LBL-38432
UC-401
Preprint

Lawrence Berkeley Laboratory

UNIVERSITY OF CALIFORNIA

CHEMICAL SCIENCES DIVISION

Submitted to Biotechnology and Bioengineering

Salting Out of Aqueous Proteins: Phase Equilibria in Two-Protein Systems

C.J. Coen, J.M. Prausnitz, and H.W. Blanch

February 1996

REFERENCE COPY
Does Not
Circulate
Bldg. 50 Library.
LBL-38432
Copy 1

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

DISCLAIMER

This document was prepared as an account of work sponsored by the United States Government. While this document is believed to contain correct information, neither the United States Government nor any agency thereof, nor the Regents of the University of California, nor any of their employees, makes any warranty, express or implied, or assumes any legal responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by its trade name, trademark, manufacturer, or otherwise, does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof, or the Regents of the University of California. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof or the Regents of the University of California.

LBL-38432
UC-401

Salting Out of Aqueous Proteins: Phase Equilibria in Two-Protein Systems

C. J. Coen, J. M. Prausnitz and H. W. Blanch

Department of Chemical Engineering
University of California
and
Chemical Sciences Division
Lawrence Berkeley Laboratory
University of California
Berkeley, CA 94720

February 1996

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 No. DE-AC03-76SF00098.

Salting Out of Aqueous Proteins: Phase Equilibria in Two-Protein Systems

C. J. Coen[1], J. M. Prausnitz[1,2], H. W. Blanch[1]

(1) Department of Chemical Engineering
University of California, Berkeley

(2) Chemical Sciences Division
Lawrence Berkeley Laboratory
University of California, Berkeley
Berkeley, CA 94720

Phone: (510) 642-1387
FAX: (510) 642-4778

Running Title: Protein Salting Out

Keywords: salting out, precipitation, protein separation, protein association, lysozyme, α-chymotrypsin, ovalbumin

Summary

The phase behavior of two aqueous binary protein mixtures, lysozyme-chymotrypsin and lysozyme-ovalbumin, was determined in ammonium-sulfate solutions. Protein concentrations were determined in both phases as a function of pH and ionic strength. For lysozyme-chymotrypsin mixtures, the observed phase behavior is similar to that for each individual protein; the presence of the second protein has little influence. The phase behavior of lysozyme-ovalbumin mixtures, however, is dramatically different from that of the respective single-protein systems. Lysozyme and ovalbumin are found together in egg whites; their association is both pH and ionic-strength dependent. The association of proteins is a key determinant of protein solubility in salt solutions.

Introduction

Protein precipitation provides a common method for concentrating and purifying proteins from solution. A variety of precipitating agents can be used to precipitate proteins selectively. Different precipitation methods, including addition of salts, hydrophilic polymers, polyelectrolytes, dyes, and metal-ion affinity ligands are described in several reviews.[3, 22, 27] Precipitation by addition of salts, in particular ammonium sulfate, remains one of the most widely used methods for crude purifications of proteins from solution.

Several studies have investigated the phase behavior of single-protein solutions at high salt concentrations.[1, 6, 7, 12, 23, 28, 29] Data on multi-protein salting-out systems, however, are rare. Dixon and Webb[9] described a simple method for optimizing the purification of a target protein from other proteins assuming that no cross-protein interactions exist and that phase behavior for all proteins is consistent with the Cohn equation:

$$\log \frac{S}{S_0} = \beta - K_s I \qquad (1)$$

where S is the protein solubility in salt solution, S_0 is the protein solubility in water, I is the ionic strength, and β and K_s are empirical parameters fit to experimental supernatant-phase protein-concentration data. Richardson et al.[24] developed a method to salt out alcohol dehydrogenase from clarified yeast extract using ammonium sulfate. They provide guidelines to estimate the achievable selectivity by salting-out.

Toward better understanding of how salting-out separations may be used selectively, we investigate the salting-out phase behavior for two aqueous binary mixtures: lysozyme and chymotrypsin, and lysozyme and ovalbumin. The first of these mixtures does not naturally occur in biological systems; however, the second mixture (hen-egg-white lysozyme and hen-egg ovalbumin) is a combination that exists in egg whites. Approximately 60% of egg-white protein is ovalbumin and about 4% is lysozyme.[2, 13] Furthermore, these two proteins are known to have specific interactions with each other in solution; they have been extensively studied, especially in

regard to processing of eggs in the food-processing industry[18, 26] under conditions such as high temperatures that denature proteins. The interactions of the native forms of lysozyme and ovalbumin have also been studied and are reviewed below. Initial studies of the effect of the interaction of lysozyme and bovine serum albumin[31] and of lysozyme and ovalbumin[16] on protein solubility suggest that association may play a major role in determining the phase behavior of protein mixtures.

Although the precise nature of the lysozyme-ovalbumin association is not well understood, several studies have measured the apparent equilibrium binding constant for a 1:1 lysozyme-ovalbumin association at neutral pH and low ionic strength. The equilibrium constant was found by ultracentrifugation to be approximately 10^5 M^{-1} at $I = 0.02$ M and pH 6.8-6.9.[5, 15] As the protein concentrations increased, aggregates larger than the 1:1 complex were observed.[15] In addition to the 1:1 association, some authors postulate that the association of lysozyme and ovalbumin at neutral pH may proceed to longer chains of lysozyme and ovalbumin which may also be crosslinked.[16] At a somewhat lower pH (pH 5.8), a smaller equilibrium binding constant of 2×10^3 M^{-1} at $I = 0.02$ M[19] was found by membrane osmometry. At this pH, aggregates larger than the 1:1 complex were not observed. The association was also found to be a strong function of pH in the range pH 5-9 with maximum association observed at neutral pH.[21] At a higher ionic strength ($I = 0.2$ M), lysozyme-ovalbumin association was not observed using ultracentrifugation.[5] This suggests that the association is primarily driven by the electrostatic interactions of the oppositely charged lysozyme and ovalbumin for which the net charges are reported as +7.5 for lysozyme and −14 for ovalbumin for pH 6.8.[16] These electrostatic attractions are screened at moderate ionic strengths.

This work reports measurements for salting-out phase behavior in ammonium-sulfate solutions as a function of solution pH and ionic strength for the following systems: 1) for single-protein systems containing lysozyme or ovalbumin and 2) for the binary-protein systems

lysozyme-chymotrypsin and lysozyme-ovalbumin. Attention is given to selectivity for protein salting-out phase separations.

Experimental

Materials

Bovine α-chymotrypsin (C-4129), hen-egg-white lysozyme (L-6876), hen ovalbumin (A-5503), and phenyl-methyl-sulfonyl fluoride (PMSF) were purchased from Sigma, St. Louis, MO. A.C.S.-grade ammonium sulfate was used. Distilled water was de-ionized and filtered (0.20 μm) by a NANOpure system prior to use.

Methods

Protein concentrations were determined by absorbance at 280 nm using a Milton-Roy 1201 Spectrophotometer. Extinction coefficients are: 2.04 L/(g cm) for chymotrypsin,[11] 2.635 L/(g cm) for lysozyme,[30] and 0.705 L/(g cm) for ovalbumin.[11] pH was measured using a Corning General-Purpose Combination electrode with a Sargent-Welch model LSX pH meter. All experiments were carried out at 25±0.1°C.

Protein Salting-Out Phase Equilibria

The procedure of Shih et al.[29] 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[10] with 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 experiments with $(NH_4)_2SO_4$). No buffers were required. 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.

Samples of the equilibrated supernatant and dense phases were diluted and analyzed by UV absorbance (280 nm) to determine protein concentration in mg protein/g water. Partitioning of salt between the dense and supernatant phases was uniform for all single-protein experiments reported here, in agreement with earlier results.[6] For most two-protein experiments, the salt partition coefficient was taken as 1.0 and was not measured. The water content of the dense phase was measured by weight loss after freeze drying for 40 hours at 40 mtorr. Mass balances on the phases closed to within ±5%. The typical experimental uncertainty of the partition coefficient, K, defined as the ratio of protein concentrations in the dense and supernatant phases, was ±10%.

$$K = \frac{c_{dp}}{c_{sp}} \tag{2}$$

For lysozyme-chymotrypsin mixtures, protein concentrations of both phases 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 Tris-HCl buffer at pH 7.3 and 0.05% (w/v) sodium azide as a preservative. 0.1 M NaCl eluted chymotrypsin and 0.5 M NaCl eluted 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 contents were measured as discussed above.

For phase separation of mixtures of lysozyme and ovalbumin, protein concentrations of both phases were determined by size-exclusion HPLC with UV detection at 280 nm. A Bio-Rad Bio Gel SEC 40-XL size-exclusion column (Bio-Rad, Richmond, CA) was used with the above HPLC system using the same Trizma buffer at pH 7.3. To prevent lysozyme-ovalbumin

associations which would interfere with the analysis, all elution buffers contained 0.5 M NaCl. This intermediate ionic strength may be important for obtaining reliable results from size-exclusion chromatography.[14,20] To obtain a linear detector response, dense-phase samples were diluted up to 100 fold. Due to the difficulty in resolubilizing the dense phase in either low or high-ionic-strength solutions, an aliquot of 5 m ionic strength ammonium sulfate solution was first mixed with the dense phase sample and then water was added dropwise until the solution clarified. At the final ionic strength it was assumed that the lysozyme-ovalbumin association was minimized so that the size-exclusion separation would give accurate results. Higher ionic strengths are also typically used in separations of lysozyme and ovalbumin from egg whites.[2]

Results and Discussion

Phase-Equilibrium Measurements: Single-Protein Systems

Figure 1 illustrates salting-out phase equilibria for lysozyme in ammonium-sulfate solutions. Supernatant (c_{sp}) and dense phase (c_{dp}) protein concentrations along with the resulting protein partition coefficients (K) are shown. The initial lysozyme concentration, c_{init} was varied from 20 to 50mg/g water without affecting lysozyme phase behavior. The current results are in agreement with our previous work.[6]

Figure 2 illustrates the ovalbumin phase equilibria in ammonium-sulfate solutions. The initial protein concentration was fixed at 30 mg/g water. Ammonium sulfate partitioned uniformly between the supernatant and dense phases. Dense-phase protein concentrations were nearly invariant with respect to pH and ionic strength. The partition coefficient is influenced primarily by the dependence of the supernatant protein concentration on pH and ionic strength. The supernatant concentrations follow the classical ionic-strength salting-out behavior described by Eq. 1. Ovalbumin phase equilibria exhibit a stronger pH dependence than lysozyme or chymotrypsin phase equilibria. Ovalbumin supernatant phase concentrations decrease as pH decreases, similar to the behavior of lysozyme and chymotrypsin, except that a minimum in

ovalbumin supernatant concentration is observed at pH 4 and ionic strengths from 7 to 9 molal. Additional ovalbumin phase-equilibria measurements at pH between 4 and 5 confirmed that the maximum partitioning to the dense phase occurred at pH 4 (or lower) rather than at its isoelectric point of pH 4.6.

Phase-Equilibrium Measurements: Two-Protein Mixtures

Ammonium-sulfate salting-out equilibria were measured for mixtures of lysozyme and chymotrypsin. The initial protein concentrations were 20 mg/g water for both proteins. For both lysozyme and chymotrypsin, Figure 3 shows supernatant protein concentrations and Figure 4 shows protein partition coefficients as a function of pH with ionic strength as a parameter. Phase behavior with respect to pH and ionic strength appears similar to that of the single-protein salting-out phase equilibria given in our previous work.[6]

However, upon quantitative comparison of the two-protein salting-out data with the individual single-protein salting-out data for lysozyme and chymotrypsin, it is apparent that there are systematic differences in the phase behavior of each protein when the other protein is present in solution. Figure 5 shows the ratio of the two-protein partition coefficient ($K_{2\text{-}protein}$) to single-protein partition coefficient ($K_{1\text{-}protein}$) as a function of pH and ionic strength. Within experimental error, the ratio of partition coefficients for both proteins is independent of both solution pH and ionic strength. However, lysozyme partitioning is decreased in the presence of chymotrypsin while chymotrypsin partitioning is enhanced by the presence of lysozyme in solution. Generally, it has been observed that the solubility of a protein is reduced in the presence of other proteins,[17] although studies have shown that single-protein and mixed-protein systems had nearly identical salting-out behavior for the case of dog hemoglobin and horse serum albumin.[9, 25] It appears that the larger protein (molecular weights: chymotrypsin, 25,000 g/mol;[11] lysozyme, 14,300 g/mol[8]) partitions more strongly to the dense phase from a mixture of two proteins.

In Figure 5, the chymotrypsin single-protein results at an initial protein concentration of 30 mg/g water were corrected to an initial protein concentration of 20 mg/g water using experimental data on the effect of initial protein concentration on protein partition coefficients. Chymotrypsin single-protein partition coefficients at c_{init} = 30 mg/g water are about 90% of the partition coefficients at c_{init} = 20 mg/g water. Other studies[9, 24] have examined the effects of initial protein concentration on mixed-protein solution salting-out behavior and have observed significant changes in salting-out only when initial protein concentrations differed by a factor of 10.

For the lysozyme-chymotrypsin system, Figure 6 shows that partitioning of lysozyme to the dense phase is favored over chymotrypsin partitioning at all solution conditions investigated. For pH 5.5-8.3, the selectivity is approximately constant at 2. However, at pH 4, the selectivity is ionic strength dependent and ranges from 2 to 12. At pH 4, the tradeoff between purity of the target protein and the fraction of the target protein recovered is evident. At pH 4 and low ionic strength, lysozyme is partitioned strongly to the dense phase and chymotrypsin remains primarily in the supernatant phase. Lysozyme is selectively salted out under these conditions, yet a small amount of lysozyme remains in the supernatant solution. On the other hand, salting out at pH 4 and a higher ionic strength recovers more of the lysozyme from the supernatant phase while at the same time increasing the amount chymotrypsin partitioned to the dense phase.

Phase-equilibrium measurements were also carried out for mixtures of lysozyme and ovalbumin salted out by ammonium sulfate. For both lysozyme and ovalbumin, the initial protein concentration was 20 mg/g water. Figures 7 and 8 show supernatant protein concentrations and protein partition coefficients, respectively, for both lysozyme and ovalbumin. Protein concentration measurements were reproducible, except for ovalbumin at pH 4 and high ionic strengths. While Figures 7 and 8 indicate that ovalbumin's phase behavior in a mixture with lysozyme is similar to that of ovalbumin alone, these figures indicate that lysozyme phase behavior in the presence of ovalbumin is less pH-dependent than that of lysozyme alone.

In Figure 9, the ratios of two-protein to single-protein partition coefficients for lysozyme and ovalbumin reveal the dramatic effect of ovalbumin on the salting-out behavior of lysozyme. Ovalbumin single-protein results at c_{init} = 30 mg/g water were normalized to c_{init} = 20 mg/g water. Ovalbumin single-protein partition coefficients at c_{init} = 30 mg/g water are about 95% of the partition coefficients at c_{init} = 20 mg/g water. Figure 9(a) shows that, at high pH, lysozyme partitioning is similar to partitioning measured in the absence of ovalbumin; however, at pH 3, lysozyme partition coefficients are reduced to one-fifth of the single-protein partition coefficients. For ovalbumin, on the other hand, Figure 9(b) shows a slight enhancement of partitioning to the dense phase in the presence of lysozyme across the entire measured pH range. Within experimental error, ovalbumin salting-out phase behavior is not as strongly affected by the presence of lysozyme in solution as lysozyme phase behavior is affected by the presence of ovalbumin.

Below pH 4, where interactions between lysozyme and ovalbumin are reduced,[21] lysozyme partitioning is decreased by the presence of ovalbumin in solution. This decrease is similar to the effect of chymotrypsin on lysozyme phase behavior. In the lysozyme-ovalbumin mixture, partitioning of the larger protein (ovalbumin 43,000 g/mol[4]) is enhanced by the presence of lysozyme, while partitioning of lysozyme is decreased in the presence of the larger protein. Also, ovalbumin's partitioning is enhanced by the presence of lysozyme just as chymotrypsin's partitioning is enhanced in the presence of lysozyme. However, above pH 4, where lysozyme and ovalbumin have opposite net charges and are observed to associate in low ionic strength solutions, lysozyme partitioning is no longer reduced by the presence of ovalbumin.

Figure 10 shows that the selectivity for lysozyme from lysozyme-ovalbumin mixtures has a more complicated dependence on pH than does selectivity for the lysozyme-chymotrypsin mixture. For all ionic strengths, the highest selectivity for lysozyme is at pH 7. However, at pH 3, lysozyme is selectively partitioned at I = 7m, while ovalbumin is selectively partitioned at

$I = 9$m. As observed at lower pH in the lysozyme-chymotrypsin system, selectivity is a function of ionic strength for the lysozyme-ovalbumin system at lower pH's.

At a ratio of 3 moles of lysozyme to 1 mole of ovalbumin, a maximum in solution turbidity was found[16] for low ionic strength at neutral pH. The authors postulated that a complex containing a ratio of three lysozymes for each ovalbumin would thus give the lowest solubility. This may correspond to an indefinite association of lysozyme and ovalbumin, where four lysozymes are coordinated with each ovalbumin and lysozyme is bi-functional and can bind to two ovalbumin molecules. The experiments reported here were conducted at equal weight concentrations of lysozyme and ovalbumin, corresponding to a three-to-one mole ratio of lysozyme to ovalbumin. Thus, our experimental observations may give a maximum in salting out of protein due to this three-to-one complex.

Dixon and Webb[9] do not consider specific protein interactions to be significant at high ionic strengths due to electrostatic screening. However, our two-protein phase equilibria data show that specific protein interactions may persist even at the high ionic strengths used in salting-out separations. It appears that, despite high ionic strengths, the close proximity of protein molecules (the result of both high protein concentrations and hydrophobic attractions among proteins that are encouraged by the sequestering of water by salt ions) may allow protein-protein surface electrostatic interactions to play a role in determining protein salting-out behavior.

Conclusions

In binary protein mixtures, it appears that, relative to single-protein phase behavior, larger proteins partition more strongly to the dense phase. At the same time, partitioning of the smaller protein decreases. For lysozyme-ovalbumin mixtures that are known to associate at low ionic strengths (apparently via electrostatic attractions), it appears that pH-dependent protein-protein interactions affect salting-out phase equilibria even at very high ionic strengths. Because salting

out provides a reasonable degree of selectivity, it provides a useful method that not only concentrates proteins, but that also purifies a target protein from an aqueous mixture of proteins.

Acknowledgments

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 No. DE-AC03-76SF00098.

Notation

c_{init}	initial protein concentration, mg/g water
c_{dp}	protein concentration in dense phase, mg/g water
c_{sp}	protein concentration in supernatant phase, mg/g water
I	Ionic strength, m (mol/kg water)
K	protein partition coefficient = c_{dp} / c_{sp}
$K_{2\text{-}protein}$	protein partition coefficient measured for a protein salted out of a 2-protein mixture
$K_{1\text{-}protein}$	protein partition coefficient measured for a protein salted out of a single-protein solution
K_s	salting-out parameter, m^{-1}
S	protein solubility, mg/g water
S_0	protein solubility in salt solution, mg/g water
β	salting-out parameter

References

1. Arakawa, T., Timasheff, S. N. 1985. Theory of Protein Solubility. Methods in Enzymology. 114: 49.

2. Awadé, A. C., Moreau, S., Mollé, D., Brulé, G., Maubois, J.-L. 1994. Two-Step Chromatographic Procedure for the Purification of Hen Egg White Ovomucin, Lysozyme, Ovotransferrin and Ovalbumin and Characterization of Purified Proteins. J. Chromatogr., A. 677: 279.

3. Bell, D. J., Hoare, M., Dunnill, P. 1983. The Formation of Protein Precipitates and Their Centrifugal Recovery. Adv. in Biochem. Eng. Technol. 26: 1.

4. Castellino, F. J., Barker, R. 1968. Examination of the Dissociation of Multichain Proteins in Guanidine Hydrochloride by Membrane Osmometry. Biochemistry. 7: 2207.

5. Clarke, R. G., Howlette, G. J. 1979. Determination of the Molecular Weight of Proteins in Heterogeneous Mixtures: Use of an Air-Driven Ultracentrifuge for the Analysis of Protein-Protein Interactions. Arch. Biochem. Biophys. 195: 235.

6. Coen, C. J., Blanch, H. W., Prausnitz, J. M. 1995. Salting Out of Aqueous Proteins: Phase Equilibria and Intermolecular Potentials. AIChE J. 41: 996.

7. Cohn, E. J., Edsall, J. T., Ed., 1943. Proteins, Amino Acids and Peptides. Reinhold, New York.

8. Dayhoff, M. O., Eck, R. V. 1967. Atlas of Protein Sequence and Structure. National Biomedical Research Foundation, Silver Spring, MD.

9. Dixon, M., Webb, E. C. 1961. Enzyme Fractionation by Salting-Out: A Theoretical Note. Adv. Prot. Chem. 16: 197.

10. Fahrney, D. E., Gold, A. M. 1963. Sulfonyl Fluorides as Inhibitors of Esterases. I. Rates of Reaction with Acetylcholinesterase, α-Chymotrypsin and Trypsin. J. Amer. Chem. Soc. 85: 997.

11. Fasman, G. D., Ed., 1989. Practical Handbook of Biochemistry and Molecular Biology. CRC Press, Boca Raton.

12. Green, A. A. 1931. Studies in the Physical Chemistry of the Proteins. VIII. The Solubility of Hemoglobin in Concentrated Salt Solutions. A Study of the Salting Out of Proteins. J. Biol. Chem. 93: 495.

13. Hegg, P.-O. 1979. Precipitation of Egg White Proteins Below Their Isoelectric Points by Sodium Dodecyl Sulfate and Temperature. Biochim. Biophys. Acta. 579: 73.

14. Herold, M., "Size Exclusion Chromatography of Proteins: Influence of Salt Concentration in the Mobile Phase," *Hewlett-Packard Application Note*, publication number 12-5091-2735E (1991).

15. Howlette, G. J., Nichol, L. W. 1973. A Sedimentation Equilibrium Study of the Interaction between Ovalbumin and Lysozyme. J. Biol. Chem. 248: 619.

16. Jeffrey, P. D., Nichol, L. W., Teasdale, R. D. 1979. Studies of Macromolecular Heterogeneous Associations Involving Cross-Linking: A Re-Examination of the Ovalbumin-Lysozyme System. Biophys. Chem. 10: 379.

17. Kennedy, J. F., Ed., 1987. Enzyme Technology. VCH Publishers, New York.

18. Matsudomi, N., 1991. Significance of Lysozyme in Heat-Induced Aggregation of Egg White Protein, pp. 73. In: N. Parris, and R. Barford, (eds.) Interactions of Food Proteins, American Chemical Society, Washington, D.C.

19. McCarty, B. W., E. T. Adams, J. 1987. Osmotic Pressure Measurements of Ovalbumin and Lysozyme Mixtures. Biophys. Chem. 28: 149.

20. Mikes, O. 1988. High Performance Liquid Chromatography of Biopolymers and Biooligomers, Part B. Elsevier Science Publisher B. V., Amsterdam, Netherlands.

21. Nakai, S., Kason, C. M. 1974. A Fluorescence Study of the Interaction Between κ- and α_{s1}-Casein and Between Lysozyme and Ovalbumin. Biochim. Biophys. Acta. 351: 21.

22. Niederauer, M. Q., Glatz, C. E. 1992. Selective Precipitation. Adv. in Biochem. Eng. Technol. 47: 159.

23. Przybycien, T. M., Bailey, J. E. 1989. Solubility-Activity Relationship in the Inorganic Salt-Induced Precipitation of α-Chymotrypsin. Enzyme Microb. Technol. 11: 264.

24. Richardson, P., Hoare, M., Dunnill, P. 1990. A New Biochemical Engineering Approach to the Fractional Precipitation of Proteins. Biotechnol. Bioeng. 36: 354.

25. Roche, J., Derrien, Y. 1946. Caractères de Solubilité des Mélanges Protéiques Artificiels en Présence de Sels Neutres. Bull. Soc. Chim. Biol. 28: 838.

26. Rossi, M., Schiraldi, A. 1992. Thermal Denaturation and Aggregation of Egg Proteins. Thermochimica Acta. 199: 115.

27. Rothstein, F., 1994. Differential Precipitation of Proteins, pp. 115. In: R. G. Harrison, (eds.) Protein Purification Process Engineering, Marcel Dekker, New York.

28. Salahuddin, A., Waseem, A., Khan, M. Y., Qasim, M. A., Sibghatullah. 1983. A Possible Relation Between the Salting-Out Behaviour of Proteins and Their Surface Hydrophobicity. Ind. J. Biochem. Biophys. 20: 127.

29. Shih, Y.-C., Prausnitz, J. M., Blanch, H. W. 1992. Some Characteristics of Protein Precipitation by Salts. Biotechnol. Bioeng. 40: 1155.

30. Sophianopoulos, A. J., Rhodes, C. K., Holcomb, D. N., Van Holde, K. E. 1962. Physical Studies of Lysozyme. J. Biol. Chem. 237: 1107.

31. Steiner, R. F. 1953. Reversible Association Processes of Globular Proteins: II. Electrostatic Complexes of Plasma Albumin and Lysozyme. Arch. Biochem. Biophys. 47: 56.

Figures

Figure Captions

Figure 1 Supernatant-phase protein concentration (c_{sp}) and partition coefficient (K) of lysozyme in ammonium-sulfate solutions. Ionic strength varies from 5.0 to 9.0 molal. Initial protein concentrations were varied from 20 to 50 mg/g water without affecting lysozyme phase behavior.

Figure 2 Supernatant-phase protein concentration (c_{sp}) and partition coefficient (K) of ovalbumin in ammonium-sulfate solutions. Ionic strength varies from 7.0 to 12.0 molal. Initial protein concentration was 30 mg/g water.

Figure 3 Supernatant protein concentrations of lysozyme and chymotrypsin mixtures as a function of pH and ionic strength. Initial protein concentrations were 20 mg/g water for lysozyme and 20 mg/g water for chymotrypsin.

Figure 4 Protein partition coefficients for lysozyme and chymotrypsin mixtures as a function of pH and ionic strength. Initial protein concentrations were 20 mg/g water for lysozyme and 20 mg/g water for chymotrypsin.

Figure 5 Protein partition-coefficient ratios for lysozyme and chymotrypsin mixtures. Chymotrypsin single-protein results at c_{init} = 30 mg/g water were normalized to c_{init} = 20 mg/g water. (Chymotrypsin single-protein data are from Coen et al.[6])

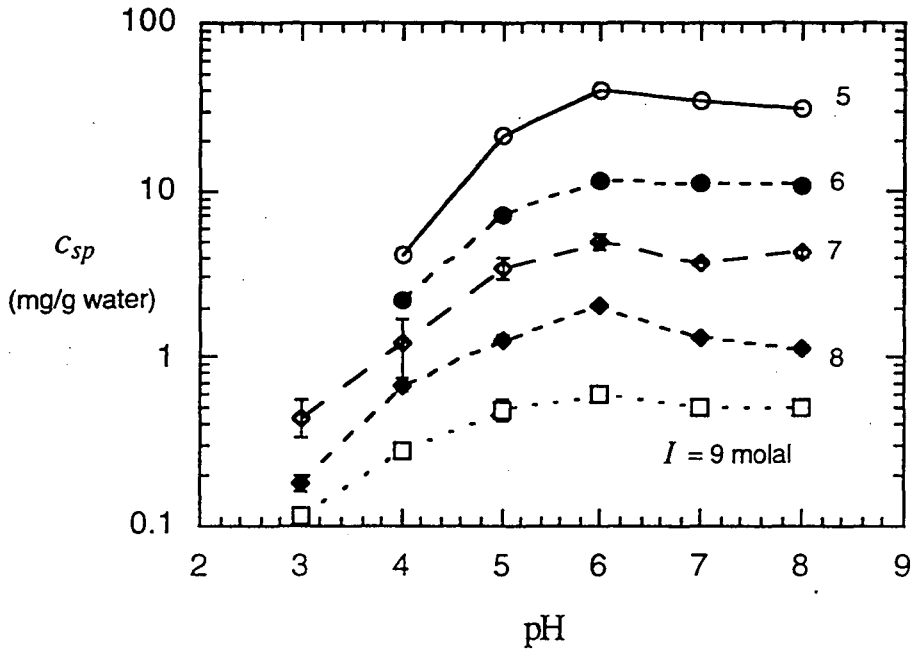
Figure 6 Selectivity of salting-out phase separations based on partition coefficients for lysozyme from mixtures of lysozyme and chymotrypsin.

Figure 7 Supernatant protein concentrations of lysozyme and ovalbumin mixtures as a function of pH and ionic strength. Initial protein concentrations were 20 mg/g water for lysozyme and 20 mg/g water for ovalbumin.

Figure 8 Protein partition coefficients for lysozyme and ovalbumin mixtures as a function of pH and ionic strength. Initial protein concentrations were 20 mg/g water for lysozyme and 20 mg/g water for ovalbumin.

Figure 9 Protein partition-coefficient ratios for lysozyme and ovalbumin mixtures. Ovalbumin single-protein results at c_{init} = 30 mg/g water were normalized to c_{init} = 20 mg/g water.

Figure 10 Selectivity of salting-out phase separations based on partition coefficients for lysozyme from mixtures of lysozyme and ovalbumin.


100
10
1
0.1
c_{sp}
(mg/g water)
5
6
7
8
I = 9 molal
2
3
4
5
6
7
8
9
pH

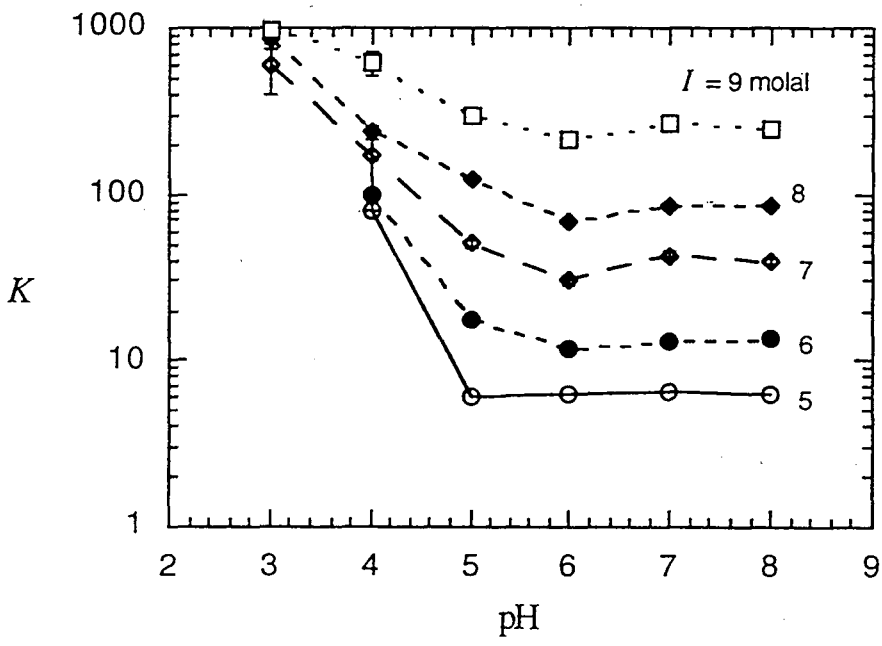
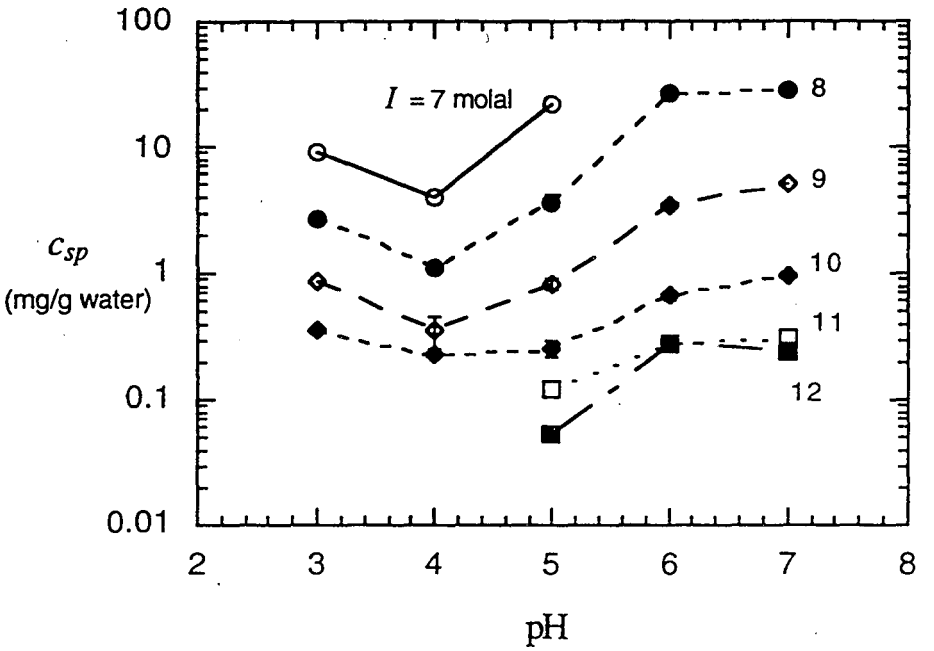

1000
100
10
1
K
I = 9 molal
8
7
6
5
2
3
4
5
6
7
8
9
pH

Figure 1


100
10
1
0.1
0.01
c_{sp}
(mg/g water)
I = 7 molal
8
9
10
11
12
2
3
4
5
6
7
8
pH

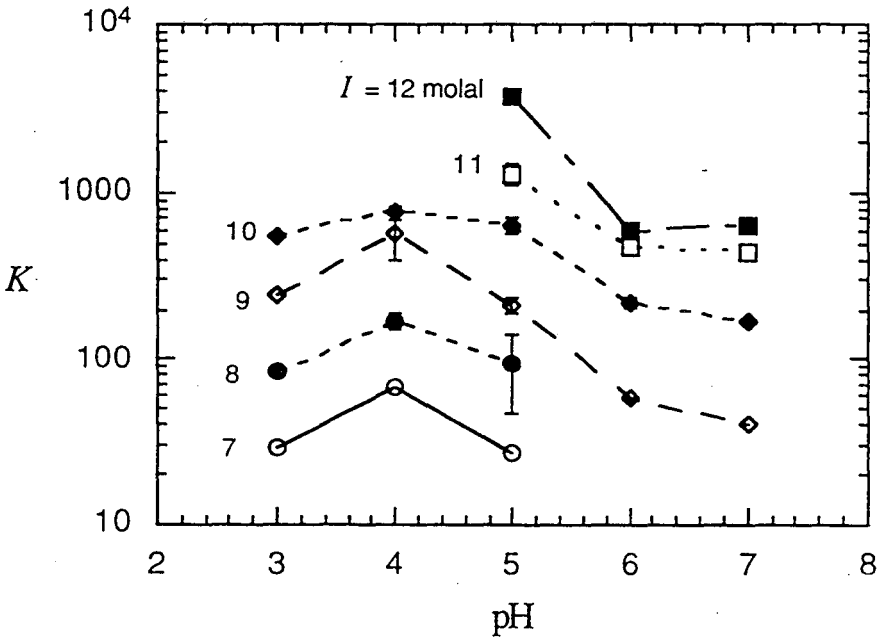
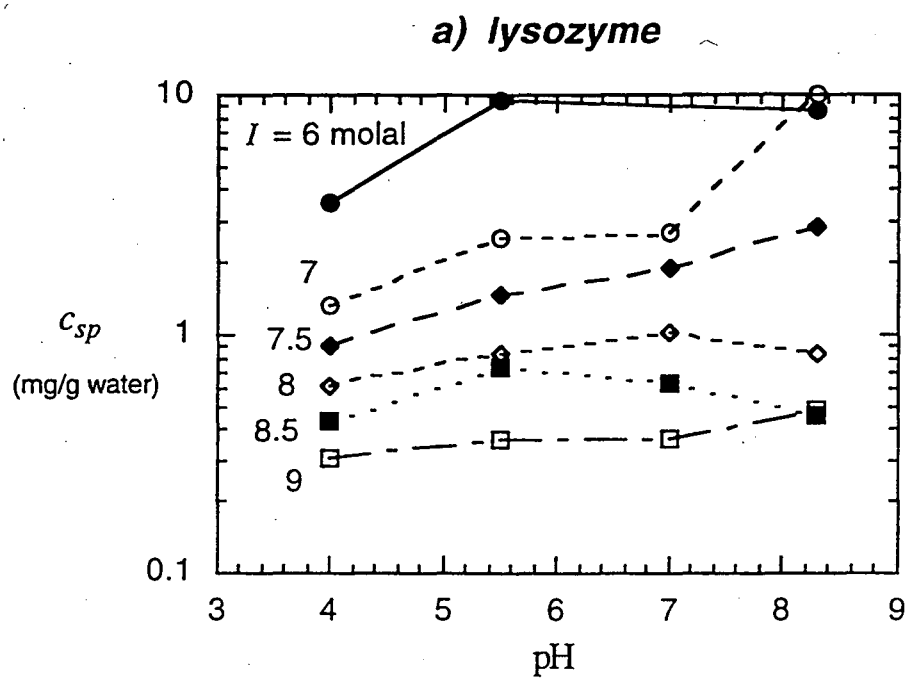

10^4
1000
100
10
K
I = 12 molal
11
10
9
8
7
2
3
4
5
6
7
8
pH

Figure 2


a) lysozyme
10
1
0.1
c_{sp}
(mg/g water)
I = 6 molal
7
7.5
8
8.5
9
3
4
5
6
7
8
9
pH

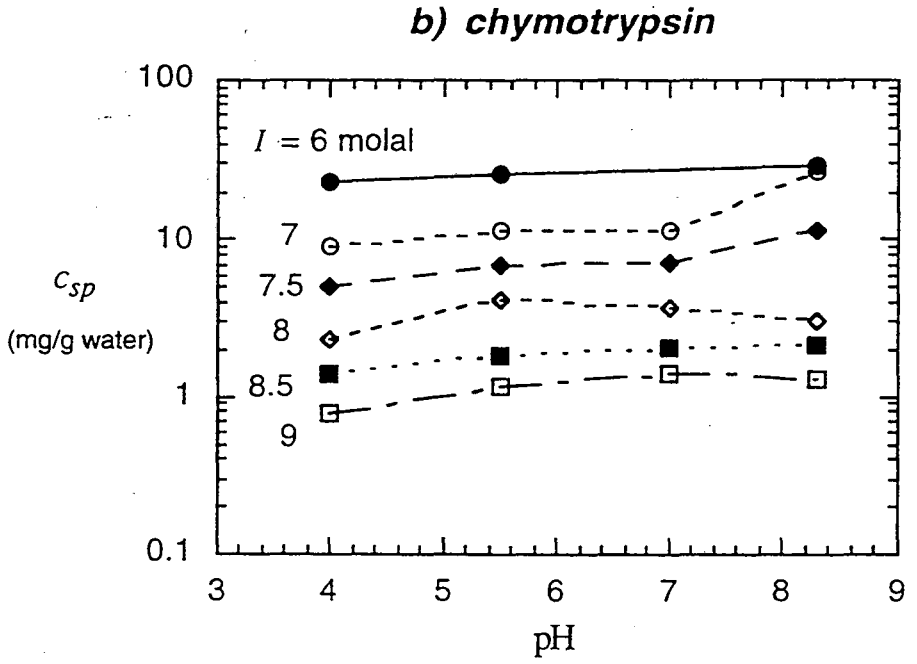
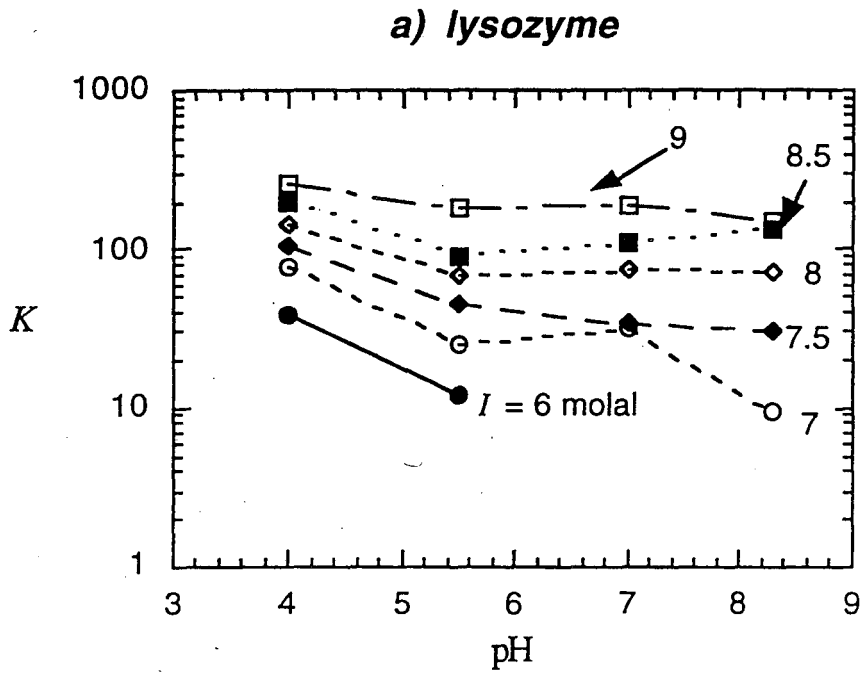

b) chymotrypsin
100
10
1
0.1
c_{sp}
(mg/g water)
I = 6 molal
7
7.5
8
8.5
9
3
4
5
6
7
8
9
pH

Figure 3


a) lysozyme
1000
100
10
1
K
3
4
5
6
7
8
9
pH
9
8.5
8
7.5
7
I = 6 molal

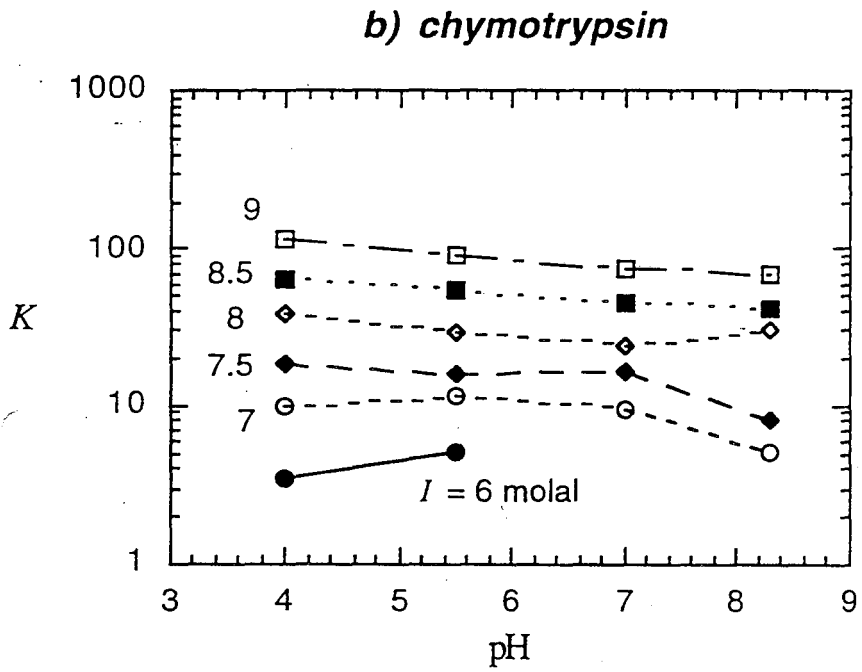
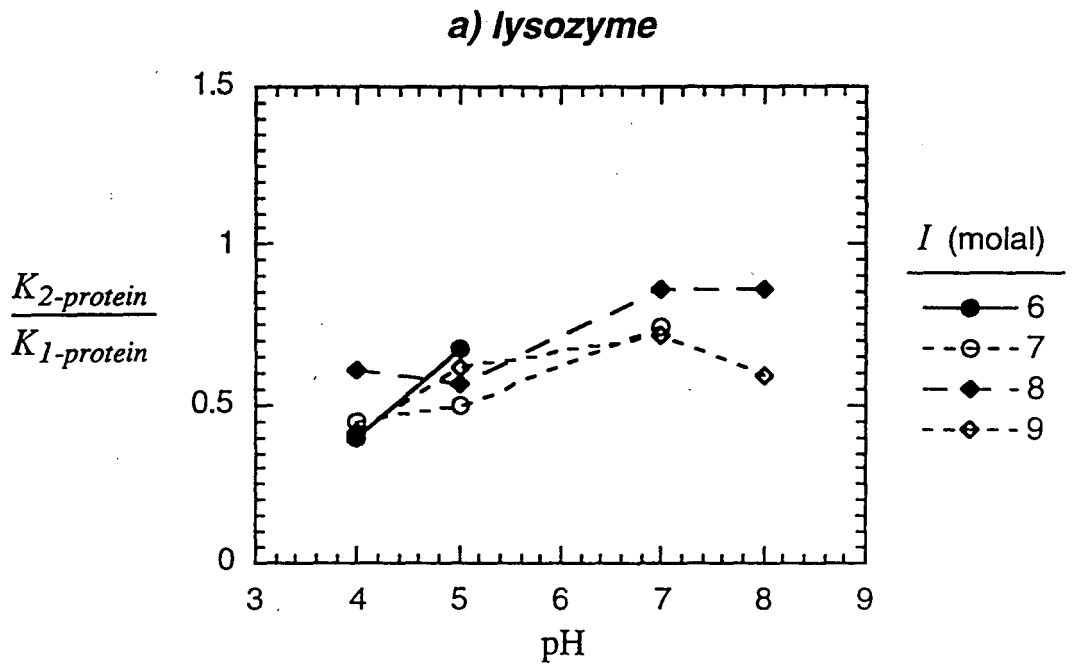

b) chymotrypsin
1000
100
10
1
K
3
4
5
6
7
8
9
pH
9
8.5
8
7.5
7
I = 6 molal

Figure 4


a) lysozyme
1.5
1
0.5
0
3
4
5
6
7
8
9
pH
$\frac{K_{2\text{-protein}}}{K_{1\text{-protein}}}$
I (molal)
6
7
8
9

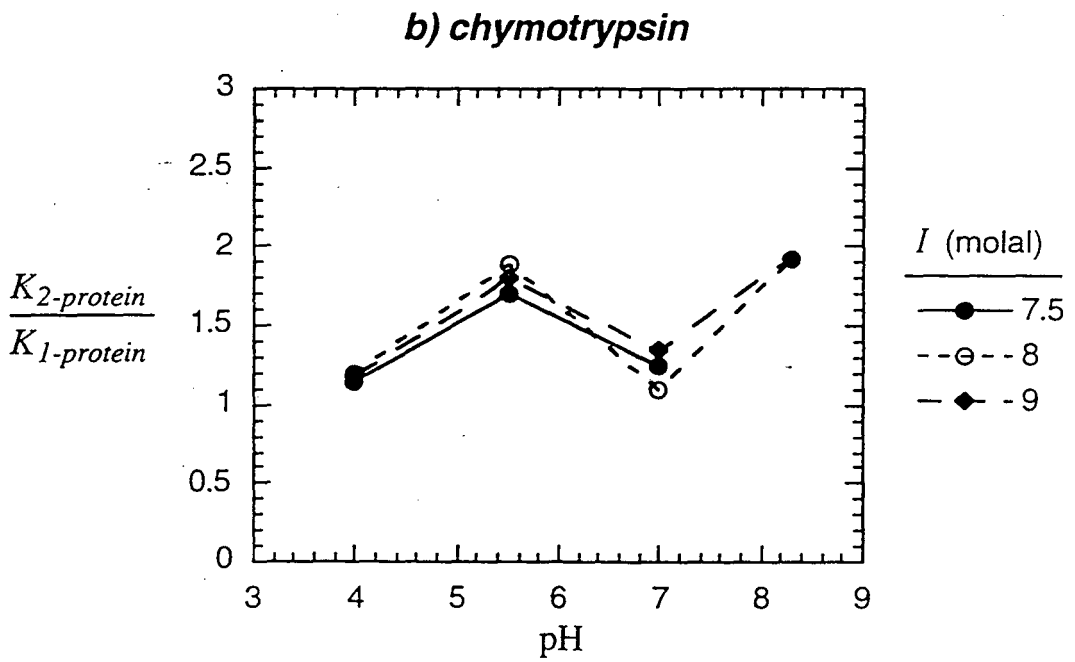

b) chymotrypsin
3
2.5
2
1.5
1
0.5
0
3
4
5
6
7
8
9
pH
$\frac{K_{2\text{-protein}}}{K_{1\text{-protein}}}$
I (molal)
7.5
8
9

Figure 5

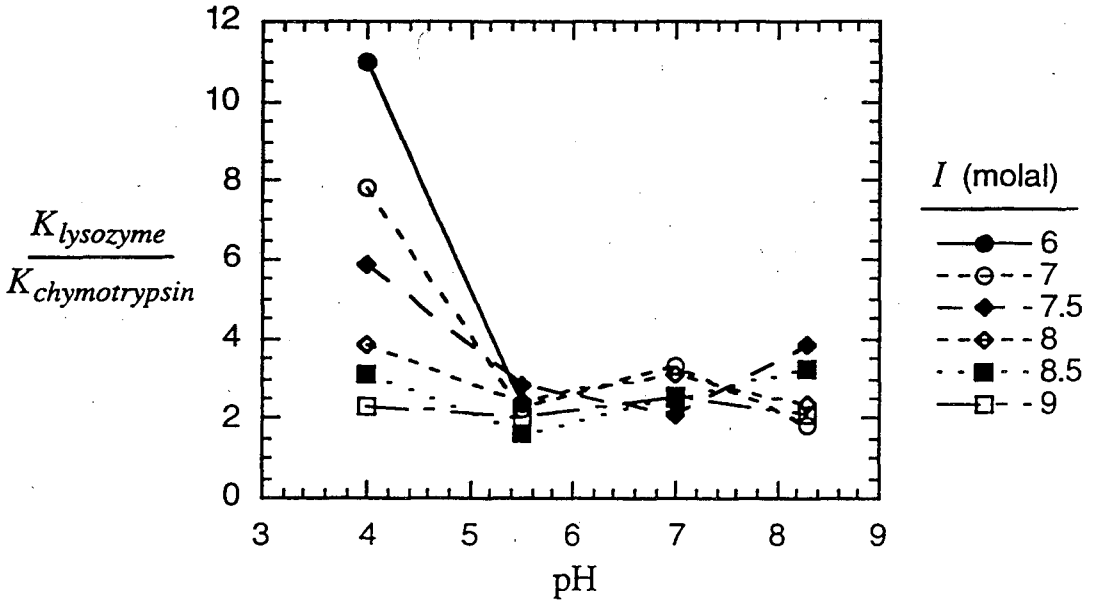
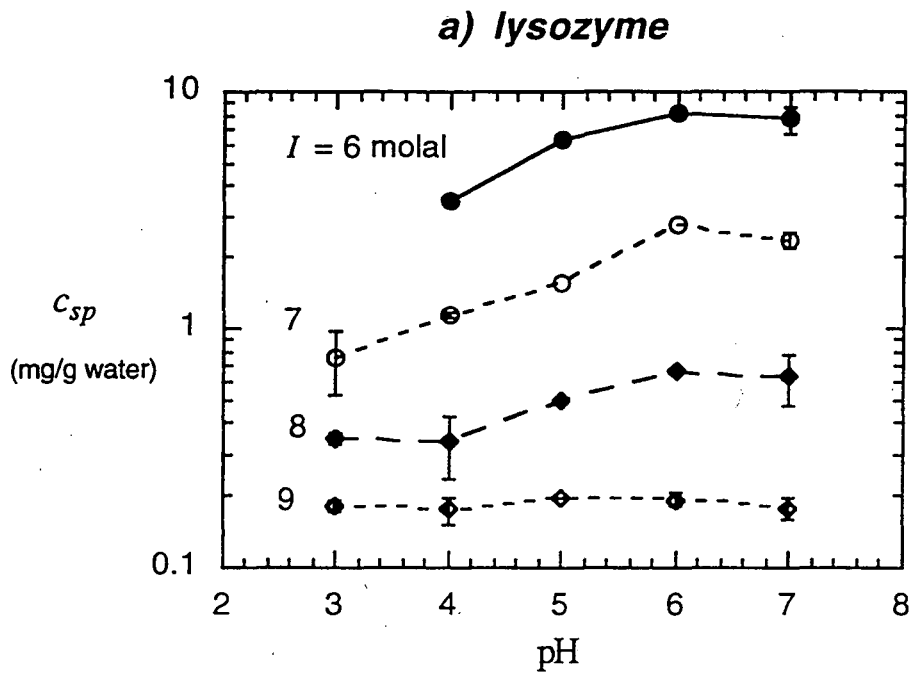

12
10
8
6
4
2
0
3
4
5
6
7
8
9
pH
$\frac{K_{lysozyme}}{K_{chymotrypsin}}$
I (molal)
6
7
7.5
8
8.5
9

Figure 6


a) lysozyme
10
1
0.1
c_{sp}
(mg/g water)
I = 6 molal
7
8
9
2
3
4
5
6
7
8
pH

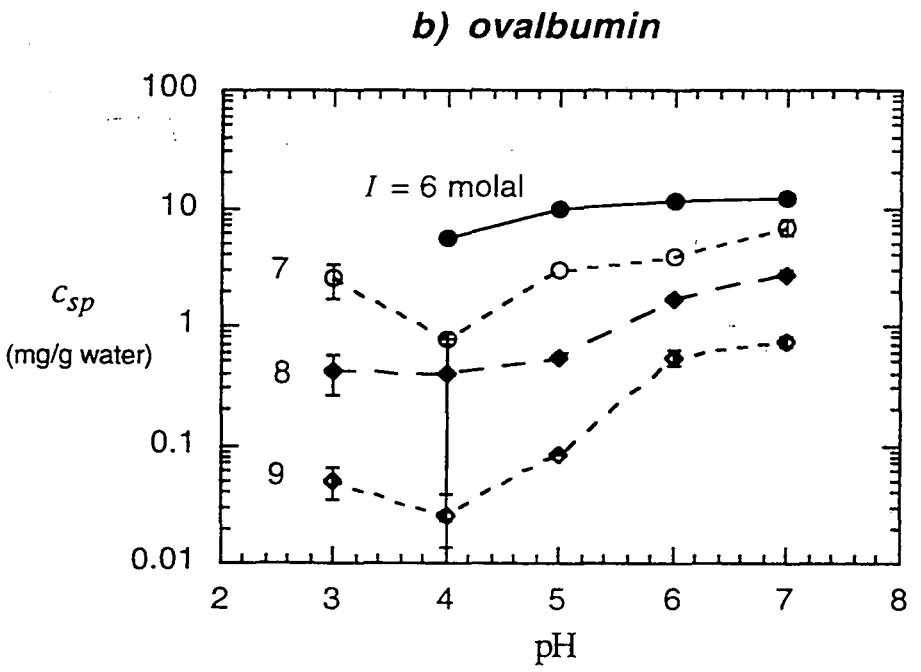
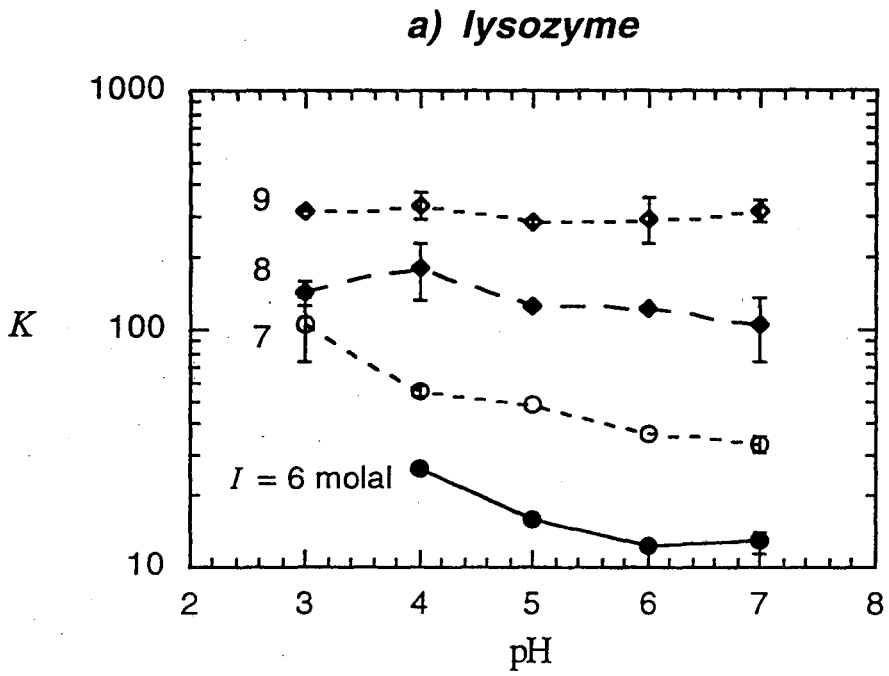

b) ovalbumin
100
10
1
0.1
0.01
c_{sp}
(mg/g water)
I = 6 molal
7
8
9
2
3
4
5
6
7
8
pH

Figure 7


a) lysozyme
1000
100
10
K
9
8
7
I = 6 molal
2
3
4
5
6
7
8
pH

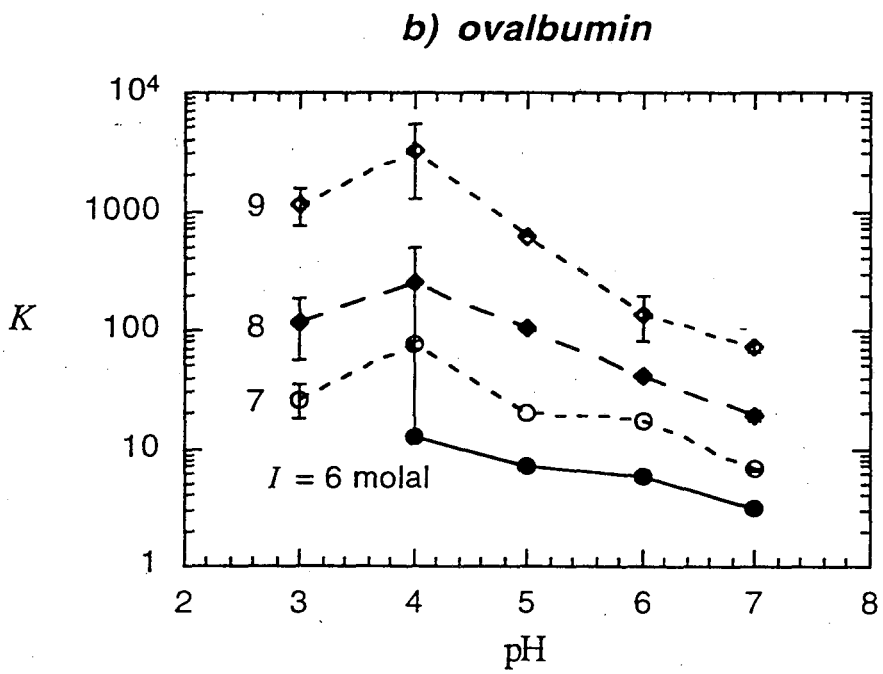
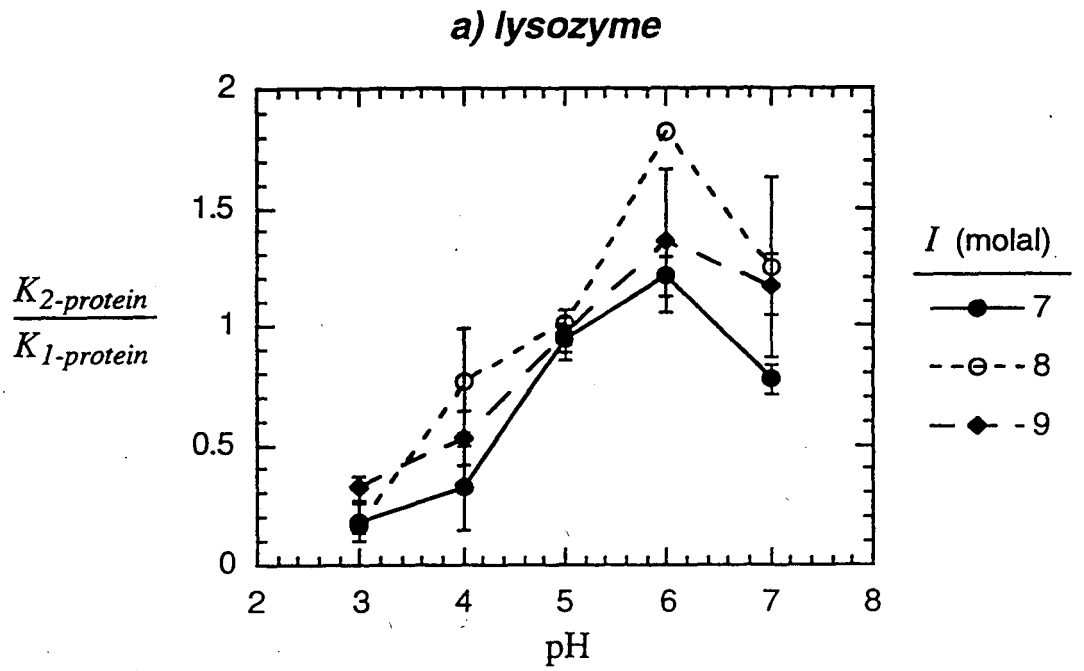

b) ovalbumin
10⁴
1000
100
10
1
K
9
8
7
I = 6 molal
2
3
4
5
6
7
8
pH

Figure 8


a) lysozyme
2
1.5
1
0.5
0
2
3
4
5
6
7
8
pH
$\frac{K_{2\text{-}protein}}{K_{1\text{-}protein}}$
I (molal)
7
8
9

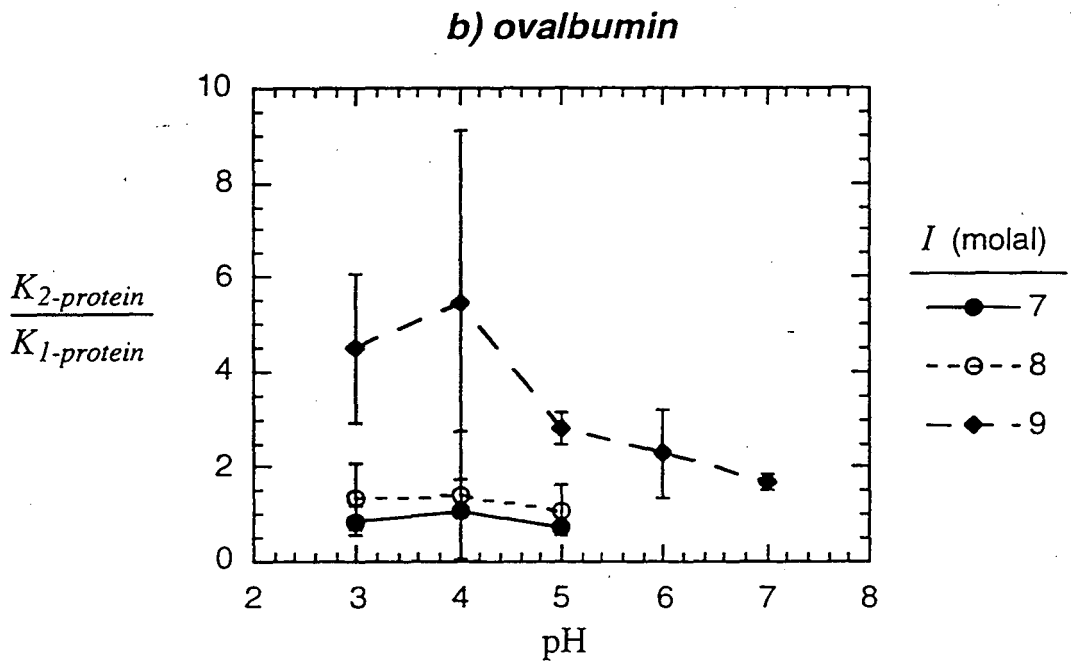

b) ovalbumin
10
8
6
4
2
0
2
3
4
5
6
7
8
pH
$\frac{K_{2\text{-}protein}}{K_{1\text{-}protein}}$
I (molal)
7
8
9

Figure 9

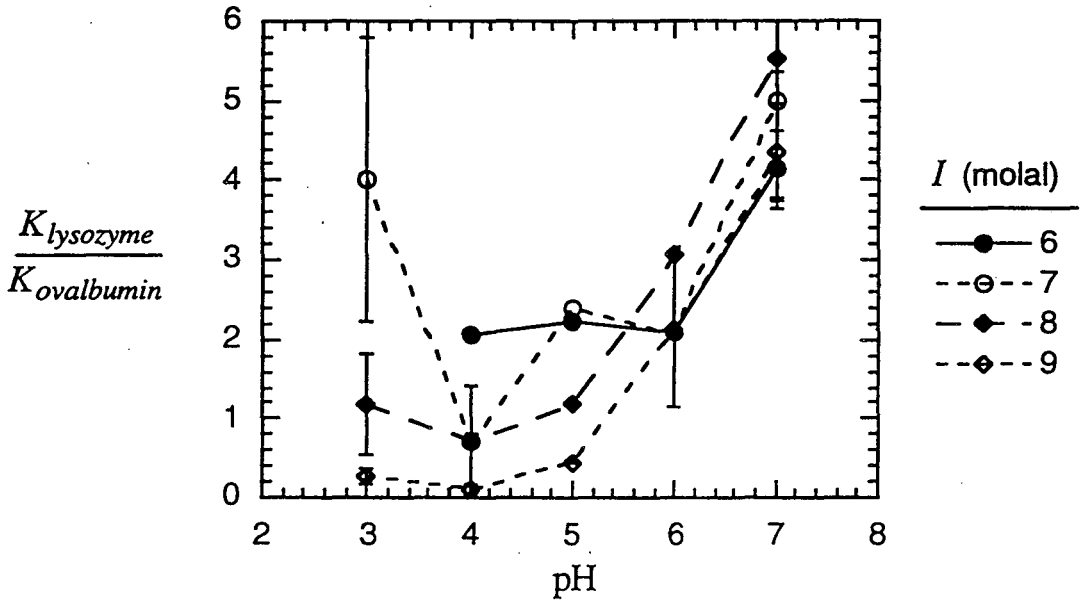


Figure 10

LAWRENCE BERKELEY NATIONAL LABORATORY
UNIVERSITY OF CALIFORNIA
TECHNICAL & ELECTRONIC INFORMATION DEPARTMENT
BERKELEY, CALIFORNIA 94720