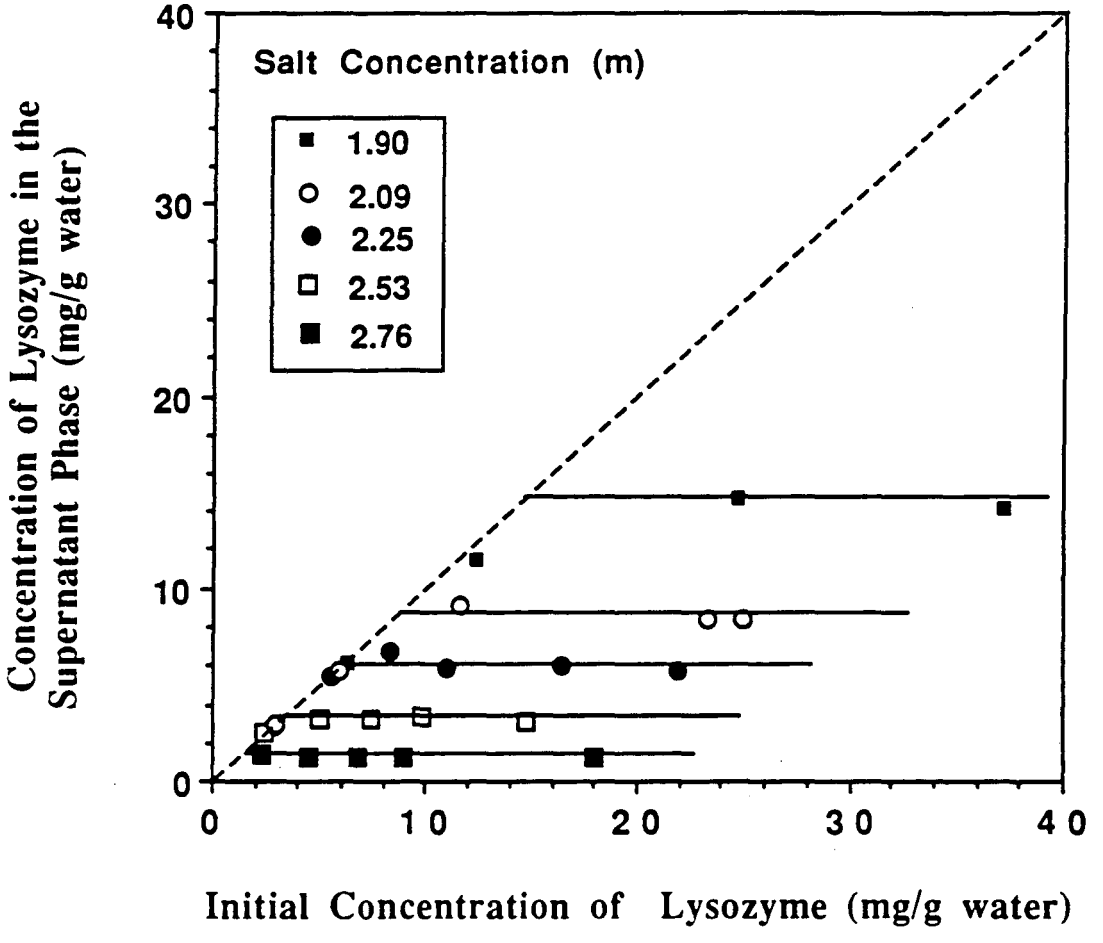

100
10
1
.1
0
1
2
3
4
pH
5
6
8
10
Concentration of Lysozyme in the Supernatant phase (mg/g water)
Ionic Strength (m)

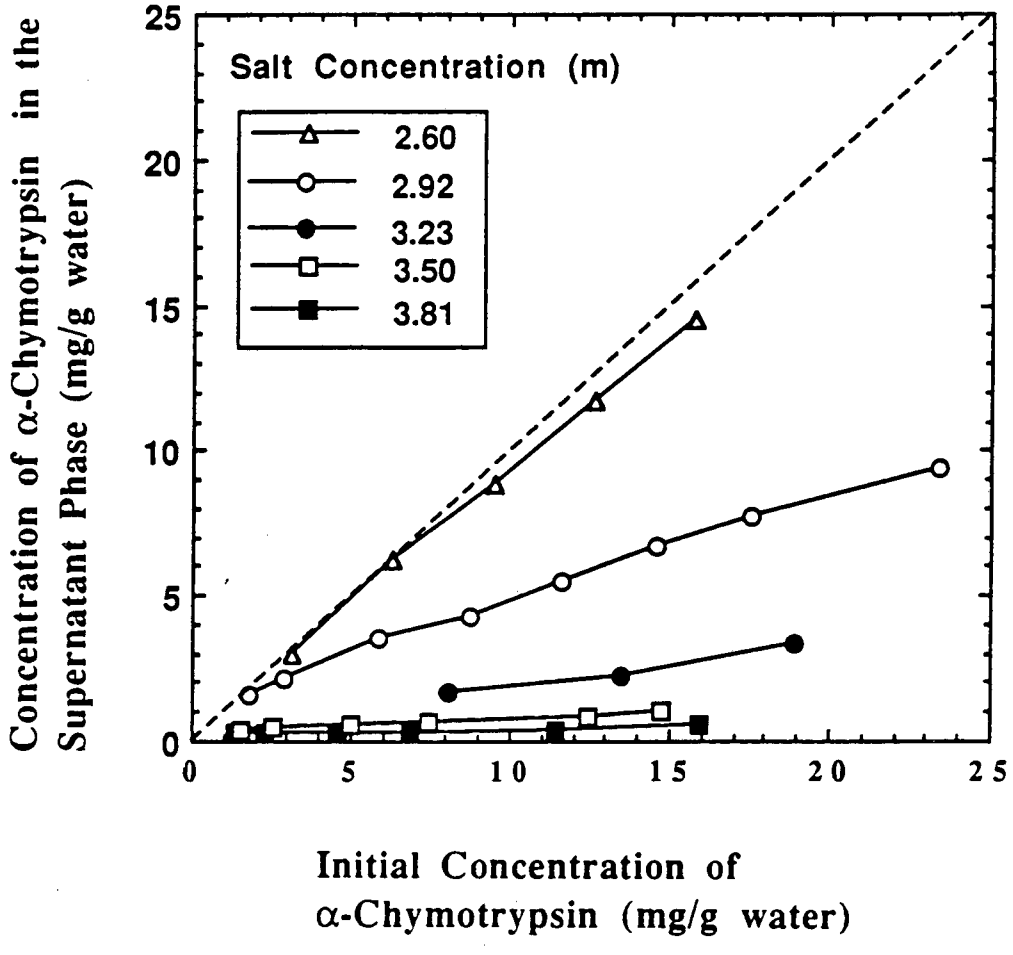

100
10
1
0.4
0.6
0.8
1.0
1.2
1.4
1.6
Crystalline
Amorphous
Solubility of Lysozyme (mg/g water)
Ionic Strength (m)

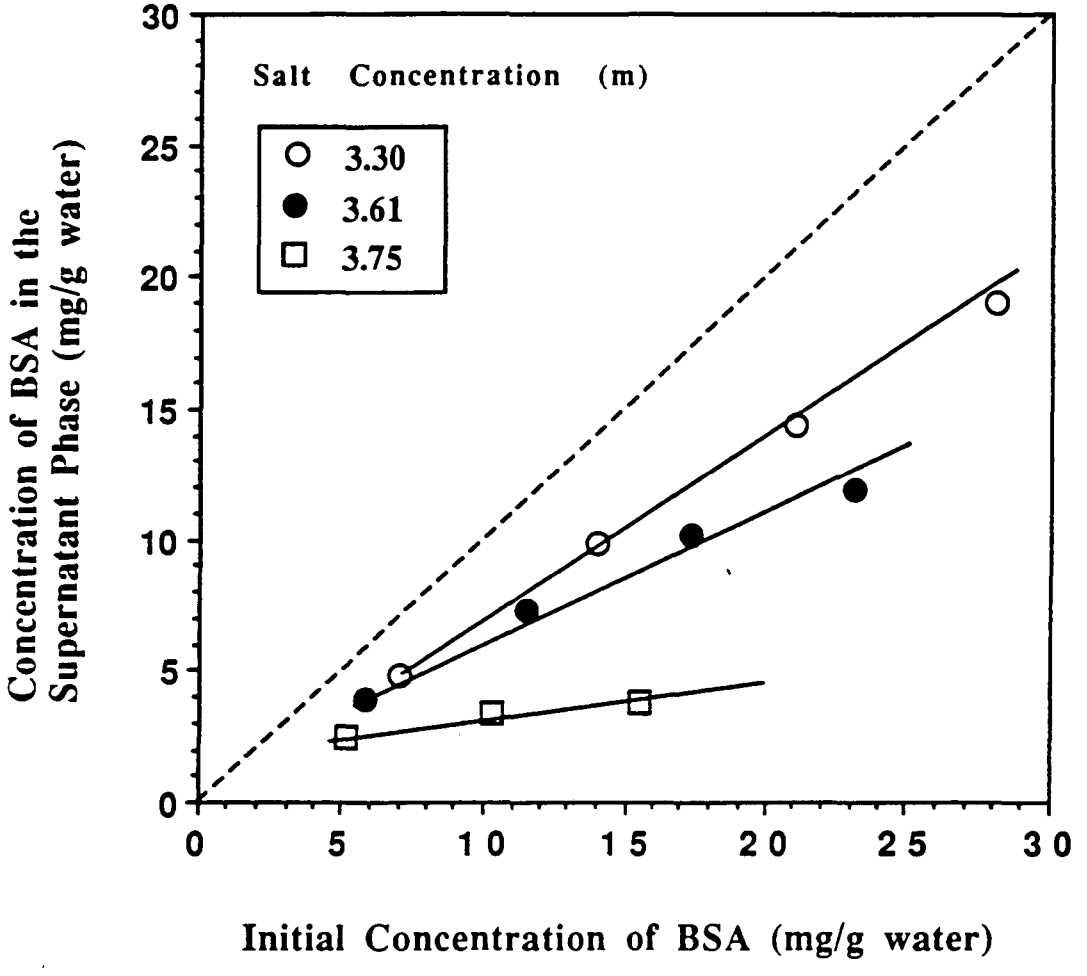

Ionic strength (m)
1.0
1.5
2.0
2.5
3.0
100
10
1
.1
3
4
5
6
7
8
9
10
11
12
pH
Concentration of Lysozyme in the Supernatant Phase (mg/g water)

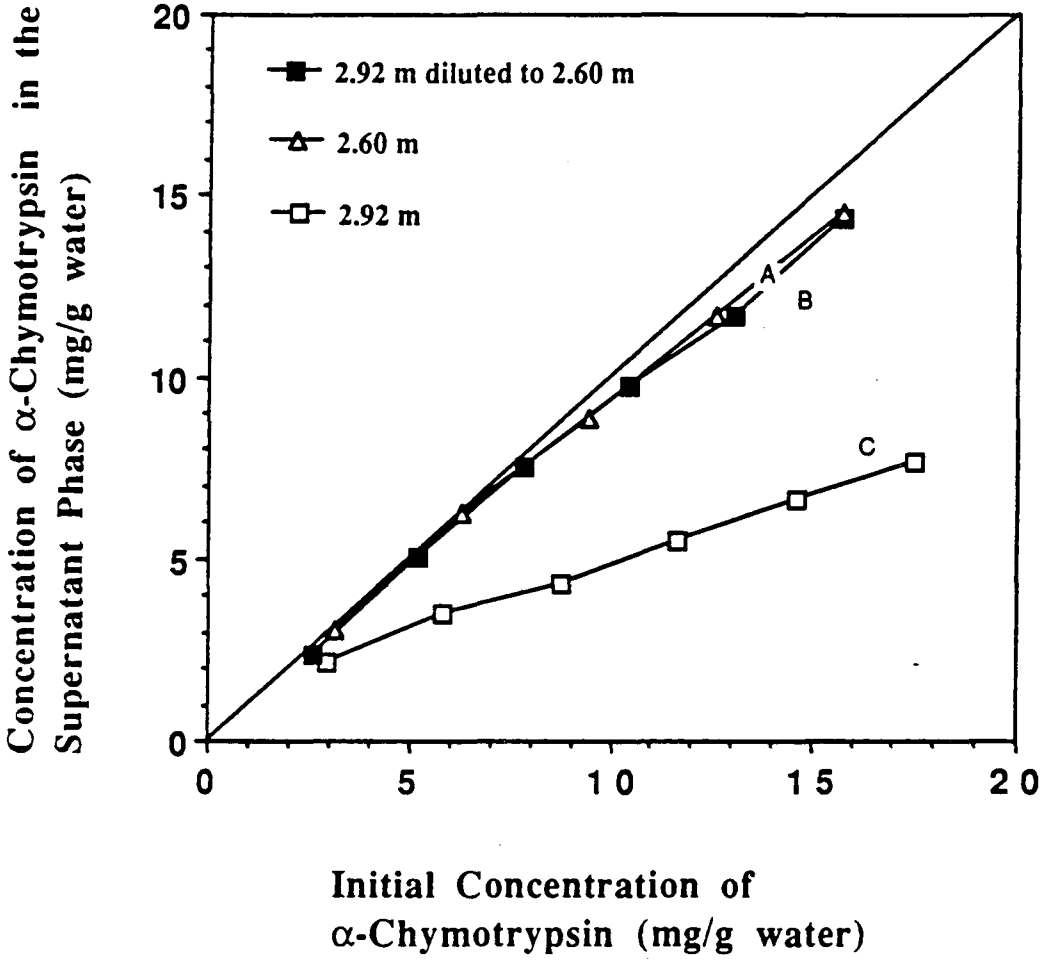

Concentration of BSA in the Supernatant Phase (mg/g water)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0
20
40
60
80
100
Time (min)

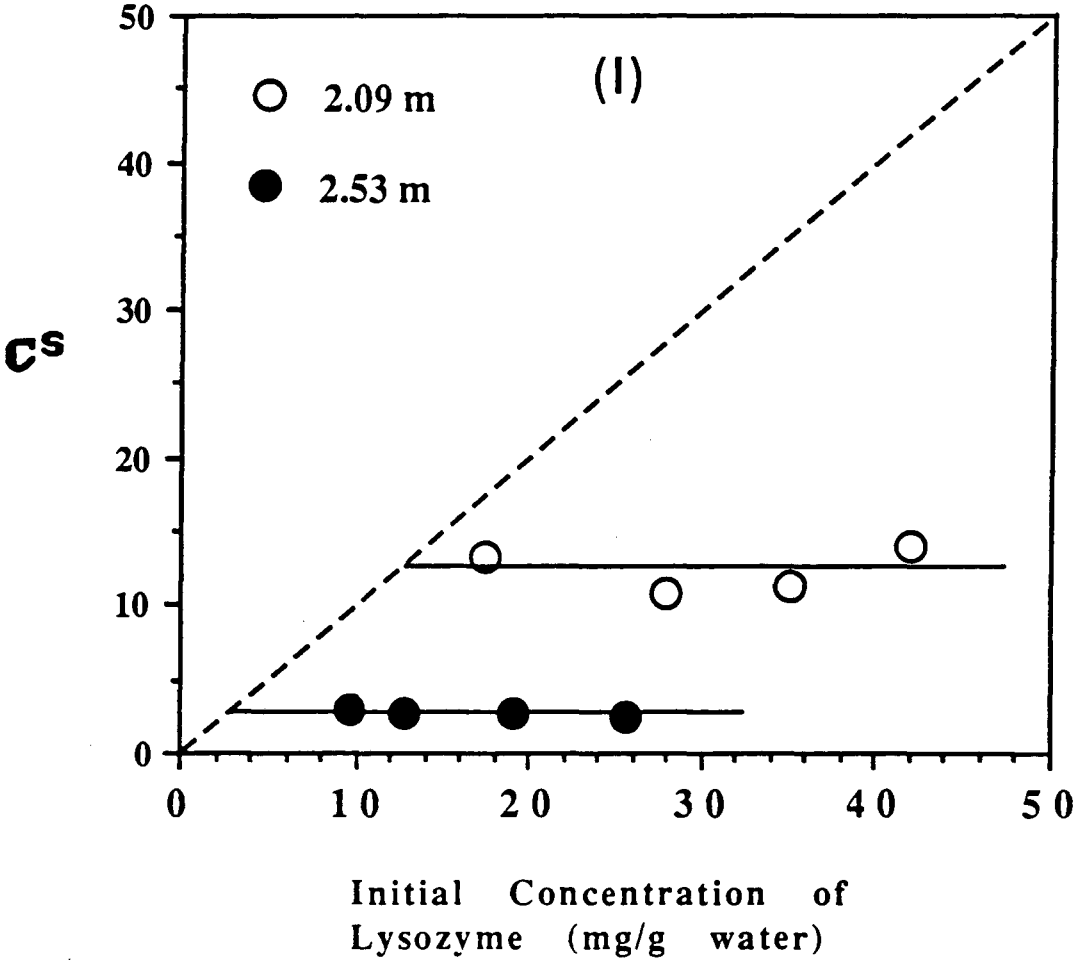

1.4
1.3
1.27
1.2
1.1
1.0
0.9
Density of Sodium Sulfate Solution (g/ml)
protein aggregates
precipitate after centrifugation
protein aggregates
float after centrifugation
1.5
2.0
2.5
2.63
3.0
Concentration of Sodium Sulfate (m)

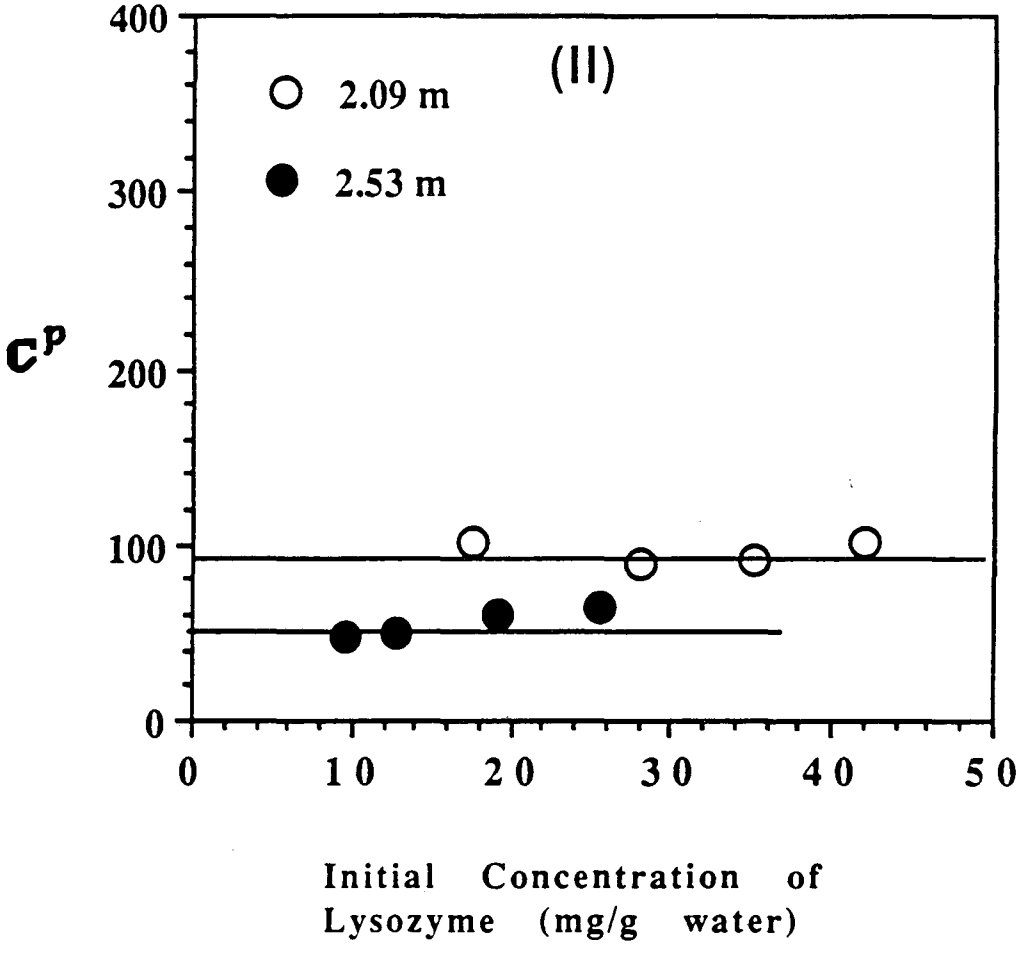

Salt Concentration (m)
1.90
2.09
2.25
2.53
2.76
Concentration of Lysozyme in the Supernatant Phase (mg/g water)
Initial Concentration of Lysozyme (mg/g water)
0
10
20
30
40

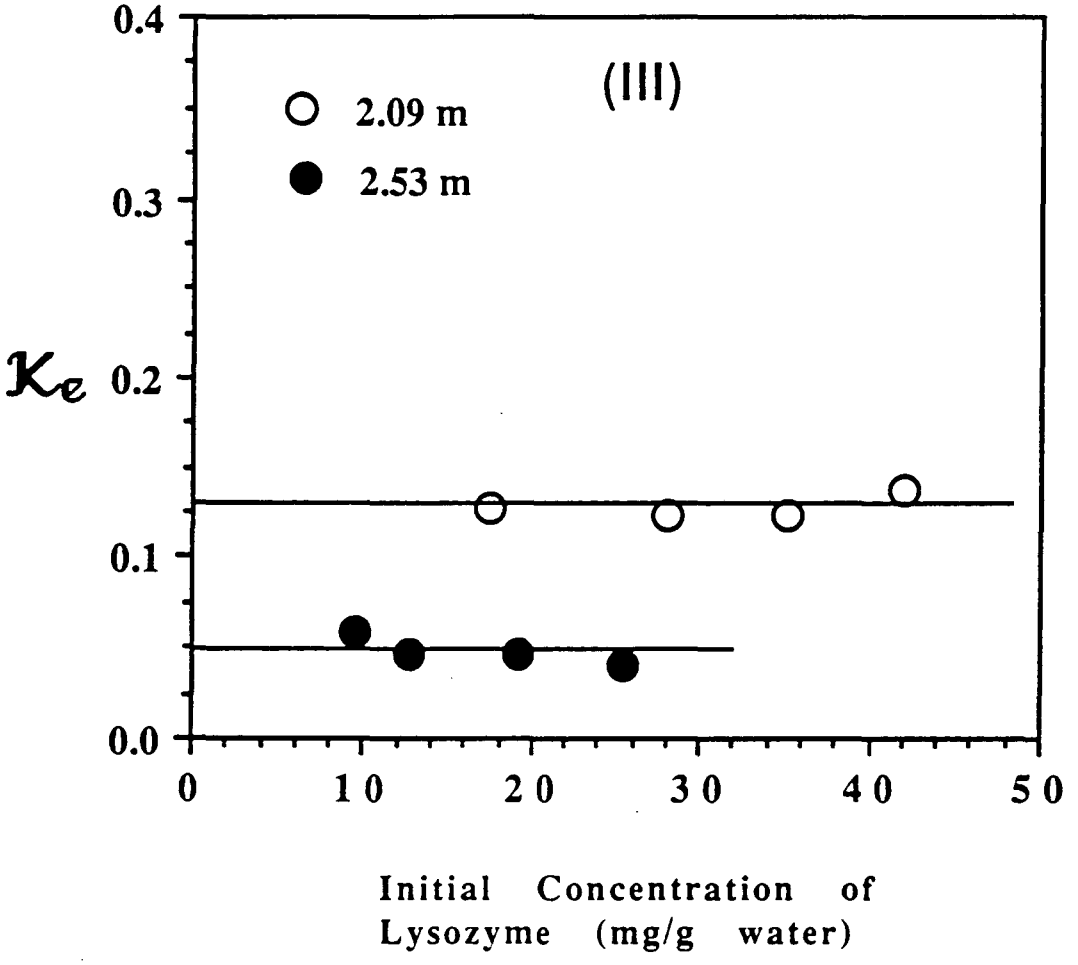

Salt Concentration (m)
2.60
2.92
3.23
3.50
3.81
Concentration of α-Chymotrypsin in the Supernatant Phase (mg/g water)
Initial Concentration of α-Chymotrypsin (mg/g water)
0
5
10
15
20
25

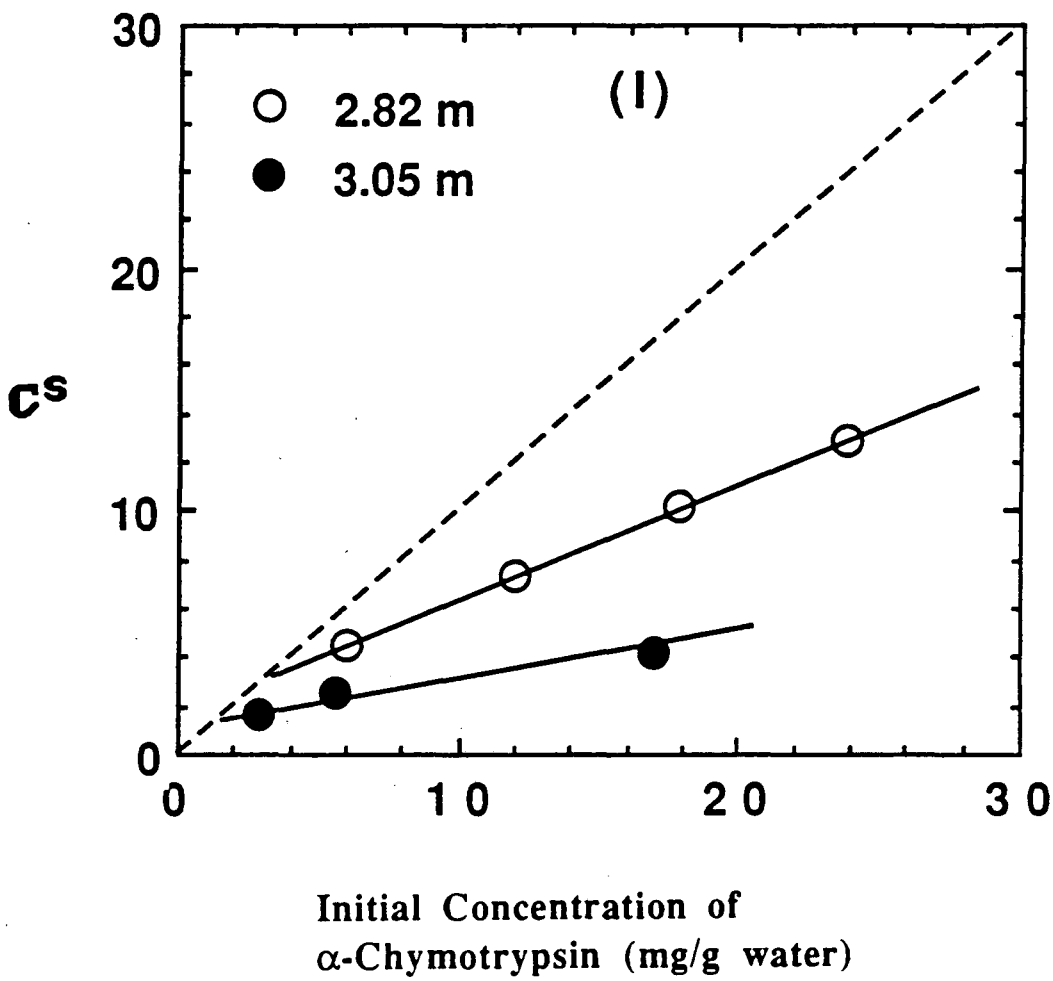

Salt Concentration (m)
3.30
3.61
3.75
Concentration of BSA in the Supernatant Phase (mg/g water)
Initial Concentration of BSA (mg/g water)
0
5
10
15
20
25
30

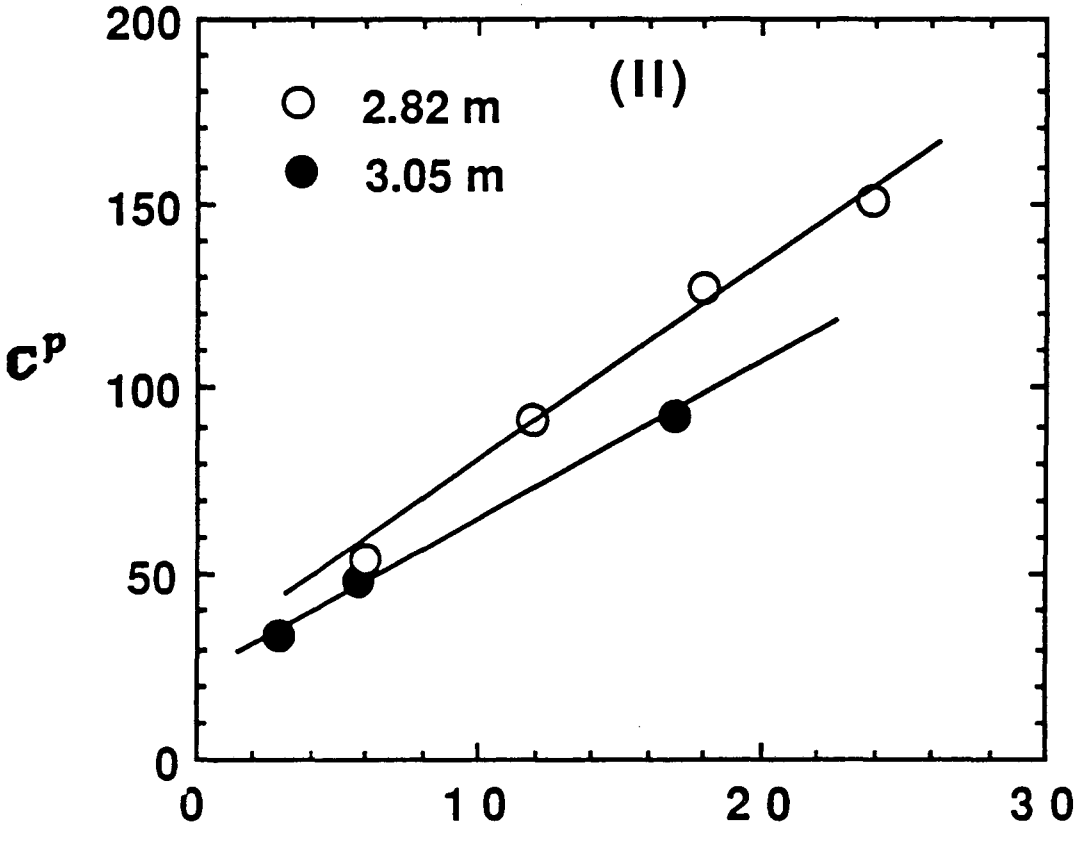

Concentration of α-Chymotrypsin in the Supernatant Phase (mg/g water)
20
15
10
5
0
2.92 m diluted to 2.60 m
2.60 m
2.92 m
A
B
C
0
5
10
15
20
Initial Concentration of α-Chymotrypsin (mg/g water)


50
40
30
20
10
0
$c^s$
(I)
2.09 m
2.53 m
0
10
20
30
40
50
Initial Concentration of
Lysozyme (mg/g water)

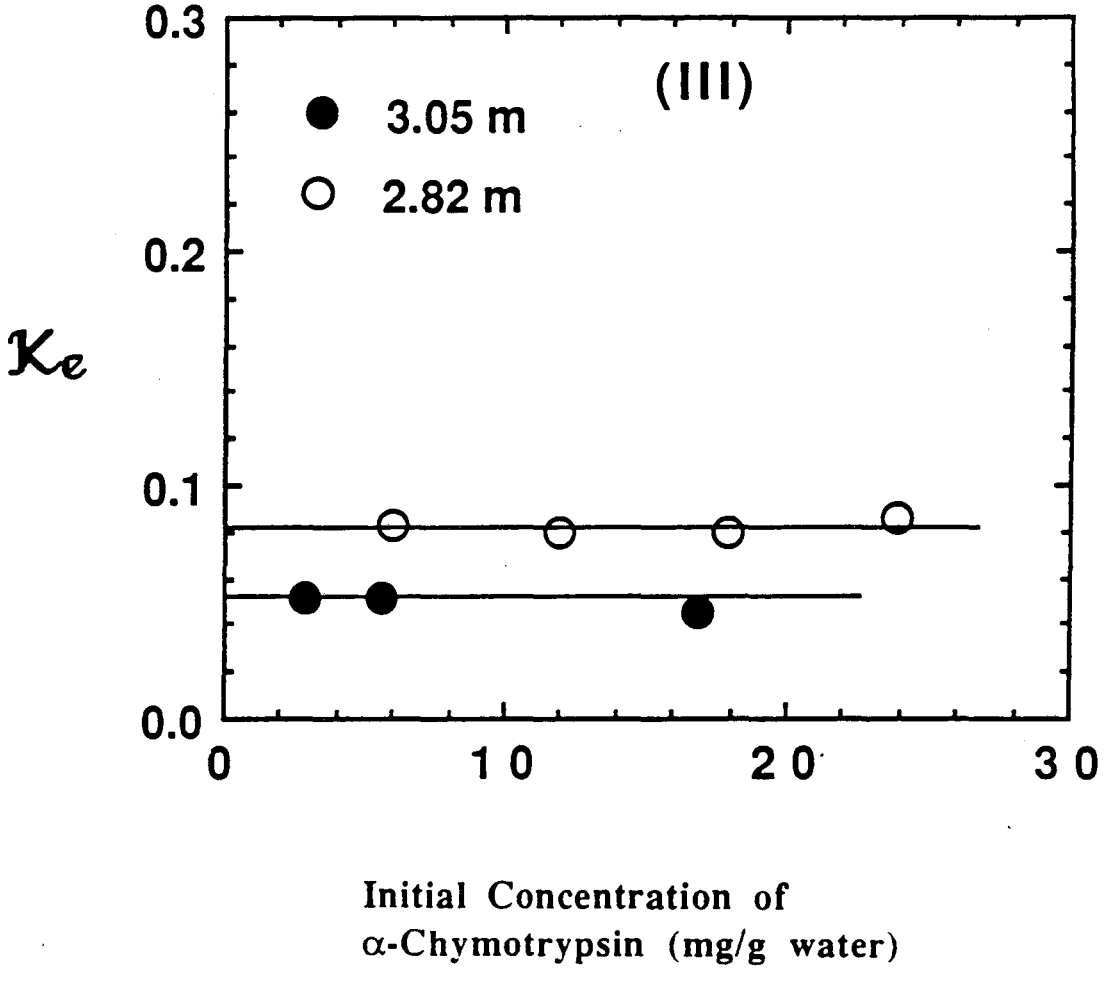

400
300
200
100
0
$C^p$
(II)
2.09 m
2.53 m
0
10
20
30
40
50
Initial Concentration of
Lysozyme (mg/g water)

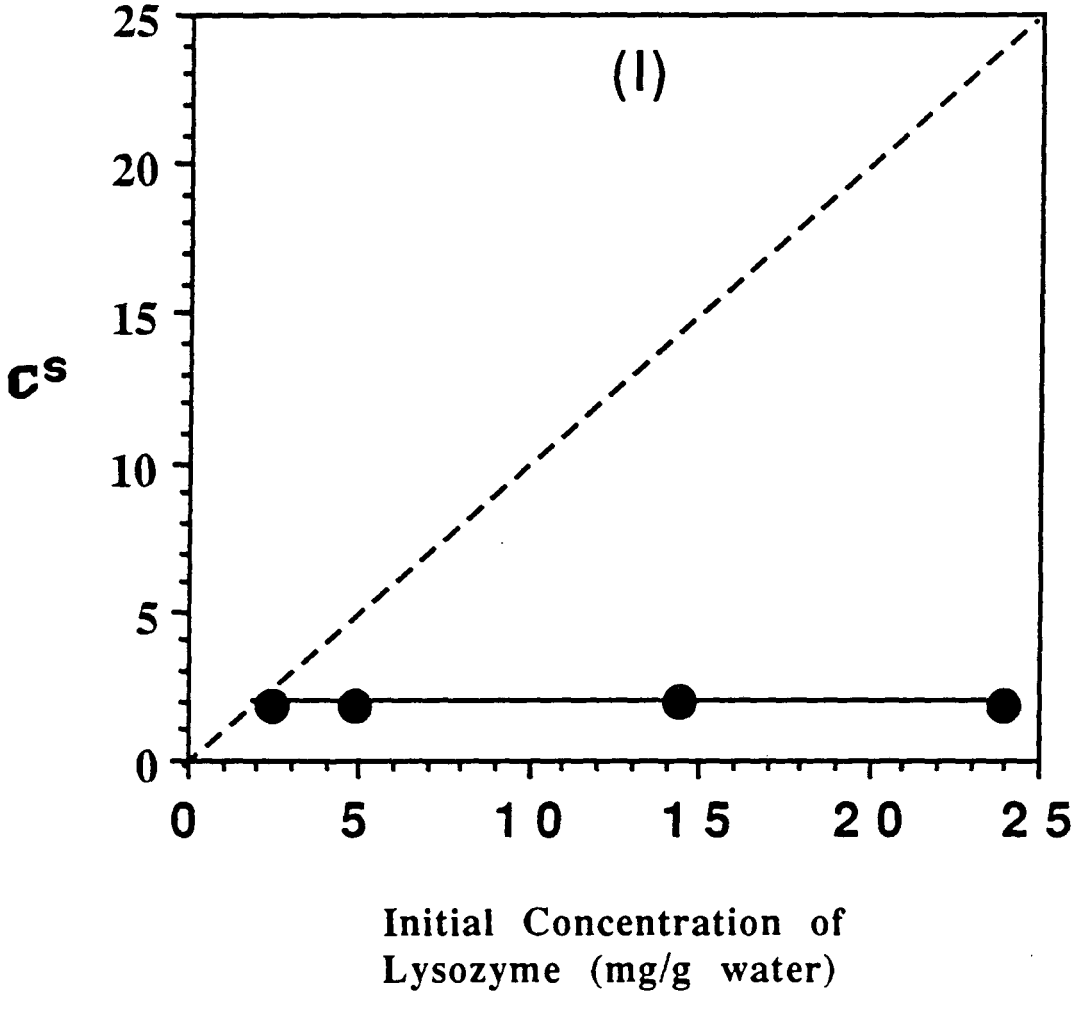

0.4
0.3
0.2
0.1
0.0
$K_e$
(III)
2.09 m
2.53 m
0
10
20
30
40
50
Initial Concentration of
Lysozyme (mg/g water)

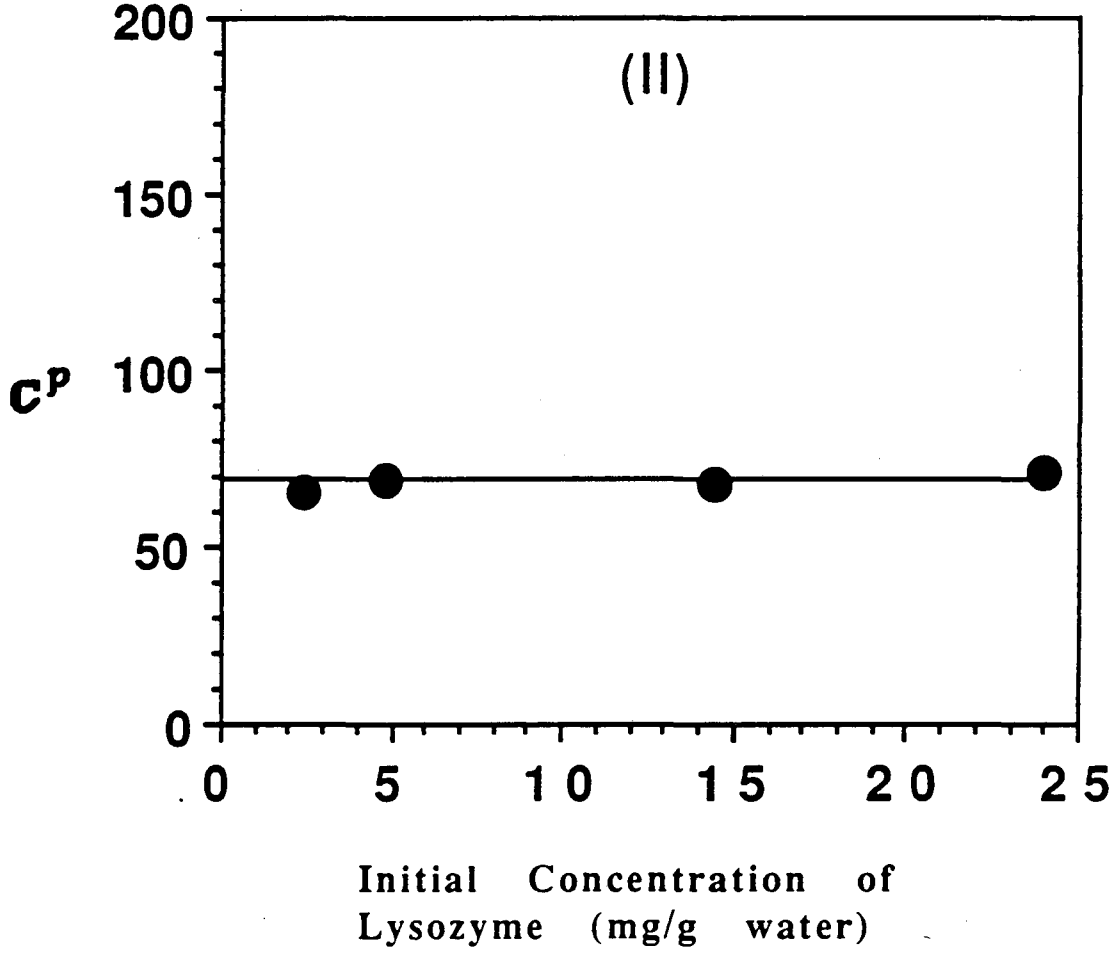

30
20
10
0
0
10
20
30
$c^s$
2.82 m
3.05 m
(I)
Initial Concentration of
α-Chymotrypsin (mg/g water)

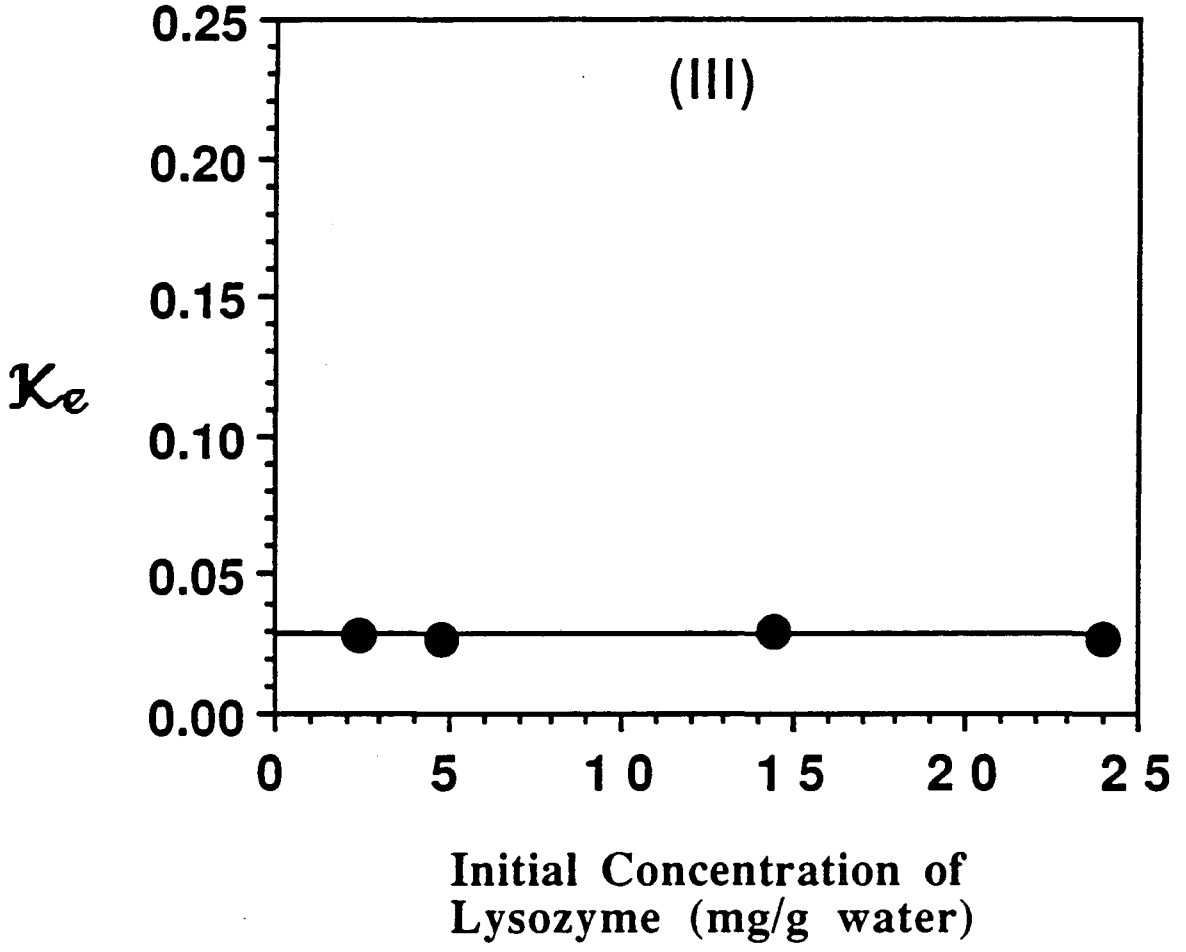


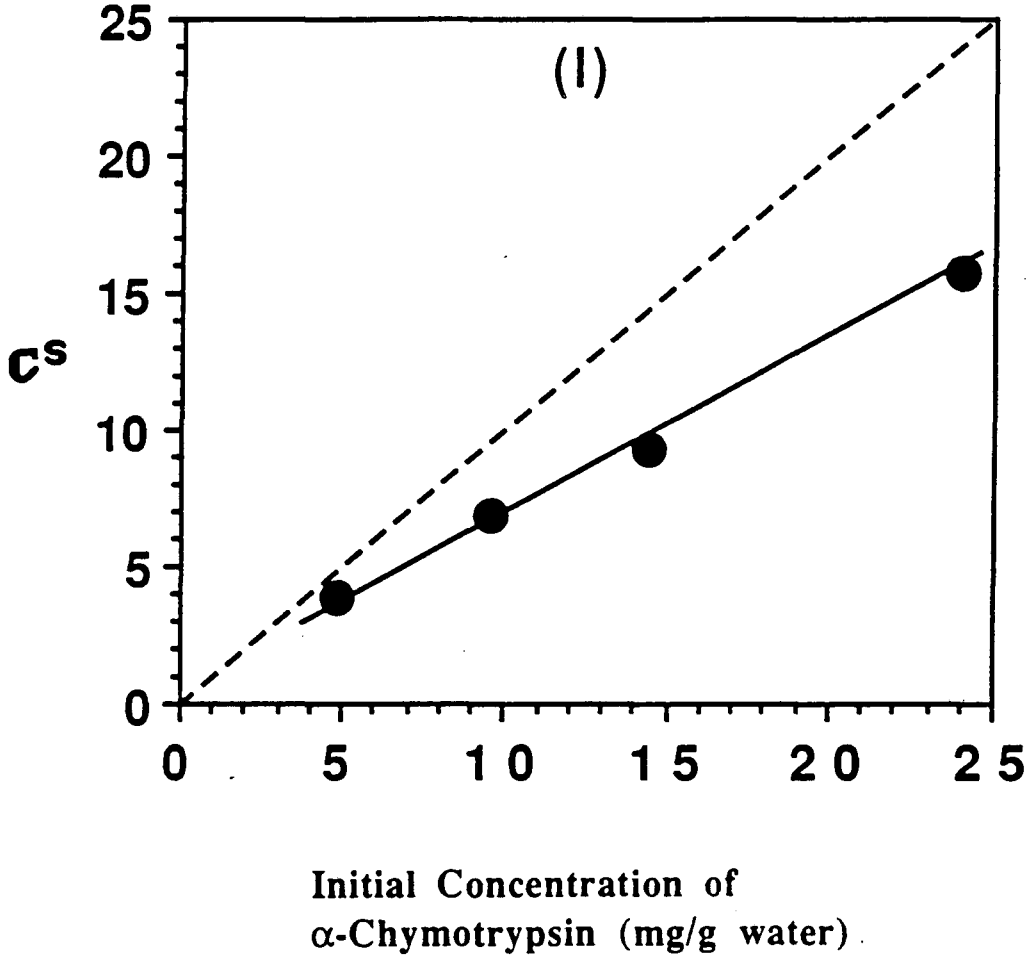

Initial Concentration of
α-Chymotrypsin (mg/g water)

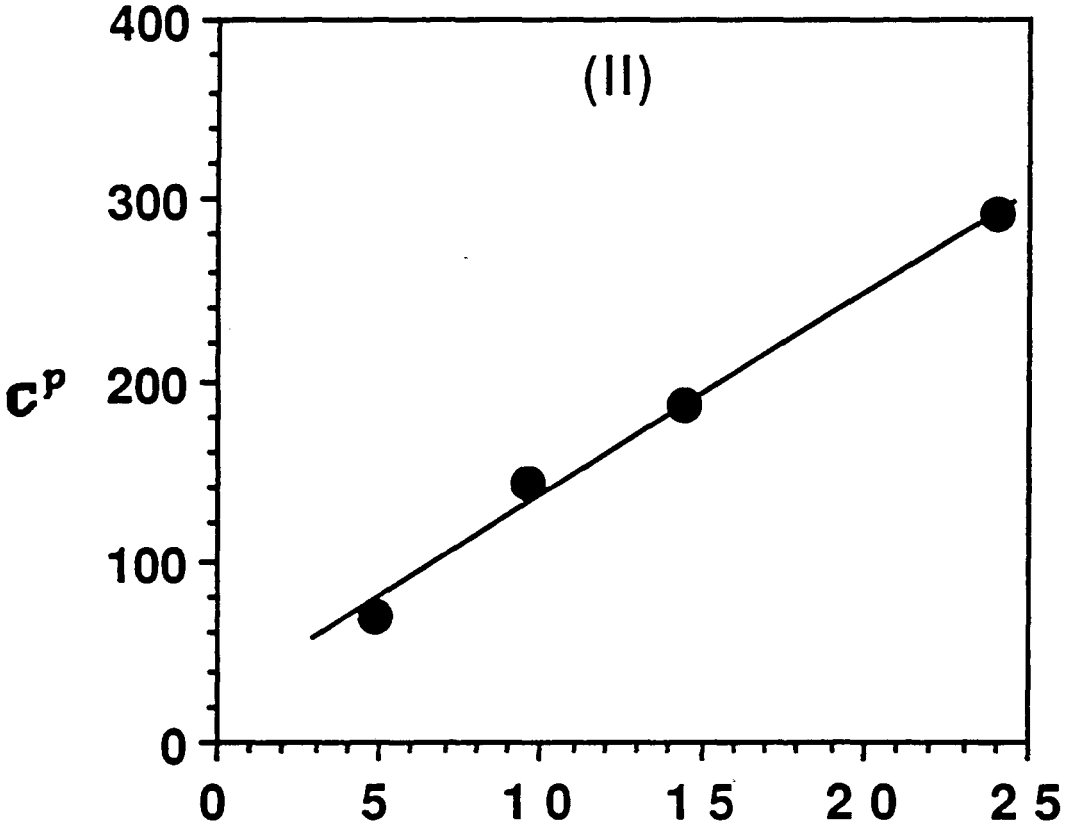

0.3
0.2
0.1
0.0
$K_e$
(III)
3.05 m
2.82 m
0
10
20
30
Initial Concentration of
α-Chymotrypsin (mg/g water)


(I)
$c^s$
25
20
15
10
5
0
0
5
10
15
20
25
Initial Concentration of
Lysozyme (mg/g water)

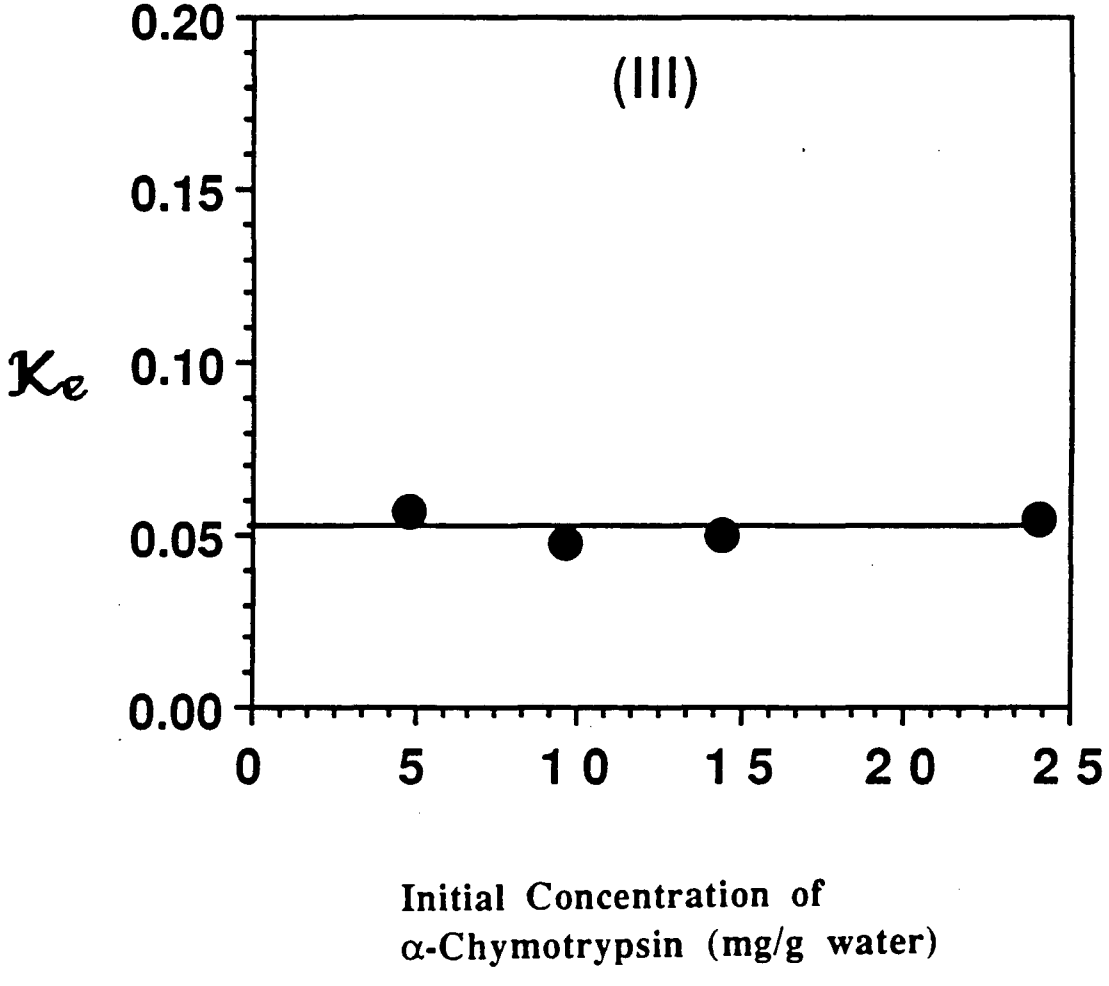

(II)
200
150
100
50
0
$C^p$
0
5
10
15
20
25
Initial Concentration of
Lysozyme (mg/g water)


(III)
0.25
0.20
0.15
0.10
0.05
0.00
$K_e$
0
5
10
15
20
25
Initial Concentration of
Lysozyme (mg/g water)


(I)
$c^s$
25
20
15
10
5
0
0
5
10
15
20
25
Initial Concentration of
α-Chymotrypsin (mg/g water)




Initial Concentration of
α-Chymotrypsin (mg/g water)


(III)
0.20
0.15
0.10
0.05
0.00
$K_e$
0
5
10
15
20
25
Initial Concentration of
α-Chymotrypsin (mg/g water)

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