Experimental Results and Thermodynamic Modeling of the Partitioning of Lysozyme, Bovine Serum Albumin, and α-Amylase in Aqueous Two-Phase Systems of PEG and (K_2HPO_4 or Na_2SO_4)

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Partitioning of lysozyme, bovine serum albumin, and α-amylase in aqueous two-phase systems of PEG + K_2HPO_4 + water and PEG + Na_2SO_4 + water at 298 K was investigated. The effects of pH and salt concentration on protein partitioning were studied. The protein partitioning depends on the protein net charge that was determined experimentally by combining titration and isoelectric focusing. The experimental data are correlated using the VERS model, that is, an osmotic virial equation with relative surface fractions to express the concentrations. The protein is split into different patch groups, and the number of patches is calculated from the diameter of the hard sphere protein molecule. The comparisons between the correlation results and the experimental data reveal a good agreement.

Introduction

Aqueous two-phase extraction is a technique for separation, concentration, and purification of proteins, cell organells, and other biological products. Aqueous two-phase systems (ATPSs) are particularly suited for separation and purification of proteins from crude materials like cell extracts, downstream broth, and culture filtrate, because insoluble cell debris often accumulates in one of the two phases. Aqueous two-phase systems are observed when, for example, two hydrophilic but incompatible polymers, such as poly(ethylene glycol) (PEG) and dextran, or a hydrophilic polymer and a strong electrolyte are simultaneously dissolved in water. Polymer/salt aqueous two-phase systems have some advantages, such as, for example, a low price, a low viscosity, and a short time required for phase splitting.

Beijerinck[1] was the first who described aqueous two-phase systems in 1896 formed by agar and gelatin. Albertson[2] discovered that polymers and electrolytes form aqueous two-phase systems. In recent years the separation of proteins and other biomolecules was investigated by many researchers, in particular by the group of Kula. Kula et al.[3−5] developed processes for protein extraction and purification in aqueous two-phase systems. Huddleston et al.[6−9] investigated the recovery of proteins by aqueous two-phase systems and studied the effect of parameters such as pH and polymer concentration on protein partitioning. Asenjo et al.[10−13] investigated the partitioning of α-amylase, subtilisin, trypsin inhibitor, and some enzymes in aqueous two-phase systems of PEG and phosphate, and also studied the effect of sodium chloride on protein partitioning in these systems. Kaul et al.[14] studied the kinetics of phase separation. Moreover, applications for the extraction of recombinant proteins by ATPSs have been developed.[15,16] A brief review on protein partitioning using aqueous two-phase systems was given by Walter.[17]

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The partitioning of proteins in ATPSs depends, for example, on the net charge as well as on the pH difference between the phases. It is common practice to correlate the partitioning of a protein to an ATPS by applying the concept of an electrical potential difference $\Delta\Phi$ between the top and bottom phases:

$$\ln K = \ln K_0 + \frac{Fz\Delta\Phi}{RT} \tag{1}$$

where K is the partition coefficent of the protein (concentration in the top phase/concentration in the bottom phase), K_0 is the partition coefficient in the absence of an electrical potential difference, F is Faraday's constant, z is the charge number of the protein, R is the gas constant, and T is the temperature. When the protein carries a sufficiently high net charge and K_0 is close to unity as well as $\Delta\Phi$ is on the order of a few millivolts, the partitioning of a protein to an ATPS is governed by the second term on the right-hand side of eq 1. A method to determine the net charge of a protein is available in the literature, but until recently there was no method available either to measure or to calculate the electrical potential difference $\Delta\Phi$. Grossmann and Maurer[18] presented an explanation for that potential difference and proposed a method to calculate $\Delta\Phi$ from an expression for the Gibbs excess energy of the aqueous polymer/electrolyte solution.

As aqueous two-phase systems are considered to be promising and powerful methods for the separation of proteins, the computer assisted design of such extraction processes requires a thermodynamic model to describe such phase equilibrium. Experimental data on protein partitioning alone are not sufficient, as, for example, the number of parameters which influence that partitioning might be large and not yet investigated extensively. In the work presented here, the partitioning of lysozyme, bovine serum albumin (BSA), and α-amylase was studied in polymer/salt systems of PEG + K_2HPO_4 + water and PEG + Na_2SO_4 + water at 298 K with particular emphasis on the effects of pH and salt concentration. As the partitioning of a protein

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Table 1. Equilibrium Compositions as Mass Fraction *w* and the Partitioning of Lysozyme (4) in PEG 4000 (1) + K_2HPO_4 (2) + Water (3) Aqueous Two-Phase Systems at 298 K and pH = 9.1

feed			upper phase			lower phase		
$100w_2$	$100w_1$	$100w_4$	$100w_2$	$100w_1$	$100w_4$	$100w_2$	$100w_1$	$100w_4$
10.82	11.25	0.0194	3.36	29.10	0.0085	16.27	0.65	0.0295
10.33	11.20	0.0194	4.08	24.42	0.0092	15.79	0.22	0.0295
9.93	11.30	0.0194	4.23	25.30	0.0097	14.46	0.58	0.0296
9.38	11.38	0.0196	4.62	23.41	0.0111	13.82	0.90	0.0256
9.03	11.36	0.0196	4.70	22.11	0.0136	13.32	1.45	0.0264

Table 2. Equilibrium Compositions as Mass Fraction (*w*) and the Partitioning of Lysozyme (4) in PEG 4000 (1) + K_2HPO_4 (2) + Water (3) Aqueous Two-Phase Systems at 298 K and pH = 7.2

feed			upper phase			lower phase		
$100w_2$[a]	$100w_1$	$100w_4$	$100w_2$[a]	$100w_1$	$100w_4$	$100w_2$[a]	$100w_1$	$100w_4$
10.81	11.25	0.0185	5.23	24.10	0.0102	16.46	0.47	0.0300
10.18	11.39	0.0185	5.38	22.83	0.0108	16.17		0.0296
9.29	11.49	0.0185	6.99	17.73	0.0144	14.06	1.77	0.0281
8.89	11.56	0.0185	7.86	14.71	0.0181	12.67	4.09	0.0258

[a] The mixture of K_2HPO_4 and KH_2PO_4 is used.

Table 3. Equilibrium Compositions as Mass Fraction (*w*) and the Partitioning of Lysozyme (4) in PEG 1500 (1) + K_2HPO_4 (2) + Water (3) Aqueous Two-Phase Systems at 298 K and pH = 9.1

feed			upper phase			lower phase		
$100w_2$	$100w_1$	$100w_4$	$100w_2$	$100w_1$	$100w_4$	$100w_2$	$100w_1$	$100w_4$
11.19	14.01	0.0500	3.72	30.41	0.0566	17.47	1.40	0.0497
10.26	14.00	0.0500	4.36	27.15	0.0521	14.03	4.53	0.0530
10.18	16.01	0.0500	3.79	30.76	0.0550	14.92	4.43	0.0488
9.36	14.04	0.0500	5.63	21.89	0.0512	12.05	6.82	0.0532
9.28	15.99	0.0500	4.48	26.46	0.0515	13.39	5.47	0.0528
9.17	17.98	0.0500	3.74	29.69	0.0533	14.84	4.37	0.0526
8.25	18.00	0.0500	4.33	26.62	0.0516	13.81	4.26	0.0505
8.17	20.02	0.0500	3.80	30.33	0.0533	14.72	4.29	0.0509

in these systems depends on the protein's net charge, the influence of pH on the net charge of the proteins was also determined. The VERS model of Grossmann et al.[19] is adopted to describe the liquid−liquid phase equilibrium of these systems.

Experimental Section

Materials. Two poly(ethylene glycol)s, with molecular weights of 4000 and 1500, respectively, dipotassium hydrogen phosphate, sodium sulfate, sodium chloride, potassium chloride, lysozyme (from egg white, M = 14 300), bovine serum albumin (M = 68 000), and α-amylase (from basilus subtilis M = 54 000) were purchased from Merck. KH_2PO_4 was purchased from Sigma. The poly(acryl amide) gel (PAG), needed for determining the isoelectric point of a protein, was purchased from Servalyte (Heidelberg, Germany).

Quantitative Analysis. The concentration of a salt (K_2HPO_4, KH_2PO_4, and Na_2SO_4) was determined by using atomic absorption spectroscopy (AAS) with a Philips PU 91178X model. The relative experimental uncertainity in the concentration of a salt is less than 5%. The concentration of PEG was determined by refractive index measurements at 298 K using a Kruss Abbe refractometer model AR3D. Since the refractive index of an aqueous phase depends not only on the concentration of PEG but also on that of the salt, extensive calibrations were performed to separate the influence of PEG and salt on the refractive index of an aqueous solution. The refractive index depends linearly on both the mass fractions of the polymer and of the salt—at least in the small range of salt concentration investigated. The relative experimental uncertainity for the PEG concentration is <3.5% with a minimum absolute uncertainty of 0.05 mass %.

The concentration of lysozyme was determined by UV/vis spectrophotometry at 280 nm using an UV-3000 Shimadzu spectrometer. At that wavelength the salts and the polymer also absorb light. Therefore, the absorbance coefficients of the salts and of PEG were also determined during the calibration procedure. It was assumed (and confirmed by test measurements) that the total absorbance is the sum of the absorbances caused by the protein, the salt, and the polymer.

The pH of an aqueous solution was measured with a precision pH meter (type 744 from Deutsche Metrohm, Filderstadt, Germany).

Isoelectric focusing experiments were carried out to determine the isoelectric points of the proteins. The equipment consisted of a horizontal electrophoresis cell (type Bio-Phoreses) with a power supply unit (Power Pac 3000), all by Bio-Rad Laboratories, Hercules, CA.

An automatic titration processor (Titrino DMS716, Deutsche Metrohm, Filderstadt, Germany) was used for titrating the aqueous protein solutions.

Experimental Procedure. Aqueous two-phase systems were prepared from stock solutions of the single solutes polymer, salt, and protein. The concentrations of PEG and salt in the stock solutions were (30 and 20) mass %, respectively. The concentration of a protein in a stock solution was 2 g/L. In PEG + K_2HPO_4 + water two-phase systems the pH was adjusted with mixtures of KH_2PO_4 and K_2HPO_4 for pH < 9.2. For pH > 9.2, as well as in aqueous two-phase systems of PEG and Na_2SO_4, a 1 M aqueous sodium hydroxide solution was used to adjust the pH. Aqueous two-phase systems with various concentrations of salt and PEG were prepared from stock solutions of PEG, salt, lysozyme, and buffer, by weighing the appropriate amounts of the stock solutions in graduated 10 mL test

Table 4. Experimental Results for the Partition Coefficient of Lysozyme in PEG 4000 (1) + K_2HPO_4 (2) + Water (3) Aqueous Two-Phase Systems at 298 K

feed	pH	
$100w_2$	8.2[a]	10.2[b]
10.77	0.22	0.66
10.27	0.23	0.69
9.91	0.27	0.73
9.33	0.34	0.74
8.97	0.45	0.77

[a] pH was adjusted by the mixture of K_2HPO_4 and KH_2PO_4. [b] pH was adjusted by NaOH.

tubes. The mixture was shaken for about 2 min and afterward centrifuged at 2400 rpm for 10 min. Then, the tubes were placed in a water bath at (25 ± 0.1) °C for 24 h to achieve final phase equilibrium. The top and bottom phases were carefully withdrawn, leaving a layer of at least 5 mm thickness near the interface. Samples of each phase were analyzed for protein, salt, and PEG.

The net charge of a protein in an aqueous solution was determined by combining acid/base titration and isoelectric focusing (IEF). The change of the protein's net charge with pH was determined in a titration experiment. IEF was applied to determine the isoelectric point, that is, the pH at which the protein carries no net charge. The titration experiments were performed with 0.1 M aqueous NaOH and 0.1 M aqueous HCl (for back-titration).[21]

Experimental Results

The experimental results for the partitioning of lysozyme in PEG + KH_2PO_4 + water systems at 298 K are summarized in Tables 1−4. Tables 1 and 2 show the results for PEG 4000 at pH 9.1 and 7.2, respectively. For the aqueous two-phase systems with PEG 1500 at pH 9.1, the corresponding results are shown in Table 3. Table 4 presents the results for the partition coefficient of lysozyme at various pH values and salt concentrations. As can be seen from these tables, the partition coefficient decreases with increasing salt concentration.

The experimental results for the partitioning of bovine serum albumine in PEG 1500 + KH_2PO_4 + water systems are given in Table 5. The results for the partitioning of α-amylase in PEG 4000 + K_2HPO_4 + water systems are given in Tables 6−8 for pH = 7.1 (Table 6), pH = 6.8 (Table 7), and pH = 9.1 (Table 8).

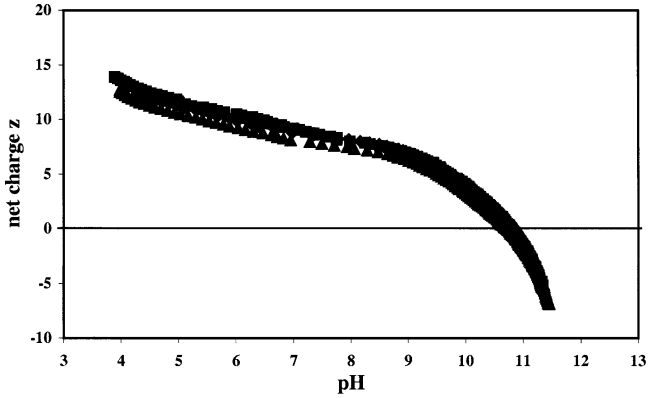

Figure 1. Influence of pH on the net charge number of lysozyme in aqueous solutions at 298 K: ◆, NaOH titration; ■, back-titration; ▲, NaOH second titration.

The results for the partitioning of lysozyme in PEG + Na_2SO_4 + water systems are given in Tables 9−11. Tables 9 and 10 present the phase compositions for systems with PEG 4000 and PEG 1500 at pH = 9.1 and pH = 4.6, respectively. The experimental results for the partition coefficient of lysozyme at various pH values and salt concentrations are given in Table 11. Furthermore, Table 12 presents the experimental results for the partitioning of α-amylase in the PEG 4000 + Na_2SO_4 + water system at pH = 5.4. Both partition coefficients (that of lysozyme and that of α-amylase) also decrease with increasing salt concentration.

Titration and isoelectric focusing were used to determine the influence of pH on the net charge of lysozyme, bovine serum albumin, and α-amylase. The result for lysozyme is shown in Figure 1. For each protein the titration experiments were repeated two to three times by both forward and backward titration, using HCl and NaOH as titrant. As is shown in the figure, the results from forward and

Table 5. Equilibrium Compositions as Mass Fraction (w) and the Partitioning of Bovine Serum Albumin (4) in PEG 1500 (1) + K_2HPO_4 (2) + Water (3) Aqueous Two-Phase Systems at 298 K and pH = 9.1

feed			upper phase			lower phase		
$100w_2$	$100w_1$	$100w_4$	$100w_2$	$100w_1$	$100w_4$	$100w_2$	$100w_1$	$100w_4$
11.15	13.99	0.0500	4.13	29.64	0.0097	15.09	3.90	0.0868
10.26	13.98	0.0500	4.39	26.56	0.0093	13.33	4.80	0.0943
10.19	15.98	0.0500	3.79	30.62	0.0105	14.83	4.73	0.1448
9.34	13.95	0.0500	5.97	22.49	0.0034	12.05	5.70	0.0567
9.25	15.98	0.0500	4.56	26.38	0.0163	13.98	5.07	0.1108
9.16	17.98	0.0500	3.71	29.87	0.0122	14.71	4.16	0.0821
8.26	17.97	0.0500	4.55	25.83	0.0322	14.02	4.47	0.1358
8.16	20.00	0.0500	3.91	29.52	0.0097	15.18	3.80	0.1341

Table 6. Equilibrium Compositions as Mass Fraction (w) and the Partitioning of α-Amylase (4) in PEG 4000 (1) + K_2HPO_4 (2) + Water (3) Aqueous Two-Phase Systems at 298 K and pH = 7.1

feed			upper phase			lower phase		
$100w_2$[a]	$100w_1$	$100w_4$	$100w_2$[a]	$100w_1$	$100w_4$	$100w_2$[a]	$100w_1$	$100w_4$
10.74	8.88	0.0890	4.68	23.22	0.1017	12.99	3.12	0.0702
10.34	9.52	0.0890	4.87	22.60	0.1008	13.55	2.07	0.0699
9.47	9.56	0.0890	6.20	17.78	0.0859	11.48	3.76	0.0722
9.42	10.89	0.0890	5.42	20.70	0.0873	13.97	1.44	0.0682
8.98	10.93	0.0890	6.18	17.59	0.0816	11.53	4.43	0.0749
8.49	12.29	0.0890	5.91	18.64	0.0807	11.85	3.97	0.0700

[a] The mixture of K_2HPO_4 and KH_2PO_4 is used.

Table 7. Experimental Results as Mass Fraction (w) for the Partitioning of α-Amylase (4) in PEG 4000 (1) + K_2HPO_4 (2) + Water (3) Aqueous Two-Phase Systems at 298 K and pH = 6.8

feed			w_4		
$100w_2$[a]	$100w_1$	$100w_4$	upper phase	lower phase	K
11.96	9.55	0.0450	0.0668	0.0407	1.64
11.16	9.57	0.0450	0.0636	0.0513	1.24
10.29	9.52	0.0450	0.0628	0.0554	1.13
10.28	10.90	0.0450	0.0584	0.0487	1.20
10.16	12.28	0.0450	0.0614	0.0494	1.24

[a] The mixture of K_2HPO_4 and KH_2PO_4 is used.

Table 8. Experimental Results as Mass Fraction (w) for the Partitioning of α-Amylase (4) in PEG 4000 (1) + K_2HPO_4 (2) + Water (3) Aqueous Two-Phase Systems at 298 K and pH = 9.1

feed			w_4		
$100w_2$	$100w_1$	$100w_4$	upper phase	lower phase	K
9.42	10.93	0.0890	0.1475	0.0507	2.91
8.57	10.90	0.0890	0.1296	0.0692	1.87
8.49	12.27	0.0890	0.1271	0.0467	2.72
8.24	10.92	0.0890	0.1250	0.0669	1.87
7.66	12.28	0.0890	0.0900	0.0516	1.74
7.59	13.64	0.0890	0.0943	0.0601	1.57

backward titrations overlap, indicating that the conformation of the proteins was not changed during the titration.

The starting point for the titration of an aqueous lysozyme solution was at pH = 4 (cf. Figure 1). The solution was first titrated with NaOH up to pH ≈ 11.5, then "back"-titrated with HCl ("back 1"), and finally titrated once again to pH ≈ 11.5 ("back 2"). Aqueous solutions of BSA were titrated with NaOH (starting at pH = 7.2) up to pH ≈ 10.5, followed by a titration with HCl to pH ≈ 4, and completed by a second titration with NaOH again to pH ≈ 10.5. The starting point for the titration of α-amylase was at pH = 5.6. The solution was titrated with NaOH to pH ≈ 11, then it was titrated with HCl to pH ≈ 4, and finally it was titrated with NaOH to pH ≈ 11. In addition, that experiment was repeated with a new solution of α-amylase, but no difference between the results was observed.

Thermodynamic Modeling. The VERS model of Grossmann et al.[19] is used to describe the liquid−liquid equilibrium in PEG + salt + water systems. The VERS (virial equation with relative surface fraction) model is a semiemprical model based on the osmotic virial equation on one side and Pitzer's equation for aqueous electrolyte solutions on the other side. The excess properties are defined on an asymmetric normalization: the reference state for water is the pure liquid, whereas for a solute it is a hypothetical 1 m solution in pure water. The equations for the activity of water and a solute are

$$\ln a_w = -\frac{M_w}{1000}\left[\sum m_i - 2A_\Phi \frac{I^{1.5}}{1+b\sqrt{I}}\right] - \frac{1000}{M_w}\sum_{i\neq w}\sum_{j\neq w}\frac{\theta_i\theta_j}{\theta_w\theta_w}[A_{ij}^0 + A_{ij}^1 \exp(-\alpha\sqrt{I})] - 2\left(\frac{1000}{M_w}\right)^2 \sum_{i\neq w}\sum_{j\neq w}\sum_{k\neq w}\frac{\theta_i\theta_j\theta_k}{\theta_w\theta_w\theta_w}B_{ijk} \quad (2)$$

$$\ln a_i^* = \ln m_i - A_\Phi z_i^2\left[\frac{\sqrt{I}}{1+b\sqrt{I}} + \frac{2}{b}\ln(1+b\sqrt{I})\right] + 2\frac{1000}{M_w}\frac{q_i}{q_w}\sum_{j\neq w}\frac{\theta_j}{\theta_w}[A_{ij}^0 + A_{ij}^1 f_2(I)] - z_i^2 f_3(I)\left(\frac{1000}{M_w}\right)^2 \sum_{j\neq w}\sum_{k\neq w}\frac{\theta_j\theta_k}{\theta_w\theta_w}A_{jk}^1 + 3\left(\frac{1000}{M_w}\right)^2 \frac{q_i}{q_w}\sum_{j\neq w}\sum_{k\neq w}\frac{\theta_j\theta_k}{\theta_w\theta_w}B_{ijk} \quad (3)$$

where

$$f_2(I) = \frac{2}{\alpha^2 I}[1 - (1+\alpha\sqrt{I})\exp(-\alpha\sqrt{I})] \quad (4)$$

$$f_3(I) = \frac{1}{a^2 I^2}\left[1 - \left(1 + \alpha\sqrt{I} + \frac{\alpha^2 I}{2}\right)\exp(-\alpha\sqrt{I})\right] \quad (5)$$

I is the ionic strength:

$$I = \frac{1}{2}\sum m_i z_i^2 \quad (6)$$

where m_i and z_i are the molality and charge number of component i, respectively. A_ϕ is the Debye−Hückel parameter, which is a function of temperature (at 298 K A_ϕ = 0.3915). M_w is the molecular mass of water (solvent); b and α are numerical values (b = 1.2; α = 2). A_{ij} and B_{ijk} are binary and ternary interaction parameters between solute species.

Table 9. Equilibrium Compositions as Mass Fraction (w) and the Partitioning of Lysozyme (4) in PEG4000 (1) + Na_2SO_4 (2) + Water (3) Aqueous Two-Phase Systems at 298 K and pH = 9.1

feed			upper phase			lower phase		
$100w_2$	$100w_1$	$100w_4$	$100w_2$	$100w_1$	$100w_4$	$100w_2$	$100w_1$	$100w_4$
9.20	13.62	0.0190	2.01	34.71	0.0089	14.87	0	0.0228
8.73	13.62	0.0190	2.71	32.56	0.0146	13.26	1.18	0.0216
8.36	13.64	0.0190	2.52	31.33	0.0204	13.15	0.86	0.0221
7.86	13.60	0.0190	3.34	28.70	0.0153	13.78	0	0.0211
7.55	13.62	0.0190	3.12	27.85	0.0168	11.92	1.49	0.0293
6.96	13.60	0.0190	3.19	25.35	0.0177	11.17	1.70	0.0231

Table 10. Equilibrium Compositions as Mass Fraction (w) and the Partitioning of Lysozyme (4) in PEG1500 (1) + Na_2SO_4 (2) + Water (3) Aqueous Two-Phase Systems at 298 K and pH = 4.6

feed			upper phase			lower phase		
$100w_2$	$100w_1$	$100w_4$	$100w_2$	$100w_1$	$100w_4$	$100w_2$	$100w_1$	$100w_4$
9.90	10.01	0.0400	4.70	23.41	0.0400	13.07	2.89	0.0450
9.14	11.99	0.0400	4.74	23.57	0.0410	11.86	4.61	0.0460
8.40	14.01	0.0400	4.47	24.06	0.0390	12.22	4.03	0.0470
7.64	15.97	0.0400	4.54	23.92	0.0410	12.40	3.92	0.0470

Table 11. Experimental Results as Mass Fraction (w) for the Partition Coefficient of Lysozyme (4) in PEG4000 (1) + Na_2SO_4 (2) + Water (3) Aqueous Two-Phase Systems at 298 K

feed	pH		
$100w_2$	5.4	5.9	6.7
9.23	0.17	0.24	0.26
8.74	0.21	0.25	0.28
8.41	0.23	0.26	0.29
7.87	0.29		0.36
7.54	0.36	0.28	0.46
7.01		0.42	0.50

The interaction parameters are symmetric and similar to Pitzer's parameters for aqueous solutions of strong electrolytes. As proposed by Pitzer, A_{ij} is the sum of two terms: the second term is to describe the influence of the ionic strength on that binary parameter.

$$A_{ij} = A_{ij}^0 + A_{ij}^1 \frac{2}{\alpha^2}[1 - (1 + \alpha\sqrt{I}) \exp(-\alpha\sqrt{I})] \qquad (7)$$

θ_i is the surface fraction of component i:

$$\theta_i = \frac{m_i q_i}{\sum m_i q_i} \qquad (8)$$

where q_j is the surface parameter of species j. PEG is considered to consist of two types of groups: PEG end groups and PEG middle groups. The surface parameter of a PEG molecule was calculated from the group surface parameters.[19]

The phase equilibrium is calculated under the constraint that both phases are electrically neutral, as described by Grossmann and Maurer.[18] When the phase boundary allows all species to partition between both phases, the condition for phase equilibrium in terms of chemical potential becomes

$$\mu_i' - \mu_i'' = \frac{Z_i}{Z_k}(\mu_k' - \mu_k'') \quad \text{where } i = 1, 2, ..., N, \text{ but } i \neq k \qquad (9)$$

where μ_i is the chemical potential of species i, which results from the Gibbs energy G through

$$\mu_i = \left(\frac{\partial G}{\partial n_i}\right)_{T,p,n_{j\neq 1}} \qquad (10)$$

The subscript k stands for an arbitrary selected ionic species. When the same reference state is chosen for a species in both phases, the difference $\mu_k' - \mu_k''$ can be replaced by introducing the activities a_k:

$$\mu_k' - \mu_k'' = RT \ln\left(\frac{a_k'}{a_k''}\right) = z_k F \Delta\Phi \qquad (11)$$

Combining eqs 9 and 11 gives

$$\ln\left(\frac{a_i'}{a_i''}\right) = \frac{Z_i}{Z_k} \ln\left(\frac{a_k'}{a_k''}\right) = z_i \frac{F\Delta\Phi}{RT} \qquad (12)$$

The above equation is used to describe the liquid−liquid equilibria for ionic species; however, for nonionic species, eq 12 reduces to the more common equation:

$$a_i' = a_i'' \qquad (13)$$

Results and Discussion

The VERS model is applied to describe the phase equilibrium in PEG + K_2HPO_4 + water and PEG + Na_2SO_4 + water systems. It was assumed that both salts dissociate completely in water. When potassium phosphate is dissolved in an aqueous phase, the following chemical equilibrium reactions have to be considered:

$$HPO_4^{2-} + H_2O \rightleftharpoons PO_4^{3-} + H_3O^+ \qquad (14)$$

$$H_2PO_4^- + H_2O \rightleftharpoons HPO_4^{2-} + H_3O^+ \qquad (15)$$

$$H_3PO_4 + H_2O \rightleftharpoons H_2PO_4^- + H_3O^+ \qquad (16)$$

Furthermore, the dissociation of water has to be taken into account:

$$2H_2O \rightleftharpoons OH^- + H_3O^+ \qquad (17)$$

In systems with Na_2SO_4, the formation of bisulfate was additionally taken into account:

$$HSO_4^- + H_2O \rightleftharpoons SO_4^{2-} + H_3O^+ \qquad (18)$$

The chemical equilibrium constants for these reactions (on a molality scale) were taken from the literature[19] (cf. Table 13).

The surface parameter of water ($q_w = 1.40$) was also assigned to all ions; thus, for an aqueous solution of a low molecular weight, strong electrolyte, the model reduces to Pitzer's equation. It was assumed that PEG 4000 and PEG 1500 consist of 89 and 32, respectively, PEG middle groups ($-CH_2-O-CH_2-$) and two end groups ($HO-CH_2-$) with the surface parameters $q_{PEG,middle} = 1.32$ and $q_{PEG,end} = 1.74$, resulting in $q_{PEG4000} = 120.96$ and $q_{PEG1500} = 45.7$.

The binary and ternary interaction parameters for the protein free aqueous two-phase systems were adopted from Grossman et al.[19] and Brenneisen[22] (cf. Tables 14 and 15).

The protein molecules are treated as hard spheres with neutral and charged surface patches. The total number of surface patches is calculated from literature data for the diameter of a globular protein molecule, assuming that one

Table 12. Experimental Results as Mass Fraction (w) for the Partitioning of α-Amylase (4) in PEG4000 (1) + Na_2SO_4 (2) + Water (3) Aqueous Two-Phase Systems at 298 K and pH = 5.4

feed			upper phase			lower phase		
$100w_2$	$100w_1$	$100w_4$	$100w_2$	$100w_1$	$100w_4$	$100w_2$	$100w_1$	$100w_4$
8.10	8.86	0.0900	3.80	22.29	0.0602	10.24	1.94	0.0913
7.81	8.88	0.0900	4.30	19.64	0.0625	11.86	0.62	0.0880
7.75	9.57	0.0900	4.02	21.36	0.0635	10.04	2.15	0.0857
7.48	8.86	0.0900	5.02	17.12	0.0655	8.87	4.06	0.0856
7.46	9.54	0.0900	4.54	19.39	0.0642	9.39	3.17	0.0873
7.06	10.92	0.0900	4.48	19.17	0.0631	11.00	1.30	0.0906

Table 13. Equilibrium Constants for Reactions 15−19

reaction	K
15	1.0096×10^{-12}
16	6.2951×10^{-8}
17	7.3961×10^{-3}
18	1.0046×10^{-14}
19	1.5510×10^{-2}

Table 14. Binary Interaction Parameters for PEG + K_2HPO_4 + Water Systems at 298 K (Grossmann et al.[19])

$A^0_{K^+,H_2PO_4^-}$	−0.048 67
$A^0_{K^+,HPO_4^{2-}}$	0.009 03
$A^0_{K^+,PO_4^{3-}}$	0.551 00
$A^1_{K^+,H_2PO_4^-}$	−0.133 00
$A^1_{K^+,HPO_4^{2-}}$	1.854 70
$A^1_{K^+,PO_4^{3-}}$	9.691 30
$A^0_{K^+,PEG}$	0.044 87
$A^0_{PEG,PEG}$	0.009 14
$A^0_{PEG,HPO_4^{2-}}$	0.046 30
$A^0_{PEG,PO_4^{3-}}$	0.000 07
$A^0_{PEG,H_2PO_4^-}$	0.054 50[a]

[a] This work.

Table 15. Interaction Parameters for PEG + Na_2SO_4 + Water Systems at 298 K (Brenneisen[22])

$A^0_{Na^+,SO_4^{2-}}$	0.018 69
$A^0_{PEG,SO_4^{2-}}$	0.153 33
$A^0_{PEG,PEG}$	0.008 35
$A^1_{Na^+,SO_4^{2-}}$	1.099 40
$B_{PEG,SO_4^{2-},SO_4^{2-}}$	−0.015 37
$B_{SO_4^{2-},Na^+,Na^+}$	0.001 31

patch has the surface of a $-CH_2$ group. That area was taken from Marcus.[23]

$$\text{No. of patches} = \frac{\text{Surface of protein}}{\text{Surface of a patch}} \qquad (19)$$

Either the patches are electrically neutral or they carry a single positive or negative charge. The number of charged patches is equivalent to the number of net charges on the protein, and the number of neutral patches is the difference between the total number of patches and the net charge number of the protein.

The hard sphere diameter of the lysozyme is 30.4 Å,[24] resulting in 58 patches per lysozyme molecule. The net charge number of the lysozyme at pH = 9.1 is +6, resulting in 6 patches with a positive unit charge and 52 neutral patches. For α-amylase and BSA, the total number of patches is 144 and 222, respectively. In the frame of the VERS model, a protein is treated as a mixture of neutral and charged groups (patches). The surface parameter of a group is $q = 1.2$. The interactions between a protein and another solute are taken into account by interactions between the protein groups on one side and the solute groups an the other side. As common in the area of electrolyte solutions, interaction parameters between groups carrying electrical charges of the same sign are neglected; for example, the binary interaction parameter between K^+ and a positively charged lysozyme patch is assumed to be zero. The still very limited experimental data on the influence of pH on the partitioning of a protein do not allow us to fit all remaining interaction parameters in a reasonable way. Therefore, it was decided to use this method only for correlation/interpolation purposes. For this restricted application, it was sufficient to adjust two binary interaction parameters as long as the partitioning of lysozyme to the PEG + K_2HPO_4 + H_2O system is considered under conditions where lysozyme carries a positive net charge (pH < pI; the isoelectric point of the lysozyme is pI = 11). In the experiments for the partitioning of lysozyme (7.2 < pH < 10.2), the charge number decreases from 8 to 3. As the lysozyme molecule carries only a positive net charge in this range, the interactions of patch groups with potassium ions were neglected ($A^0_{lys,K^+} = 0$). The remaining parameters describe interactions between any (i.e. uncharged as well as charged) lysozyme group on one side and HPO_4^{2-} as well as the PEG group, respectively, on the other side ($A^0_{lys,HPO_4^{2-}}$ and $A^0_{lys,PEG}$). These parameters were fitted to the experimental results for the partitioning of lysozyme at various pH values by minimizing the following objective function:

$$OF = \sum_J \left[\frac{(a_p)^I_J - (a_p)^{II}_J}{(a_p)^{II}_J} \right]^2 \qquad (20)$$

This fitting showed that $A^0_{lys,HPO_4^{2-}}$ depends on pH; for example, decreasing the pH from 10.2 to 7.2 shifts that parameter from 0.014 to 0.043. Such an influence might be avoided by increasing the number of adjustable parameters. However, then the numerical values for those parameters are no longer unambiguous. Therefore, we perferred to pay with that deficiency for the sake of a lower number of parameters.

The isoelectric point of α-amylase is pI = 5.4, and in the pH range from 6.8 to 9.1, the charge number changes from −5 to −11. BSA also carries a negative net charge at pH = 9.1, the only pH where partitioning experiments for BSA were performed. Therefore, the binary parameters $A^0_{α\text{-}Am,HPO_4^{2-}}$ and $A^0_{BSA,HPO_4^{2-}}$ were set to zero and $A^0_{α\text{-}Am,K^+}$ and A^0_{BSA,K^+} were fitted to the experimental results for the protein partitioning. A parameter study revealed that the parameters for interactions between α-amylase and BSA on one side and PEG groups on the other side can be set to zero ($A^0_{BSA,PEG} = A^0_{α\text{-}Am,PEG} = 0$). The binary parameter for interactions between α-amylase and potassium ions proved to depend on pH, whereas the influence of pH on A^0_{BSA,K^+} could be neglected.

The modeling of the partitioning of lysozyme and α-amylase in aqueous two-phase systems of PEG and Na_2SO_4 was performed in the same way as that described for the systems with PEG + K_2HPO_4. All interaction parameters are given in Table 16 (for PEG + K_2HPO_4 + H_2O) and Table 17 (for PEG + Na_2SO_4 + H_2O). The results for the partition coefficient of lysozyme, BSA, and α-amylase in PEG + K_2HPO_4 + water systems with different molecular weights of PEG at different pH values are shown in Figures 2 and 3 (lysozyme), Figure 4 (BSA), and Figure 5 (α-amylase). These figures demonstrate the good agreement between experiment and correlation.

Table 16. Protein Interaction Parameters in PEG + K_2HPO_4 + Water Systems at 298 K

pH	$z_{protein}$	parameter	value
7.2	8	$A^0_{Lys,HPO_4^{2-}}$	0.042 57
8.2	7	$A^0_{Lys,HPO_4^{2-}}$	0.031 25
9.1	6	$A^0_{Lys,HPO_4^{2-}}$	0.023 67
10.2	4	$A^0_{Lys,HPO_4^{2-}}$	0.014 08
6.8	−5	A^0_{amy,K^+}	0.003 14
7.2	−6	A^0_{amy,K^+}	0.003 15
9.1	−11	A^0_{amy,K^+}	0.005 29
9.1	−37	A^0_{BSA,K^+}	−0.090 96
-	-	$A^0_{Lys,PEG}$	−0.001 468

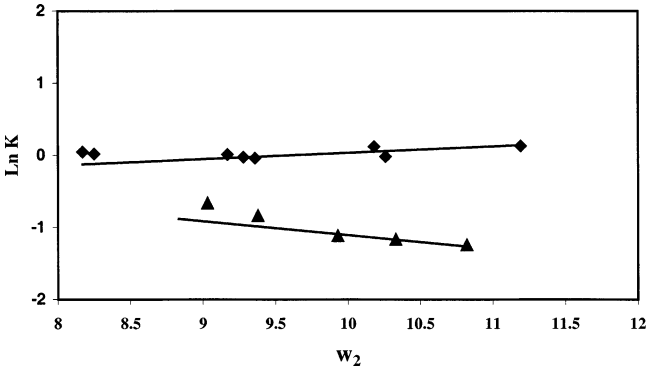

Figure 2. Comparison of lysozyme partition coefficients of the PEG (1) + K_2HPO_4 (2) + water (3) system at pH = 9.1 and 298 K: ◆, PEG 1500; ▲, PEG 4000; —, VERS model.

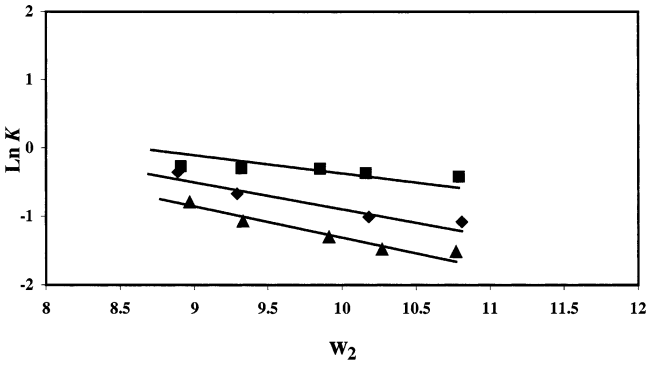

Figure 3. Comparison of lysozyme partition coefficients in the PEG 4000 (1) + K_2HPO_4 (2) + water (3) system at different pH values and 298 K: ■, pH = 10.2; ◆, pH = 7.2; ▲, pH = 8.1; —, VERS model.

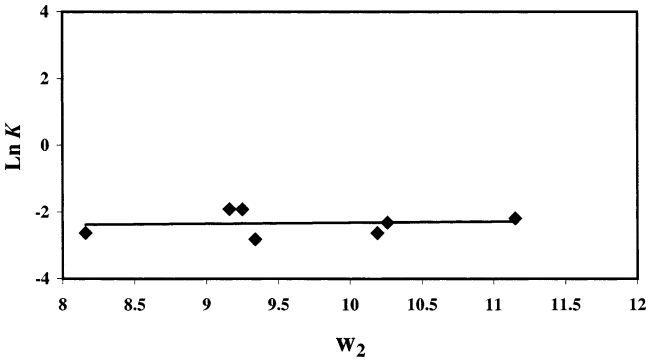

Figure 4. Comparison of BSA partition coefficients in the PEG1500 (1) + K_2HPO_4 (2) + water (3) system at 298 K and pH = 9.1: ◆, experiment; —, VERS model.

Table 17. Protein Interaction Parameters in PEG + Na_2SO_4 + Water Systems at 298 K

pH	$z_{protein}$	parameter	value
4.7	11	$A^0_{Lys,SO_4^{2-}}$	0.018 27
5.3	10	$A^0_{Lys,SO_4^{2-}}$	−0.014 27
5.9	9	$A^0_{Lys,SO_4^{2-}}$	−0.032 78
6.7	8	$A^0_{Lys,SO_4^{2-}}$	−0.051 27
9.1	6	$A^0_{Lys,SO_4^{2-}}$	−0.082 44
5.4	−1	A^0_{amy,Na^+}	−0.002 82
-	-	$A^0_{Lys,PEG}$	−0.017 17

The results of the correlation of the partitioning of lysozyme in the PEG + Na_2SO_4 + water systems are shown in Figures 6 and 7 for 4.7 ≤ pH ≤ 9.1 (corresponding to a net charge number of lysozyme 11 ≥ $z_{lysozyme}$ ≥ 6. Figure 8 shows similar comparisons for the partition coefficient of α-amylase in the PEG 4000 + Na_2SO_4 + water system at

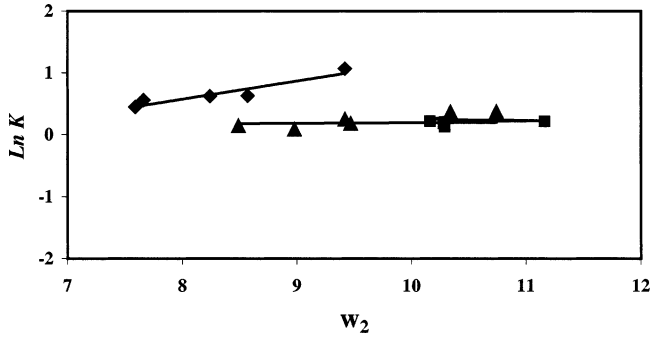

Figure 5. Comparison of α-amylase partition coefficients in the PEG4000 (1) + K_2HPO_4 (2) + water (3) system at different pH values and 298 K: ■, pH = 6.8; ◆, pH = 9.1; ▲, pH = 7.2; —, VERS model.

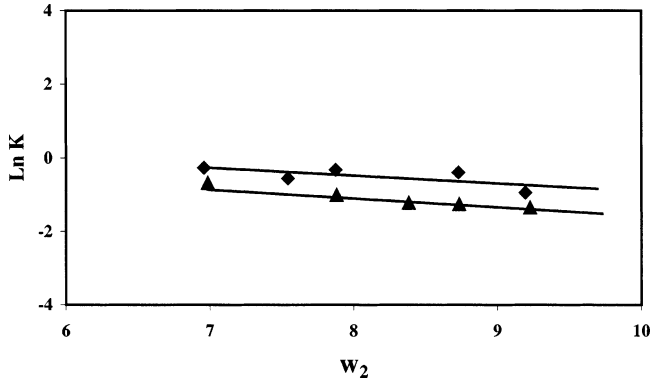

Figure 6. Comparison of lysozyme partition coefficients in the PEG4000 (1) + Na_2SO_4 (2) + water (3) system at 298 K: ◆, pH = 9.1; ▲, pH = 6.7; —, VERS model.

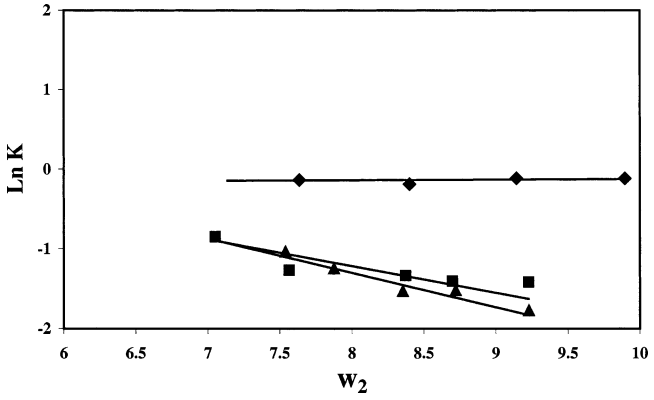

Figure 7. Comparison of lysozyme partition coefficients in the PEG (1) + Na_2SO_4 (2) + water (3) system at 298 K: ■, pH = 5.7 (PEG4000); ▲, pH = 5.3 (PEG4000); ◆, pH = 4.7 (PEG1500); —, VERS model.

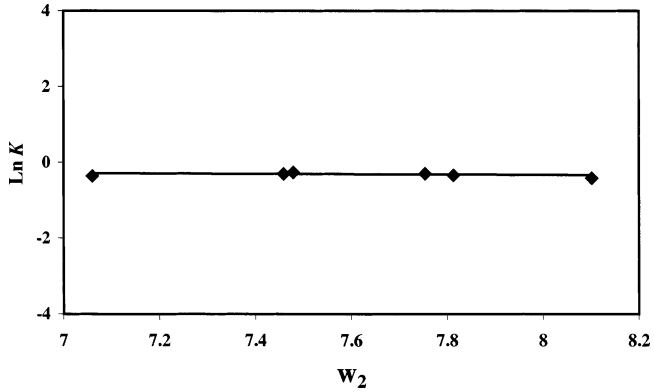

Figure 8. Comparison of α-amylase partition coefficients in the PEG4000 (1) + Na_2SO_4 (2) + water (3) system at 298 K and pH = 5.3: ◆, experiment; —, VERS model.

298 K and pH = 5.3. The results from the correlation are always in good agreement with the experimental data.

Conclusion

Experimental results are presented for the partitioning of lysozyme, BSA, and α-amylase in aqueous two-phase systems of PEG + K_2HPO_4 + water and PEG + Na_2SO_4 + water at 298 K at various pH values. The experimental results are successfully correlated with an osmotic virial equation of state. Such correlations also require us to know the influence of pH on the electric net charge of the protein molecules. This influence was determined by combining isoelectric focusing and titration experiments. However, the calculations revealed that a good correlation can only be achieved assuming that some interaction parameters of the osmotic virial equation of state depend on the pH of the aqueous phase.

Symbols

K = partition coefficient
K_0 = partition coefficient in the absence of an electrical potential difference
F = Faraday's constant
z = electric charge number
R = universal gas constant
T = absolute temperature
a = activity
M = molecular mass
m = molality
A_φ = Debye−Huckel parameter of water
I = ionic strength
b = parameter (=1.2 (mol/kg))
A_{ij} = binary interaction parameter
$A_{ij}(\nu)$ = binary interaction parameters (ν = 0; 1)
B_{ijk} = ternary interaction parameter
q = surface parameter
pI = pH at the isoelectric point
IEF = isoelectric focusing
w = mass percent
OF = objective function

Subscripts

w = water
i, k = component, species
p = protein

Superscripts

I = phase I
II = phase II
* = unsymmetrical convention

Greek Letters

Δ = difference
Φ = electrical potential
θ = surface fraction
α = constant
μ = chemical potential

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