

Effects of pH, salt type, and ionic strength on the second virial coefficients of aqueous bovine serum albumin solutions

YoonKook Park*[†] and Ginkyu Choi**

*Department of Chemical System Engineering, **Department of Electronic and Electrical Engineering, Hongik University, Jochiwon-up, Yongi-gu 339-701, Korea
(Received 8 August 2008 • accepted 28 October 2008)

Abstract—The osmotic pressures of aqueous bovine serum albumin (BSA) solutions were measured at different pH (3.6, 4.6, 5.6, and 7.6) in combination with different concentrations (0.01 M, 0.1 M, 1 M, and 3 M) of salts (sodium, potassium, and lithium) by using a Wescor colloid membrane osmometer. The osmotic second virial coefficients for BSA were determined from the experimental osmotic pressure. Predominant forces between protein molecules were measured at the various pH, ionic strength, and type of salt. These experimental data were utilized to determine the depth of square-well potential, which accounts for specific interactions between protein molecules at various conditions.

Key words: Second Virial Coefficients, Specific Interaction, SBA, Osmotic Pressure, PMF

INTRODUCTION

Protein aggregation is a significant problem in the biochemical, biomedical, and pharmaceutical industries. The formation of protein aggregates has been blamed for numerous diseases, including both Alzheimer's and Parkinson's disease [1,2]. Additionally, the production and delivery of protein drugs are also often complicated by the association process. As a result, protein association is of considerable interest to the biotechnology industry. In spite of the critical importance of protein aggregation, very little is presently known about the details of the process, particularly, the effects of association on the structural properties and conformational stability of protein [3].

Furthermore, understanding weak protein interactions is also important for treating biological disorders [4], structure-based drug design [5], purifying protein mixtures [6], understanding protein diffusion in concentrated solutions [7], and stabilizing protein-based therapeutic formulations. However, these interactions are typically too weak to be characterized in quantitative terms such as association constants. Association constants are normally used for strong protein interactions that can be measured by using methods like surface plasmon resonance or fluorescence polarization. Instead, weak protein interactions are often characterized in terms of the osmotic second virial coefficient, B_{22} . This is mainly because this coefficient can be measured by traditional colloidal characterization techniques, as well as newer methods such as static light scattering [8], small-angle x-ray [9], or neutron scattering [10], and membrane osmometry [11].

A number of new techniques allowing the measurement of protein interactions have recently become available. However, measurement of the osmotic pressure still remains one of the most effec-

tive approaches. The first experiments to determine osmotic pressure were performed as early as 1899 by Starling [12]. Since then, the osmometer has often been used to characterize proteins in solution. One such example is the determination of the osmotic pressure of hen egg-white lysozyme in the presence of ammonium sulfate as a salt by Moon et al. [13].

In addition to the new developments in experimental methods, many research groups have proposed different theoretical methods to investigate protein interactions in aqueous media. In 1945, McMillan and Mayer [14] treated the solvent and its constituents as a continuum, using statistical mechanics based on the assumption that their interactions with the protein molecules could be ignored. This assumption made it possible to relate the osmotic virial coefficient to the potential mean force between the protein molecules. Vilker et al. [15] applied the virial expansion model for BSA solutions, in the presence of several intermolecular potential functions (charge-charge, charge-dipole, dipole-dipole, charge-induced dipole, dipole-induced dipole). It has also been shown that repulsive charge-charge interactions and attractive dispersion interactions are very similar to the DLVO theory [16]. However, this method is not valid for concentrated proteins, as water interaction in hydration is distinctly different from its behavior in bulk.

Table 1 provides a selected list of the experimental conditions used and methods applied by previous researchers to investigate the effects of different salt concentrations and pHs on the osmotic pressure of the proteins in water. As indicated in Table 1, the colloidal osmometer and various scattering techniques seem to be the predominant methods used in studies of protein molecules interactions.

In this study, osmotic pressures at various BSA concentrations were measured with different salts by using an osmometer. Osmotic pressure experimental data was used to determine the second virial coefficient of BSA at various experimental conditions. The effects of salt, salt type, ionic strength, and pH on the second virial coefficients were investigated. The potential of mean force was applied to determine the depth of the square-well depth potential in order to account for the specific interaction between BSA molecules.

[†]To whom correspondence should be addressed.

E-mail: parky@hongik.ac.kr

[‡]This article is dedicated to Professor Chul Soo Lee in commemoration of his retirement from Department of Chemical and Biological Engineering of Korea University.

Table 1. List of experimental conditions and methods studied

Protein	Protein conc. salt [g/Lit]	Salt(s)	Salt conc.	pH	Method	Ref.
α -Chymotrypsin	40	Potassium sulfate	0.05-0.3 M	3-12	Osmometer LALLS ^a	37
	10	Sodium phosphate Sodium chloride			SLS ^b SANS ^c	10
Lysozyme	10	Sodium chloride	0.005-0.5 M	3-9	SLS ^b SANS ^c	10
	100	Ammonium sulfate	1 and 3 M	4-8	Osmometer	13
	450	Sodium chloride	0.15 M	7	Osmometer	38
	5	Potassium chloride				
		Ammonium sulfate	0.1 and 1 M	5-8	LALLS ^a	22
		Sodium chloride				
		Potassium isothiocyanate				
		Potassium chloride				
BSA ^d	450	Sodium chloride	0.15 M	4.5-7.4	Osmometer	15
	100	Ammonium sulfate	1 and 3 M	4-8	Osmometer	13
Ovalbumin	450	Sodium chloride	0.15 and 0.5 M	7	Osmometer	38
	5	Ammonium sulfate	0.1 and 1 M	5-8	LALLS ^a	22
		Sodium chloride				
		Potassium isothiocyanate				
		Potassium chloride				

^aLALLS: low angle laser light scattering^bSLS: static light scattering^cSANS: small-angle neutron scattering^dBSA: bovine serum albumin

EXPERIMENTAL

1. Materials

Bovine serum albumin (BSA) was purchased from Sigma-Aldrich and used as received. BSA solutions were prepared with deionized water from a Millipore Milli-Q system. Sodium chloride, potassium chloride and lithium chloride were used as salts in the BSA aqueous solutions. Mono- and di-basic sodium phosphate (0.1 M citric acid) were used to make the buffer solution. 0.1 N sodium hydroxide and 0.1 N hydrochloric acid solutions were used to adjust the pH of the BSA aqueous solution. Sodium azide was used as an anti-bacterial agent. All of the above materials were purchased from Sigma-Aldrich.

2. Experimental Methods

The osmotic pressure of bovine serum albumin (BSA) was measured by using a commercially available Wescor colloid osmometer (model 4420). The osmometer was purchased from Millipore and was equipped with cellulose membranes with a 10,000 molecular-weight cut-off. 0.15 M sodium chloride aqueous solution was prepared by using the sodium phosphate buffer solution, and the pH was adjusted to 4.5, 5.4, or 7.4. The pH was measured with a pH meter (Mettler, model MP 220). A BSA aqueous solution with a concentration of about 100 [g/Lit] was prepared by using the 0.15 M sodium chloride solution. The resulting BSA solution was diluted with 0.15 M sodium chloride aqueous solution to yield solutions with several different concentration of BSA. The solution concen-

trations were measured with a UV-vis spectrometer (Ocean Optics Inc., model USB 2000). In order to achieve the desired pH of the BSA aqueous solution, either 0.1 N hydrochloric acid or 0.1 N sodium hydroxide solution was added and vigorously mixed to prevent local denaturation of the protein. A similar experimental procedure used by previous researchers [11,15] was adopted. In the case where potassium chloride or lithium chloride was used as a salt, the same

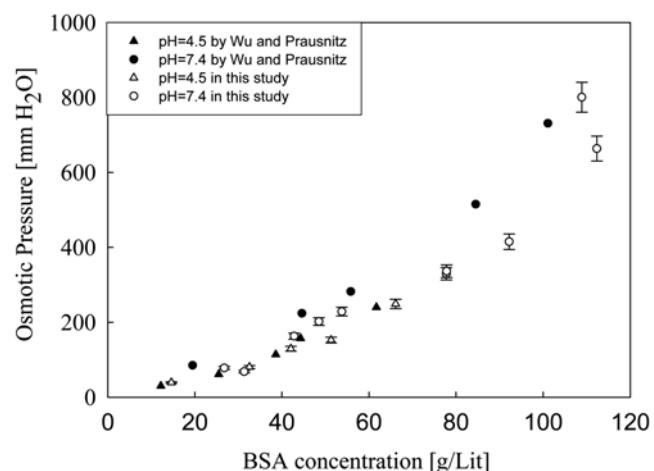


Fig. 1. Osmotic pressure of BSA aqueous solution at pH 4.5 and 7.4 and 1M sodium chloride concentration.

experimental method was applied but with a different salt concentration and pH. The system temperature was maintained at 298 ± 1 K during all experiments.

In order to confirm the experimental data, the data obtained using this osmometer in the lab was compared to published data [11]. As indicated in Fig. 1, reliable data within a reasonable margin of error were obtained. Osmotic pressure, in general, can be expressed in the form of a virial expansion. If only the second virial coefficient is assumed to be significant by neglecting the higher order contribution, then the osmotic pressure Π can be expressed as

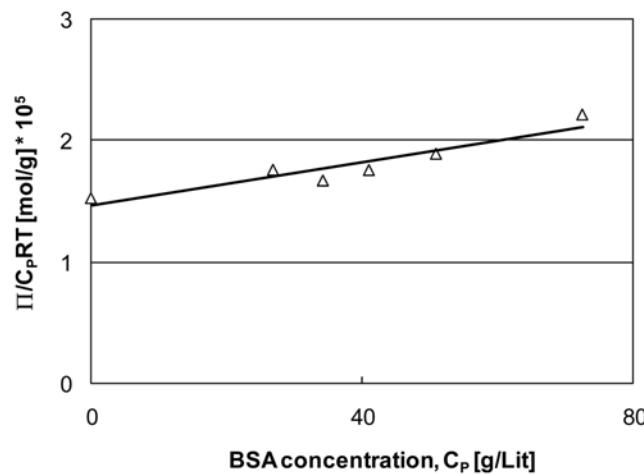


Fig. 2. Estimation of molecular weights of BSA and osmotic second virial coefficient at pH of 4.6 and 0.1 M concentration aqueous KCl solution.

Table 2. Molecular weights and osmotic second virial coefficients of BSA from osmometer measurements in various aqueous salt solutions at 298 K

Salt	Salt conc.	pH	Mw [g/mol]	$B'_{22} \times 10^4$ [mL mol/g ²]	B_{22} [m ³ /mol]
NaCl*	0.15 M	7.4	69000	1.34	0.637
		5.4	69000	1.11	0.531
		4.5	69000	1.06	0.506
KCl	0.1 M	3.6	108879	0.26	0.311
		4.6	74541	1.13	0.630
		5.6	63420	0.92	0.369
		7.6	101010	1.71	1.747
	1 M	4.6	77927	0.63	0.383
		5.6	80316	1.26	0.810
		7.6	135544	2.29	4.213
	3 M	5.6	107814	1.39	1.611
		7.6	83623	1.35	0.946
LiCl	0.01 M	4.6	107559	0.61	0.700
		5.6	93821	0.80	0.700
		7.6	82510	1.06	0.725
	0.1 M	4.6	94415	0.64	0.569
		5.6	82470	0.65	0.442
		7.6	117730	1.15	1.597

* Reference [15] Vilker et al. (1981)

$$\frac{\Pi}{c_p RT} = \frac{1}{M_p} + B_{22} c_p \quad (1)$$

where c_p is the protein concentration [g/Lit], R is the gas constant, T is the absolute temperature, and M_p is the protein molecular weight. The protein molecular weight (M_p) and the osmotic second virial coefficient (B_{22}) are obtained by plotting $\Pi/c_p RT$ against protein concentration (c_p). Fig. 2 shows the method applied to obtain the osmotic second virial coefficient experimentally. As shown, it produced an acceptably linear relation between $\Pi/c_p RT$ and protein concentration (c_p). The osmotic second virial coefficients obtained experimentally are listed in Table 2.

The osmotic second virial coefficient can be used as a means to quantify the degree of nonideality. If B_{22} is equal to 0 (along with other higher order virial coefficients), then Eq. (1) reduces to the ideal solution behavior, obeying the van't Hoff equation, as indicated in Eq. (2).

$$\Pi = \frac{c_p}{M_p} RT \quad (2)$$

As a result, the value of B_{22} prescribes the nature of the interaction between protein molecules at the molecular level. If B_{22} is positive, then the osmotic pressure is greater than that for an ideal solution, which implies that repulsive interactions are predominant in the system. The reverse is true for negative values of B_{22} , where attractive interactions dominate.

THEORETICAL APPROACH

The osmotic second virial coefficient can be related to the potential of mean force (PMF) using a statistical mechanical approach as proposed by McMillan and Mayer [14]. Specifically, B_{22} can be expressed as

$$B_{22} = -\frac{1}{2} \frac{N_A}{M_p^2} \int_0^{\infty} [e^{-W(r)/kT} - 1] 4\pi r^2 dr \quad (3)$$

where $W(r)$ is potential of mean force; k , the Boltzmann constant and r , the intermolecular center-to-center distance. Note that Eq. (3) was obtained under the assumption that the protein molecules are spheres, such that $W(r)$ is the sum of pairwise potentials:

$$W(r) = W_{hs}(r) + W_{elec}(r) + W_{disp}(r) + W_{spec}(r) \quad (4)$$

where, $W_{hs}(r)$ is the hard sphere potential; $W_{elec}(r)$, the double layer repulsion potential; $W_{disp}(r)$, the dispersion potential; and $W_{spec}(r)$, a square-well potential that accounts for hydrophobic interactions.

The hard sphere potential is given by

$$W_{hs}(r) = \begin{cases} \infty & \text{for } r \leq \sigma \\ 0 & \text{for } r > \sigma \end{cases} \quad (5)$$

where σ is the effective hard sphere diameter of BSA. The hard sphere diameter can be estimated with the following equation:

$$\sigma = 2 \left(\frac{3\sqrt{M_p}}{4\pi N_A} \right)^{1/3} \quad (6)$$

The double layer repulsive interaction $W_{elec}(r)$ is approximated by

$$W_{elec}(r) = \frac{Z^2 e^2 e^{-\kappa(r-\sigma)}}{4\pi\epsilon_0\epsilon_r \left(1 + \frac{\kappa\sigma}{2} \right)^2} \quad (7)$$

Here, Z is the charge of BSA; e , the charge of an electron; ϵ_0 , the permittivity constant; and ϵ_r , the relative dielectric constant of water. κ is the inverse Debye length related to the ionic strength of the solution, I , and temperature T by

$$\kappa^2 = \frac{2e^2 N_A I}{kT \epsilon_0 \epsilon_r} \quad (8)$$

The attractive Hamaker dispersion potential is given by Hamaker:

$$W_{disp}(r) = -\frac{H}{12} \left[\frac{\sigma^2}{(\sigma+2\kappa)^2 - \sigma^2} + \frac{\sigma^2}{(\sigma+2\kappa)^2} + 2\ln\left(1 - \frac{\sigma^2}{(\sigma+2\kappa)^2}\right) \right] \quad (9-1)$$

for $r \leq \sigma+2\kappa$

$$W_{disp}(r) = -\frac{H}{12} \left[\frac{\sigma^2}{r^2 - \sigma^2} + \frac{\sigma^2}{r^2} + 2\ln\left(1 - \frac{\sigma^2}{r^2}\right) \right] \quad (9-2)$$

for $r > \sigma+2\kappa$

where H is the effective Hamaker constant for protein-protein dispersion interactions. A good approximation for the Hamaker constant of a protein in aqueous solution is on the order of $5 kT$ [17].

The square-well potential is given by

$$W_{spec}(r) = \begin{cases} -\epsilon_{spec} & \text{for } \sigma \leq r \leq \sigma + \delta \\ 0 & \text{for } r > \sigma + \delta \end{cases} \quad (10)$$

where ϵ_{spec} is the depth of the square well and δ is the width of the square well. Positive well depths correspond to an attractive potential, and negative well depths correspond to a repulsive potential. A value of 0.134σ was chosen for δ by assuming that dimer of BSA are formed only under this experimental condition [18].

Tanford et al. [19] developed a model to estimate the net charge of BSA. Pujar and Zydny [20] showed reliable results to determine the net charge of BSA. This method was used to calculate BSA net charge and the results are shown in Table 3. It should be noted that a combination of bisection, secant, and inverse quadratic interpolation methods was used to estimate net charge value of BSA.

RESULTS AND DISCUSSION

Table 3. Net charge on BSA calculated by using the Pujar and Zydny method [20] and values for ϵ_{spec}/kT for BSA in various aqueous solutions from the PMF model

Salt	Salt conc.	pH	Z	ϵ_{spec}/kT
KCl	0.1 M	3.6	7.2	5.9
		4.6	0.8	6.4
		5.6	-4.8	5.3
		7.6	-11.4	6.8
	1 M	4.6	-0.9	5.0
		5.6	-7.5	6.9
		7.6	-15.1	5.6
	3 M	5.6	-1.0	5.1
		7.6	-18.0	6.4
LiCl	0.01 M	4.6	2.1	7.2
		5.6	-2.9	5.4
		7.6	-9.7	5.5
	0.1 M	4.6	0.8	6.3
		5.6	-4.8	5.2
		7.6	-11.4	6.8

As shown in Table 2, a series of experiments were performed using potassium chloride and lithium chloride as the salt at various pHs (3.6-7.6) to investigate the effect of pH on the BSA molecular interactions. Overall, several results were found: 1) as the pH was increased from 4.6 to 7.6, the second virial coefficient of BSA also increased; 2) the second virial coefficient of BSA decreased from 1.6 to 0.94 [m³/mol] as the pH was increased from 5.6 to 7.6 when the potassium chloride ionic strength was 3M, indicating that there is a significant effect of ionic strength on the protein-protein interaction; and 3) this behavior may be attributed to the conformational changes of protein. It appears that the effect of pH, salt, and salt concentration is quite significant in interactions between proteins.

1. Effect of pH

Several proteins can be unfolded by a decrease in pH due to Coulombic repulsion from the net positive charges of the polypeptide chain [21]. This effect can be offset by the presence of anions, which bind to these positively charged sites and screen them, leading to the formation of partially-folded intermediates [3]. Anions differ in their neutralizing effect on the net positive charges in the acid-unfolded polypeptide chain and in the amount of structure and compactness they induce. For example, the osmotic second virial coefficient of BSA, B_{22} , was 0.311 at pH 3.6, compared to 1.747 at pH 7.6 in potassium chloride 0.1 M ionic strength. Similarly, in the 0.1 M lithium chloride ionic strength B_{22} was 0.569 at pH 4.6 compared to 1.597 at pH 7.6. The effect of pH on the osmotic second virial coefficient of BSA was significant.

2. Effect of the Type of Salt

It has been reported [13,22-23] that the osmotic pressure of proteins in aqueous media is dependent on the type of the salt. In general, ions that have the greatest effect resulting in stronger interactions with water than water itself, are known as kosmotropes, while ions that have the opposite effect are known as chaotropes. The order of various ions, based on their ability to precipitate a mixture of proteins [23], known as the Hofmeister series is as given below:

Anions: SCN⁻ > NO₃⁻ > Cl⁻ > citrate > acetate > phosphate > SO₄²⁻

Cations: Ca²⁺ > Mg²⁺ > K⁺ > Na⁺ > Li⁺

Based on the Hofmeister series, sodium cations and potassium cations have a similar effect on protein precipitation (salt-out), although the potassium cation is bigger than the sodium cation and lithium cations in terms of size. As Arakawa and Timasheff [24] reported, preferential interactions of proteins (BSA) with salt have a huge impact on the solubility as well as the stability of the protein molecules. There, the experiments were performed at lower ionic strength in the case of lithium chloride (0.01 M and 0.1 M only). Specifically, smaller ions such as lithium cations reduce hydrophobic solubilities in water, causing salt-out at more than 0.1 M of lithium chloride ionic strength.

In this study, the cation in the salt was systematically changed (see Table 2), while the anion was fixed (chloride anion in the protein solution) in order to investigate the effect of the cation on self-association of the protein in aqueous media. The B_{22} values did not vary much under the same experimental conditions. At 0.1 M ionic strength, B_{22} values were 1.747 and 1.597 in potassium chloride and lithium chloride aqueous solutions, respectively. The effect of the cation on the osmotic second virial coefficient in aqueous solution seemed to be less evident.

3. Effect of the Ionic Strength

The influence of salts on polymers is quite well understood and is mainly due to the screening of electrostatic interactions by salt ions and the Donnan effect [25]. Many studies have shown that the magnitude of various salt effects varies according to the lyotropic or Hofmeister series. Both the charge and the size of the ion play important roles in determining its lyotropic effects [26-28]. Several studies suggest that ions directly interact with polymers by binding to their hydrophilic groups [29]. Other studies suggest that there is an indirect interaction with the polymer, either through a perturbation of the water environment or by inducing changes in the hydrogen bonding of water to the polar groups of the polymers [30]. However, the interaction between protein molecules in a concentrated salt solution is not well understood.

To compare the trends of the virial coefficient patterns of BSA, we systematically varied salt concentration over a wide range including 0.01 M, 0.1 M, 1 M, and 3M (see Table 2). For BSA aqueous solutions with the presence of potassium chloride as salt, B_{22} remained positive for an entire range of salt concentrations. In addition, the magnitude of the osmotic second virial coefficient increased with an increase in the ionic strength of salt. As Moon et al. [31] reported, this may be attributed to the fact that the increase in concentration of salt ions resulted in decreases in the volume available to the protein. Similar results were obtained when lithium chloride was used as a salt.

4. Net Charge Value of BSA (Z) and the Depth of the Square Well (ϵ_{spec}/kT)

For effective utilization of the results obtained, it is important that the experimental results be incorporated in a robust model which can be used for predicting the behavior of proteins in water, particularly their degree of self-association. The development of a consistent microscopic approach for these complex systems is extremely difficult due to the presence of many interactions, including protein-protein, protein-electrolyte, electrolyte-solvent, solvent-solvent and protein-solvent interactions. The net charge of BSA as calculated by Pujar and Zydny [20] in various conditions is shown in Table 3. Based on the experiment [19], the value of Z in a pH 7 solution should be approximately -10. In the presence of 0.1 M chloride ion, Z is lowered to -20 [32]. This is due to the chloride binding to the BSA protein. The estimated net charge of BSA is acceptable with a reasonable error margin.

Table 3 shows the values of the specific interaction term, ϵ_{spec}/kT , in various aqueous solutions estimated with a perturbation introduced via the PMF model. The positive well depth values obtained indicate the presence of an attractive interaction in all the conditions studied. In general, the increase in concentration of salt ions triggered a significant impact on the excluded-volume effect. According to Kuehner et al. [33], the attractive interaction at a high salt concentration gives rise to a strong (several kT), short-range attractive potential.

$W_{spec}(r)$, in Eq. (4), is responsible for hydrophobic interactions between BSA protein molecules. In accordance with the suggestions by Zhang et al. [34], positive well depth values were obtained not only in lower ionic strength ($I \leq 0.5$ M), but also in moderate ($I \approx 0.5$ M) to high ionic strength ($I > 0.5$ M). This indicates the presence of attractive interaction. The effect of protein concentration on the strength of attraction was not investigated in this study and other

references [34-36] are referred to for this issue.

CONCLUSIONS

Immense effort has been devoted towards attempts to discover the structure-property relationships in aqueous protein solutions. The effect of pH, salt type, and ionic strength on the second virial coefficient of BSA in aqueous solution is quite significant. Specific interaction terms were evaluated by treating globular proteins as hard spheres with a PMF model. In the presence of chloride ion in the aqueous solution, a strong attractive potential exists over the entire range of salt concentration. This study bridges the gap between the existing experimental data and the theoretical model, providing experimental data required for a systematic investigation of the effects of various factors known to be closely related to the changes on the protein secondary structure.

ACKNOWLEDGMENTS

This work was supported by the 2007 Hongik University Research Fund. The authors are very grateful to Mr. D. Vickers (Tuskegee University) for his technical support.

REFERENCES

1. J. Goers, S. E. Permyakov, E. A. Permyakov, V. N. Uversky and A. L. Fink, *Biochemistry*, **41**, 12546 (2002).
2. M. Ramirez-Alvarado, J. S. Merkel and L. Regan, *PNAS*, **97**, 8979 (2000).
3. V. N. Uversky, A. S. Karnoup, R. Khurana, D. J. Segel, S. Doniach and A. L. Fink, *Protein Sci.*, **8**, 161 (1999).
4. P. M. Tessier, A. M. Lenhoff and S. I. Sandler, *Biophys. J.*, **82**, 1620 (2002).
5. A. J. Oakley and M. C. Wilce, *Clin. and Exp. Pharm. Physio.*, **27**, 145 (2000).
6. Z. Sun and J. Cai, *Korean J. Chem. Eng.*, **23**, 607 (2006).
7. D. E. Kuehner, C. Heyer, C. Rämsch, U. M. Fornefeld, H. W. Blanch and J. M. Prausnitz, *Biophys. J.*, **73**, 3211 (1997).
8. C. Hitscherich Jr., J. Kaplan, M. Allaman, J. Wiencek and P. J. Loll, *Protein Sci.*, **9**, 1559 (2000).
9. A. Dueruix, J. P. Guilloteau, M. Ries-Kautt and A. Tardieu, *Journal of Crystal Growth*, **168**, 28 (1996).
10. O. D. Velev, E. W. Kaler and A. M. Lenhoff, *Biophys. J.*, **75**, 2682 (1998).
11. J. Wu and J. M. Prausnitz, *Fluid Phase Equilibria*, **155**, 139 (1999).
12. E. H. Starling, *J. Physiol.*, **24**, 317 (1899).
13. Y. U. Moon, R. A. Curtis, C. O. Anderson, H. W. Blanch and J. M. Prausnitz, *Journal of Solution Chem.*, **29**, 699 (2000).
14. W. G. McMillan Jr. and J. E. Mayer, *J. Chem. Phys.*, **13**, 276 (1945).
15. V. L. Vilker, C. K. Colton and K. A. Smith, *J. Colloid Interface Sci.*, **79**, 548 (1981).
16. E. J. W. Verwey and J. T. G. Overbeek, *Theory of stability of hydrophobic colloids*, Elsevier, Amsterdam (1948).
17. S. Nir, *Prog. Surf. Sci.*, **8**, 1 (1977).
18. M. S. Wertheim, *J. Chem. Phys.*, **85**, 2929 (1986).
19. C. Tanford, S. A. Swanson and W. S. Shore, *J. Am. Chem. Soc.*, **77**, 6414 (1955).

20. N. S. Pujar and A. L. Zydny, *J. Colloid Interface Sci.*, **192**, 338 (1997).
21. C. Tanford, *Adv. Protein Chemistry*, **23**, 121 (1968).
22. R. A. Curtis, J. M. Prausnitz and H. W. Blanch, *Biot. Bioeng.*, **57**, 11 (1998).
23. F. Hofmeister, *Arch. Exp. Pathol. Pharmakol.*, **24**, 247 (1888).
24. T. Arakawa and S. N. Timasheff, *Biochem.*, **21**, 6545 (1982).
25. B. H. Chang and Y. C. Bae, *Biomacromolecules*, **4**, 1713 (2003).
26. K. D. Collins, *Biophys. J.*, **72**, 65 (1997).
27. N. Korolev, A. P. Lyubartsev, A. Rupprecht and L. Nordenskiold, *Biophys. J.*, **77**, 2736 (1999).
28. R. Vogel, G. B. Fan, M. Sheves and F. Siebert, *Biochem.*, **40**, 483 (2001).
29. P. von Hippel and T. Schleich, *Acc. Chem. Res.*, **2**, 257 (1969).
30. H. Muta, R. Kojima, S. Kawauchi, A. Tachibana and M. Satoh, *J. Mol. Structure: THEOCHEM*, **536**, 219 (2001).
31. Y. U. Moon, C. O. Anderson, H. W. Blanch and J. M. Prausnitz, *Fluid Phase Equilibria*, **168**, 229 (2000).
32. R. A. Alberty and H. H. Marvin Jr., *J. Am. Chem. Soc.*, **73**, 3220 (1951).
33. D. E. Kuehner, H. W. Blanch and J. M. Prausnitz, *Fluid Phase Equilibria*, **116**, 140 (1996).
34. F. Zhang, M. W. A. Skoda, R. M. J. Jacobs, R. A. Martin, C. M. Martin and F. Schreiber, *J. Phys. Chem. B*, **111**, 251 (2007).
35. R. A. Curtis, J. Ulrich, A. Montaser, J. M. Prausnitz and H. W. Blanch, *Biotech. Bioeng.*, **79**, 367 (2002).
36. J. Rescic, V. Vlachy, A. Jamnik and O. Glatter, *J. Colloid Interface Sci.*, **239**, 49 (2001).
37. C. A. Haynes, K. Tamura, H. R. Korfer, H. W. Blanch and J. M. Prausnitz, *J. Phys. Chem.*, **96**, 905 (1992).
38. M. A. Yousef, R. Datta and V. G. J. Rodgers, *J. Colloid Interface Sci.*, **243**, 321 (2001).