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Partition Behavior of Bovine Serum Albumin in PEG2000-Sodium Citrate-Water Based Aqueous Two-Phase System

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Abstract: The effect of phase forming components, pH, neutral salt (NaCl) addition on partitioning of bovine serum albumin (BSA) in PEG2000-sodium citrate based aqueous two-phase system was investigated at 25°, 35°, and 45°C. The systems were prepared by varying the phase forming components concentration at a pH of 5.0, 6.0, 7.0, 8.0, and 9.0 with different NaCl salt concentrations of 0.05 M, 0.1 M, 1.5 M, 0.2 M, 0.3 M, and 0.4 M. The affinity of the BSA for the lower phase increases with increase in pH, due to increase in ratio of trivalent to divalent citrate ions in the two-phase system. It was confirmed that the partition of BSA depends on the relative hydrophobicity of the compounds as well as their charge. Optimum level of pH and NaCl concentration for the partitioning of bovine serum albumin was determined.

Keywords: Bovine serum albumin, pH, polyethylene glycol, sodium citrate, optimization

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INTRODUCTION

Aqueous two-phase system (ATPS) has attracted the attention of the biotechnological industry because of its potential for the separation and purification of bio molecules namely proteins, enzymes, antibiotics etc. (1). ATPS have drawn more interest among the other separation processes, particularly in the field of biotechnology due to the mild conditions of the process, short processing time, and ease of scale-up (2, 3). The aqueous two-phase system obtained with the polymer and salt solution is found to have low interfacial tension, fast and high phase separation rates, and low cost, which made it most viable for the down stream processing compared with the polymer-polymer ATPS.

Most of the research works on aqueous polymer salt systems were made using polyethylene glycol with salt consisting of selective anions namely, phosphate, sulphate, or carbonates. These salts, however, led to high concentration of sulphate and phosphate salts in the effluent streams, causing environmental problems. Recently, Vernau and Kula (4) and Zafarani-Moattar et al. (5), and Murugesan and Perumalsamy (6) have used citrate, as a substitute for phosphate and sulphate salts to form an aqueous two-phase system with PEG. Since citrates are biodegradable and nontoxic, PEG + citrate salts could form environmentally safe aqueous two-phase systems, which are more suitable for the extraction of biological materials. In the present study, the PEG2000-sodium citrate based aqueous two-phase system was used to study the partition behavior of BSA.

Partitioning of protein in the two-phase system is a complex phenomenon, taking into account the interaction between the partitioned substance and the concentration of the phase forming components present in each phase. Partitioning of protein depends on both chemical and physical interactions namely, hydrogen bonding, charge interaction, van der Waals forces, hydrophobic interaction, and steric effects (1). Moreover, the distribution of molecules between the two phases mainly depends upon the concentration of phase forming components, the molecular weight of polymer, salt type, pH, and temperature of the system and the concentration of added neutral salt. Thus, the distribution of proteins between the two phases is characterized by the partition coefficient, K. The partition coefficient is defined as the ratio of the concentration of the protein in the top (C_{top}) and bottom (C_{bottom}) phase, respectively.

$$K = \frac{C_{Top}}{C_{bottom}} \tag{1}$$

Thus, the different factors affecting/enhancing the system can be manipulated in order to achieve the desired effect (3). In the present study, the partitioning of BSA was altered by changing the pH of the system and addition of neutral salt. The partition coefficient K of a biomolecule varies with the electrochemical potential difference between the phases and the net charge of the partitioned biomolecule (1).

EXPERIMENTAL

Materials

Analytical grade (Merck) polyethylene glycol with a molar mass average of 2000, tribasic sodium citrate dihydrate and sodium chloride were used. Bovin serum albumin (BSA) and Commercial Brilliant Blue G dye were purchased from Sigma Aldrich Company (St. Louis, M). Double-distilled, deionized water was used for the present experiments.

Measurement of Partition Coefficient

Concentrated (35.3%, w/w) sodium citrate solutions at the required pH were prepared by mixing appropriate amounts of equimolar solutions of tri-sodium citrate dihydrate and citric acid monohydrate. Aqueous two-phase systems were prepared by mixing suitable amounts of PEG and citrate solutions, with pure protein, in 15 ml graduated tubes with conical tips. A known amount of NaCl was also added to study the neutral salt effect on the phase behavior. The final weight was adjusted to 10 g by the addition of water. The systems were well-mixed by a vortex mixer and left in a water bath (Schott-Gerate CT 52, Germany) for overnight. Samples of top phase were taken using a pipette and the samples of the bottom phase were also taken through the top phase using a syringe by maintaining a small positive pressure to avoid contamination. The protein concentration in the individual phases was determined by the method of Bradford (7). For the determination of protein concentration, samples withdrawn from each phase were diluted with a known amount of distilled water, and its ultraviolet absorbance was measured in a dual- beam spectrophotometer at 595 nm. In order to make a necessary correction to avoid the interference of PEG and citrate, an identical solution of the corresponding phase without protein was used as a blank.

RESULTS AND DISCUSSION

In the present study, partitioning of bovine serum albumin was carried out in a series of PEG2000-sodium citrate-water based aqueous two-phase systems. The systems were prepared by varying the concentrations of phase forming components at a pH of 5.0, 6.0, 7.0, 8.0, and 9.0 with different NaCl salt concentrations of 0.05 M, 0.1 M, 1.5 M, 0.2 M, 0.3 M, and 0.4 M. The effect of system pH and NaCl concentration on the partition coefficient of Bovine Serum Albumin in PEG2000-sodium citrate-water system was analyzed and the details are discussed below. True partitioning occurs when the protein concentration in the system is <1 g/liter. Since the actual concentration limit depends on the properties of the protein, initially, partitioning experiments

were conducted with a protein concentration of 2 g/liter in the system. Since the precipitation of protein at the interface was observed, further experiments were carried out with a protein concentration of 1 g/liter.

Effect of PEG Concentration on BSA Partition Coefficient

The effect of PEG2000 concentration on the partition coefficient of BSA in PEG2000-sodium citrate based aqueous two-phase system is illustrated in Fig. 1. It was observed that the partition coefficient of BSA decreases with an increase in PEG2000 concentration and temperature, which is in good agreement with the other reported literature (Asenjo et al., 1994) (8). Since BSA is hydrophilic in nature, it will partition into salt-rich bottom phase, because the relative hydrophobicity of the bottom phase is very low (Wu et al. 2000) (9). The PEG concentration was varied from 0.125 to 0.225 (wt fraction) for a fixed sodium citrate salt concentration, 0.125 (wt fraction). The minimum partition coefficient of BSA was observed at a concentration of 0.225 (wt fraction) PEG and 0.125 (wt fraction) sodium citrate at all temperature (25°, 35°, and 45°C) at pH 9 and hence further experiments were conducted using the above concentration of PEG2000 and sodium citrate aqueous two-phase system.

Effect of pH on BSA Partition Coefficient

The effect of partitioning of BSA in PEG2000-sodium citrate-water system at a pH of 5.0, 6.0, 7.0, 8.0, and 9.0 at constant NaCl concentrations at 25°, 35°,

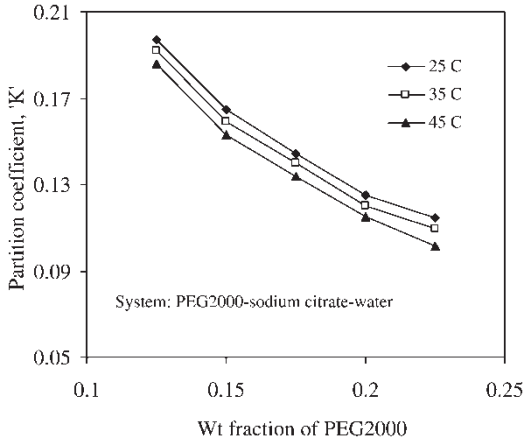

Figure 1. Effect of PEG2000 concentration on partition coefficient of BSA at different temperatures.

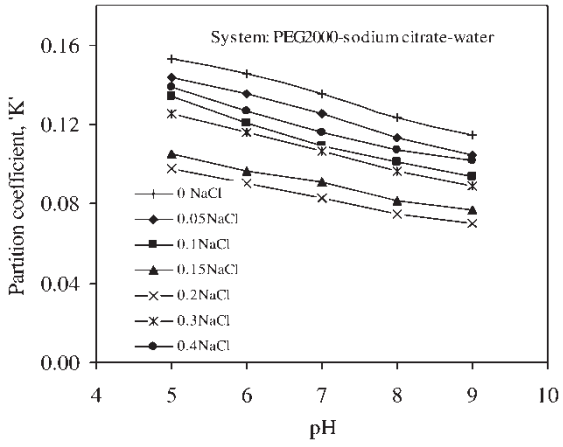

Figure 2. Effect of pH on the partitioning of BSA in PEG2000-sodium citrate-water system at 25°C.

and 45°C were shown in Figs. 2–4. From the figures it was observed that the partition coefficient of BSA decreased with an increase in the pH of the systems, at constant neutral salt (NaCl) concentration. The pH affects the partitioning, either by changing the charge of the solute or by altering the ratio of the charged species present (10). Albertsson 1986 (1) demonstrated that negatively charged materials like BSA at the working pH, have decreasing partition coefficients in the presence of neutral salts where as Walter et al. 1985 (2) showed the reverse behavior for positively charged proteins.

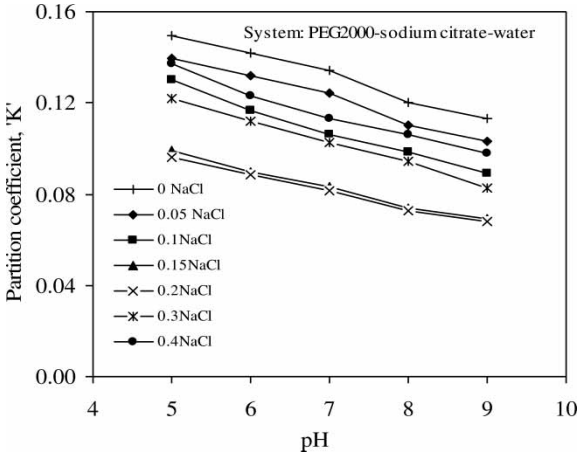

Figure 3. Effect of pH on the partitioning of BSA in PEG2000-sodium citrate-water system at 35°C.

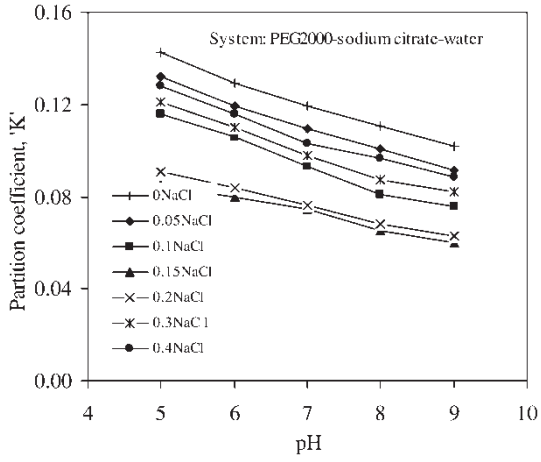

Figure 4. Effect of pH on the partitioning of BSA in PEG2000-sodium citrate-water system at 45°C.

In the present work BSA has a negative net charge at above isoelectric point (pI). The isoelectric point of protein is the pH value at which the net charge of the protein is neutral. The isoelectric point of BSA is 4.7 (Kristina Berggren et al. 1995) (11). The decrease in partition coefficient was observed when the charge of the protein changes from positive to negative. The affinity of the BSA for the lower phase increases, when the trivalent to divalent citrate ion ratio increases, which leads to more BSA partition into bottom phase, which is in good agreement with the observations of Zaslavsky 1978 and Hustedt et al. 1978 (3, 12). Moreover, it can be concluded that the enhanced protein affinity for the lower phase with increase in pH is due to an increase of the salting-out effect in the lower phase and the decrease of hydrophobic interaction between BSA and PEG in the upper phase (Franco et al. 1996) (13). These effects are due to an increased ratio of trivalent to divalent citrate ions with an increase in pH.

Effect of Neutral Salt (NaCl) on BSA Partition Coefficient

Generally, the neutral salts have the capacity to modify the water structure. The cation always decreases the partition coefficient of BSA in aqueous two-phase system (14). This behavior can be explained on the basis that the ions of the salt have different affinities for the two-phases, giving rise to an electrostatic potential difference (1, 2).

In the present work, the effect of neutral salt on BSA partition coefficient was analyzed at different NaCl concentration at different temperatures. In Figs. 5–7 the dependence of the BSA partition coefficient on the concentration

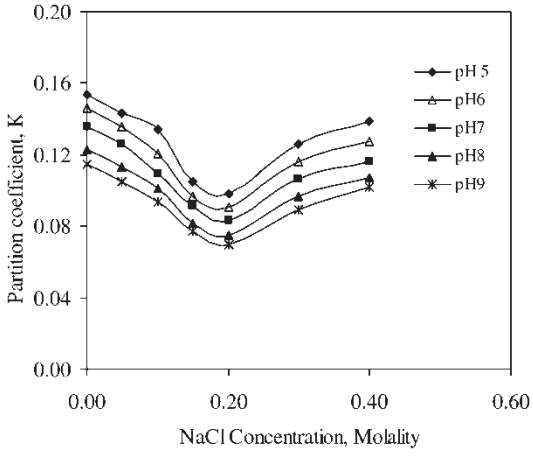

Figure 5. Effect of NaCl concentrations on the partitioning of BSA in PEG2000-sodium citrate-water system at 25°C.

of NaCl at constant pH values of 5.0, 6.0, 7.0, 8.0, and 9.0 is shown for three different temperatures 25°, 35°, and 45°C. The partition coefficient of the BSA decreases with increasing NaCl concentration at low salt concentrations up to 0.15 M NaCl. In this range of salt concentration, interactions of the protein molecules with the sodium ions and citrate molecules in the citrate-rich bottom phase increased. The partitioning of BSA was determined by its charge and its relative hydrophobicity (15). For salt concentrations above 0.2 M, higher partition coefficients of BSA have been observed at different

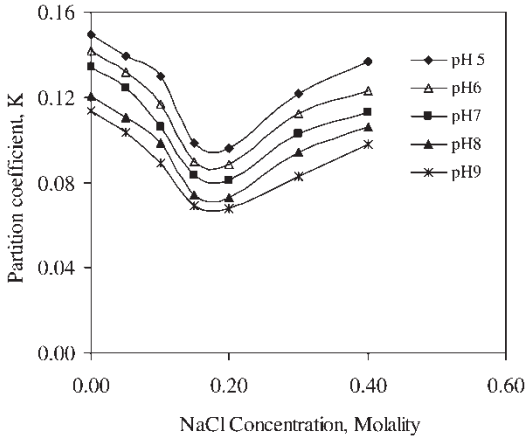

Figure 6. Effect of NaCl concentrations on the partitioning of BSA in PEG2000-sodium citrate-water system at 35°C.

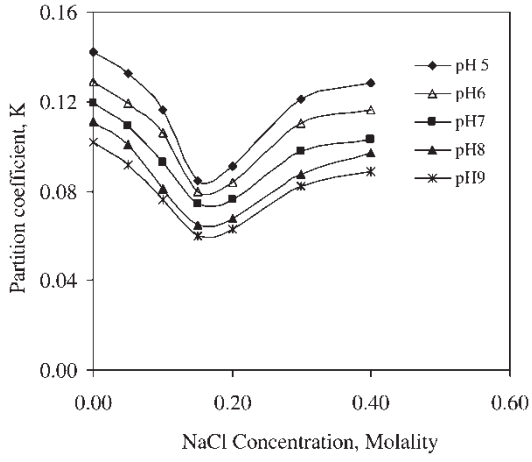

Figure 7. Effect of NaCl concentrations on the partitioning of BSA in PEG2000-sodium citrate-water system at 45°C.

pH values, where the BSA partition is determined by its hydrophobic nature. It appears likely that the partition coefficient was totally independent of its charge. The same kind of result was observed by other researchers (1, 12) for other proteins. It was confirmed that the polyethylene glycol being more hydrophobic in nature tends to strongly interact with the non-polar regions of BSA more and more. Hence the partition coefficient of BSA increases at above 0.2 M NaCl concentrations.

Effect of Temperature on BSA Partition Coefficient

The effect of temperature on protein partitioning is quite complex because the phase composition, the electrostatic interactions, and the hydrophobic interactions are all coupled to the temperature (3). The temperature has an indirect effect on the entropy of the water molecules in their interaction with PEG, (16) which drive partition of the protein. Some reports have described an increase in the partition coefficient with the temperature (17, 18) whereas some others have found that the partition coefficient showed no temperature dependence (19, 20). In the present work, the effect of temperature on the partition coefficient of BSA was studied at 25°, 35°, and 45°C and is shown in Fig. 8 for a constant pH 7. From the figure it was observed that the partition coefficient of BSA decreases with increase in temperature. This is due to the variation of the structure in the PEG phase components, which is in good agreement with the observation of Lee and Lee (1979, 1987) (21, 22). At low temperatures, the hydrophobic side chains were shielded from water as they were covered inside the proteins. When

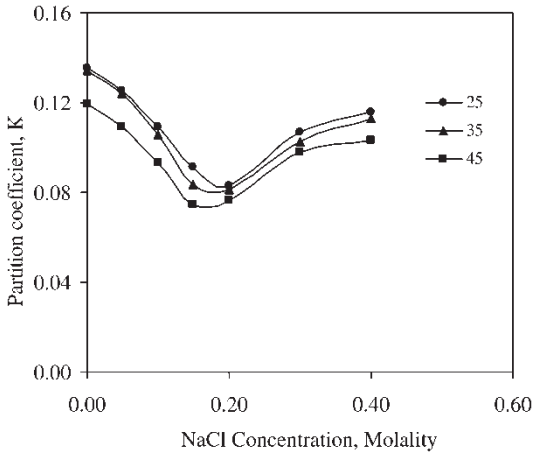

Figure 8. Effect of temperature on the partitioning of BSA in PEG2000-sodium citrate-water system at pH 7.

temperature increases, the hydrophobic side chains were exposed to the solvent and the water molecules in the solvent formed clusters around these hydrophobic residues. Hence the chance to form water clusters around these exposed side chains were increased, which leads to a decrease in partition coefficient of the BSA (Nerli et al. 2001) (14).

Optimization of BSA Partitioning

The aim of the present study is to investigate the optimum level of pH and NaCl concentration for the partitioning of pure Bovine Serum Albumin (BSA) in PEG2000-sodium citrate-water based aqueous two-phase system at different temperatures (25°, 35°, and 45°C). The concentration of NaCl and pH were considered to be the factors having influence on the partition coefficient K. An attempt was made to optimize these factors with respect to the partitioning coefficient, for which the surface response methodology (RSM) was used. The surface response method is a statistical technique used to correlate the measured responses to the input variables empirically. The objective of RSM is to determine the optimum operating conditions for a given system. Optimizing the responses using one variable at a time may not yield a good result, because it deliberately assumes that minimizing the value of one variable is independent of others, which is not usually true, because in the present case the effect of pH and NaCl on the partition coefficient is interrelated. In order to overcome this problem, the surface response methodology was used for the optimization of BSA partitioning.

A 3-level factorial design was used for the optimization of partitioning of BSA in PEG 2000-sodium citrate-water based aqueous two-phase systems. The pH (X_1) and NaCl concentration (X_2) were chosen as independent variables and the partition coefficient of BSA was the dependent response (output variable). The experiments were carried out in the range of 5.0 to 9.0 pH and 0 to 0.4 M NaCl concentrations at each temperature. The summary of the experimental design is given below.

The Summary of the experimental design

Method	Response surface
Initial design	3 Level factorial
Design model	Quadratic
Experiments	13

Response name	Obs	Minimum	Maximum	Temp(°C)
Partition coefficient, K	13	0.070	0.153	25
	13	0.068	0.149	35
	13	0.063	0.142	45

Factor name	Low actual	High actual	Low coded	High coded
pH	5.00	9.00	−1.000	1.000
NaCl (molality)	0.00	0.40	−1.000	1.000

For optimization, 13 experimental values are employed for each temperature. In order to develop a functional relationship between the dependent variable, namely the partition coefficient and the independent variables namely the pH and NaCl concentration, the linear model, the quadratic model, and the cubic model have been tested. Since the first and second order models suffer from lack of fit, the third order polynomial was used to fit the data. From the analysis of the data, the X_1^3, X_2^3 terms were not significant, the reduced third order cubic equation to predict the optimum partition coefficient was used and has the following form

$$K = A_0 + A_1X_1 + A_2X_2 + A_{11}X_1^2 + A_{22}X_2^2 + A_{12}X_1^2X_2 + A_{21}X_1X_2^2 \quad (2)$$

where K is the predicted partition coefficient, A_0 is the intercept term, A_i values are coefficients of the linear terms, and A_{ij} values are the coefficients of the quadratic terms. X_1-pH X_2-NaCl concentration, molality.

The coefficients of the polynomial were estimated using the least square method. The estimated coefficients were analyzed based on the standard error and R^2 values. The model for the estimation of partition coefficient of BSA in PEG2000-sodium citrate based aqueous two-phase system at each temperature is given below

At 25°C

$$K = 0.17877 - 0.00258X_1 - 0.47041X_2 - 0.00051X_1^2 + 1.49594X_2^2 - 0.02763X_1X_2 + 0.00378X_1^2X_2 - 0.06031X_1X_2^2 \quad (3a)$$

At 35°C

$$K = 0.15985 + 0.00170X_1 - 0.39765X_2 - 0.00076X_1^2 + 1.46979X_2^2 - 0.044346X_1X_2 + 0.00470X_1^2X_2 - 0.05837X_1X_2^2 \quad (3b)$$

At 45°C

$$K = 0.18412 - 0.009008X_1 - 0.41019X_2 + 0.93488X_2^2 \quad (3c)$$

The experimental data were analyzed using the "Design Expert" software (www.statease.com). The analysis of variance (ANOVA) for each system temperature confirms the adequacy of the chosen models. The results of the analysis of variance at 25°, 35°, and 45°C were given in Table 1. The model coefficients for each temperature are given in Tables 2–4.

The response surface of the model is shown in Figs. 9–11 for partition coefficients of BSA at 25°, 35°, and 45°C respectively. From the figures it was observed that the partition coefficient of BSA was higher at both low and high levels of NaCl concentration at any fixed value of pH. The optimum partition coefficient was observed from the surface plot. The optimum value of K was found using the Equation 3 at optimal NaCl concentration and pH values for each desired temperature and were given in Table 5.

Based on the results and analysis, it was confirmed that the surface response methodology (3-level factorial design) could be extended for the optimization of the BSA partitioning in PEG 2000-sodium citrate based aqueous two-phase systems by altering the pH and NaCl concentrations.

Description of Aqueous Two-Phase Extraction for the Large Scale Recovery of Protein

The unit operation involved in protein extraction from biological resource by aqueous two-phase extraction is shown in Fig. 12. The top and bottom phases are subjected to ultra-filtration to separate the soluble proteins from the salt and the PEG from the waste. Proteins were retained and salt and PEG were allowed to permeate through the membrane. The permeated salt and PEG are recycled back to the mixer settler tank for further extractions in order to

Table 1. Analysis of variance (ANOVA) of the model at different temperatures

Source	Sum of Square	DF	Mean Squares	F Value	Prob > F
At 25°C					
Model	8.26 E-03	7	1.18E-03	16286.14	< 0.0001
Residual	3.63E-07	5	7.25E-08		
Lack of fit	3.63E-07	1	3.63E-07		
Std. dev.	2.69E-04		R^2	1.0000	
Mean	0.10		Adjusted R^2	0.9999	
C.V	0.26		Predicted R^2	0.9949	
At 35°C					
Model	8.12E-03	7	1.16E-03	1.77E + 06	< 0.0001
Residual	3.28E-09	5	6.57E-10		
Lack of fit	3.28E-09	1	3.28E-09		
Std. Dev.	2.56E-05		R^2	09999	
Mean	0.10		Adjusted R^2	0.9999	
C.V	0.25		Predicted R^2	0.9999	
At 45°C					
Model	6.78E-03	3	2.26E-03	285.17	< 0.0001
Residual	7.13E-05	9	7.93E-06		
Lack of fit	7.13E-05	5	1.43E-05		
Std. dev.	2.82E-03		R^2	09896	
Mean	0.094		Adjusted R^2	0.9861	
C.V	3.01		Predicted R^2	0.9712	

minimize the PEG loss and improve the yield. The purity of the protein separated using the ATPS by ultra-filtration can be ascertained through HPLC. The amount of protein recovered was estimated by the Bradford method (7).

Table 2. Estimated parameters of the model at 25°C

Factor	Coefficient estimate	DF	Standard error	Mean squares	F Value	Prob > F
Intercept	0.083	1	1.118E-04			
pH	−0.014	1	1.904E-04	3.95E-04	5445.59	<0.0001
NaCl	−9.80E-03	1	1.904E-04	1.92E-04	2649.38	<0.0001
pH^2	1.00E-03	1	1.620E-04	2.76E-06	38.10	0.0016
$NaCl^2$	0.043	1	1.620E-04	5.09E-03	70274.4	<0.0001
pH.NaCl	4.75E-04	1	1.35E-04	9.03E-07	12.45	0.0168
pH^2·NaCl	3.03E-003	1	2.33E-04	1.22E-05	168.29	<0.0001
pH·$NaCl^2$	−4.83E-03	1	2.33E-04	3.10E-05	428.15	<0.0001

Table 3. Estimated parameters of the model at 35°C

Factor	Coefficient estimate	DF	Standard error	Mean squares	F Value	Prob > F
Intercept	0.081	1	1.06E-05			
pH	−0.014	1	1.81E-05	3.97E-04	6.04E05	<0.0001
NaCl	−0.011	1	1.81E-05	2.27E-04	3.45E05	<0.0001
pH^2	7.03E-04	1	1.54E-05	1.37E-06	2080.38	<0.0001
$NaCl^2$	0.042	1	1.54E-05	4.98E-03	7.58E06	<0.0001
pH.NaCl	−7.51E-04	1	1.28E-05	2.25E-06	3430.85	<0.0001
pH^2.NaCl	3.76E-03	1	2.22E-05	1.89E-05	28720.0	<0.0001
pH.$NaCl^2$	−4.67E-03	1	2.22E-05	2.91E-05	44271.0	<0.0001

Table 4. Estimated parameters of the model at 45°C

Factor	Coefficient estimate	DF	Standard error	Mean squares	F Value	Prob > F
Intercept	0.076	1	1.06E-03			
pH	− 0.018	1	1.15E-03	1.95E-03	245.75	<0.0001
NaCl	− 7.25E-03	1	1.15E-03	3.15E-04	39.76	<0.0001
pH^2	0.037	1	1.57E-03	4.52E-03	570.01	<0.0001

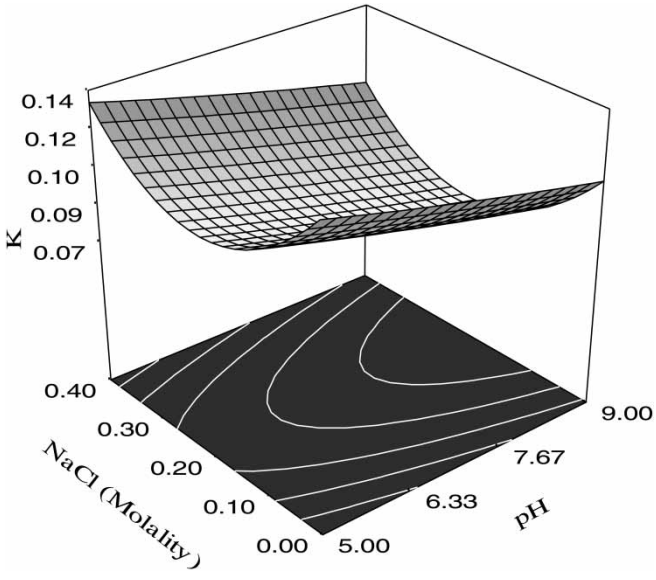

Figure 9. Response surface of BSA partition coefficient at 25°C.

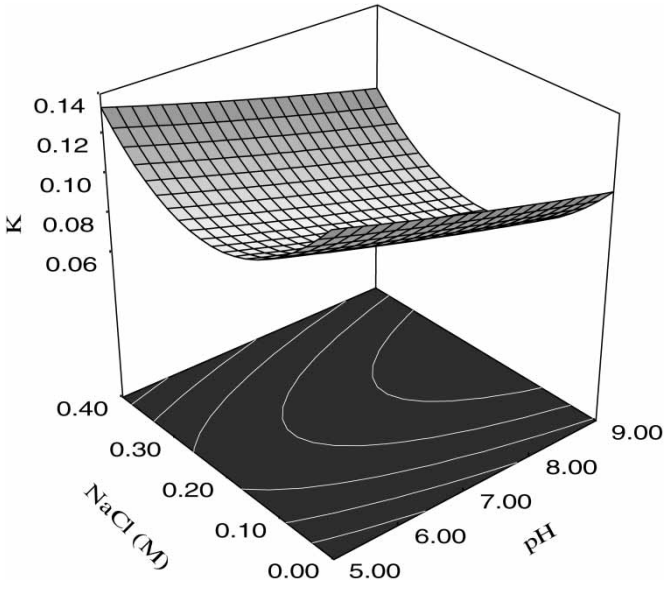

Figure 10. Response surface of BSA partition coefficient at 35°C.

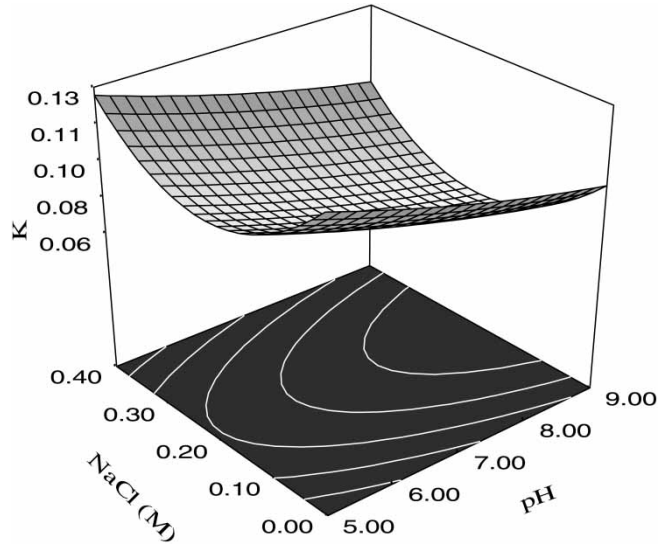

Figure 11. Response surface of BSA partition coefficient at 45°C.

Table 5. Optimum value of Partition coefficient of BSA at different temperatures

Temperature (°C)	pH	NaCl (Molality)	Partition coefficient, K
25	8.88	0.18	0.06981
35	8.78	0.19	0.06514
45	8.65	0.20	0.06229

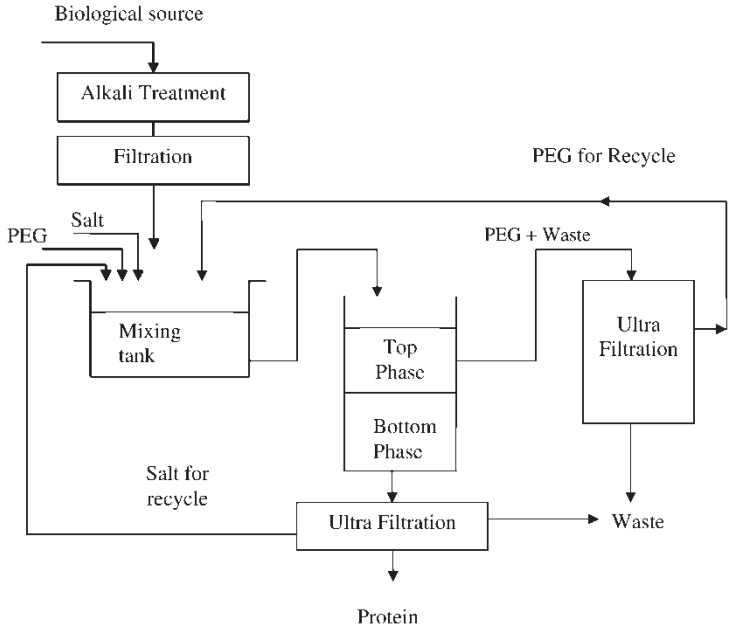

Figure 12. Representation of ATPS with ultrafiltration for the separation of protein.

CONCLUSION

The effect of PEG2000 concentration, pH, NaCl concentration, and temperature on the partition coefficient of BSA in PEG2000-sodium citrate based aqueous two-phase system was studied. It was observed that the partition coefficient of BSA decreases with increase in pH values. The affinity of the BSA for the salt-rich bottom phase increases with increase in pH, due to an increase in the ratio of trivalent to divalent citrate ions in the two-phase system. It was observed that the partition coefficient of BSA was higher at both low and high levels of NaCl concentration at any fixed value of pH. The optimum partition coefficient was observed at around the central area of the surface plot. The optimum value of the partition coefficient K was found at optimal NaCl concentration and pH values for each desired temperature. The surface response

method was satisfactorily used for the optimization of partition coefficient of BSA in PEG2000 based aqueous two-phase system.

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