Journal of Microbiology and Biotechnology
Vol. 6. No. 5, 352-357, 1996

Salting-out Effects on the Partition of Proteins in Aqueous Two-phase Systems

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The partition of proteins in the salt-rich phase of polyethylene glycol (PEG)/salt aqueous two-phase systems is limited by the salting-out effects of salt. The logarithm of the concentration of proteins partitioned in the salt-rich phase decreases linearly with increases in the concentration of salt in the salt-rich phase (salting-out). Therefore, the partition of a given protein in the salt-rich phase of aqueous two-phase systems can be estimated from the salting-out constant. The slope of the solubility line (salting-out constant) for a given protein is determined by the type of salt in the two-phase systems.

Liquid-liquid extraction of proteins using aqueous two-phase systems is simple, rapid, and scalable involving only mild conditions (2, 6, 16). Therefore, liquid-liquid extraction provides obvious advantages over conventional separation and overcomes the limitations of more conventional methods currently used in bioprocesses. Thus, it makes the studies on the partition mechanisms of proteins in aqueous two-phase systems pertinent (3, 5, 7, 13). Nevertheless, the partition mechanism of proteins is not yet known. Consequently, it is not possible to predict the partition behavior of proteins, nor to design or select the optimum aqueous two-phase systems for extraction of proteins. It is difficult to realize the full potential of liquid-liquid extraction if the practice is based on trial and error as it is today (17, 18).

The aqueous two-phase systems which have received the most attention are polyethylene glycol (PEG)/dextran systems. Recently, more interest has been shown in PEG/salt aqueous two-phase systems (10, 12, 13, 15). The PEG/salt systems or two-phase systems containing salt instead of another polymer are advantageous because they cost less and have lower viscosity, leading to lower operating cost (pumping, centrifugation etc.). Therefore, PEG/salt two-phase systems were selected for this study.

PEG/salt two-phase systems can be prepared by mixing PEG and a salt in a water exceeding certain threshold concentrations (8, 9). The resultant top phase is rich in PEG and the bottom phase is rich in salt. It is well established that the solubility of proteins is strongly affected by the concentration of salt in solutions. In fact, various salts are used to precipitate proteins from the solutions in both laboratory and industrial practices. This study examines the salting-out effects of salt on the partition of proteins in the salt-rich bottom phase of aqueous two-phase systems.

The salting-out effects of salt on the solubility of proteins have been established by Melander and Horvath (4, 14). They expressed the solubility of proteins in the salt solution as:

$$\ln C = \ln C_o + \beta + (\Lambda - \Omega\sigma)\, m_s \quad (1)$$

or

$$\ln C = \ln C_o + \beta + K_s m_s \quad (2)$$

where C = concentration of proteins in the salt solutions,
C_o = concentration of proteins in pure water,
$\Lambda = D\mu/RT$
μ = dipole moment of proteins,
R = gas constant,
T = absolute temperature,
β, D = constants,
$\Omega = [NA + 4.8N^{1/3}(\kappa^e - 1)V^{2/3}]/RT$
N = Avogadro's number,
A = hydrophobic surface area of proteins,
κ^e = correction factor for the macroscopic surface tension of solvent to molecular dimemsions,
V = molar volume of proteins,
σ = molar surface tension increment of salt,
m_s = molar concentration of salt solutions, and
$K_s = \Lambda - \Omega\sigma$, salting-out constant.

This study applies the salting-out theory to the PEG/

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Key words: salting-out, aqueous two-phase systems, liquid-liquid extraction, PEG (polyethylene glycol)

salt two-phase systems and determines the salting-out effects of salt on the partition of proteins in the salt-rich phase in order to establish the partition mechanism of proteins in aqueous two-phase systems.

MATERIALS AND METHODS

Proteins

The solutions of bovine serum albumin (BSA), ovalbumin (OA), and lysozyme (Sigma Chemical Co., St. Louis, MO) were prepared in deionized water (10%, w/v) and stored at 4°C.

Preparation of Stock Solutions

The polyethylene glycol (PEG) solutions of various molecular weights, 1,450 (PEG1450), 3,350 (PEG3350), 8,000 (PEG8000) (Sigma Chemical Co. , St. Louis, MO) were prepared by dissolving PEG in deionized water. The molar concentrations of the stock solutions for PEG 1450, PEG3350, PEG8000 were 0.34 M (50%, w/v), 0.15 M (50%, w/v), and 0.06 M (50%, w/v), respectively. All PEG solutions were kept at room temperature.

The salt solutions of ammonium sulfate ($(NH_4)_2SO_4$), magnesium sulfate ($MgSO_4$), sodium sulfate (Na_2SO_4), sodium citrate ($Na_2C_6H_5O_7$), potassium citrate ($K_2C_6H_5O_7$), and sodium tartrate ($Na_2C_4H_4O_6$) (Mallinckrodt Inc., Paris, KY) were prepared in deionized water, buffered with 10 mM potassium phosphate, and adjusted to pH 7.2. Potassium phosphate and sodium phosphate solutions (Mallinckrodt Inc., Paris, KY) were prepared by adding equal moles of monobasic and dibasic in deionized water (pH 7.2).

Phase Diagram

The phase diagram of PEG/salt two-phase systems were constructed by titration according to the method of Albertsson (1). Ten ml of PEG solution (0.06 to 0.34 M) was placed into a flask (125 ml). A salt solution was then titrated (1.8 to 4.0 M) into the flask with stirring. First a homogeneous mixture was obtained, but after a certain amount of salt had been added, one further drop of the salt solution caused turbidity creating a two-phase system. The composition of this mixture was noted. One ml of deionized water was added to confirm the dissolution by observing the clarity of the homogeneous solution. The salt solution was then added dropwise until a turbid two-phase system was formed again. The composition of this mixture was noted and more deionized water was added get a one-phase system and so forth. The titration was continued until 25 to 50 ml of the salt solution was used.

Ten ml of the salt solution (1.8 to 4.0 M) was then titrated with a PEG solution (0.02 to 0.34 M) in the same manner as above. In the way a series of compositions were obtained and the concentration of PEG was plotted against that of salt for these compositions. All titrations were conducted at 25°C. The tie lines were determined from the volume ratio of the top to the bottom phases. The critical point (K) was determined by connecting the middle points of the tie lines.

Aqueous Two-phase Systems

BSA (0.5%, w/v), OA (0.5%, w/v), and lysozyme (0.02%, w/v) in 10 ml of PEG/salt two-phase systems (PEG/potassium phosphate, PEG/sodium phosphate, PEG/ammonium sulfate, PEG/potassium citrate, PEG/sodium tartrate) were shaken for 10 min with a Thermolyne Speci-Mix (Thermolyne Co., Dubuque, IA) and centrifuged for 10 min at 700 × g.

Concentration of Proteins Partitioned in the Salt-rich Phase

A hole at the end of the bottom tip of the centrifuge tube was made with a soldering iron. The salt-rich bottom phase of PEG/salt two-phase systems was drawn through the hole and diluted 4 times. The concentration of proteins in the diluted samples was measured spectrophotometrically at 280 nm.

Concentration of Salt in Salt-rich Phases

The concentration of salt in the salt-rich phase of PEG/salt two-phase systems was determined from the phase diagram constructed.

Salting-out Constant of Proteins in Two-phase Systems

The salting-out constant of proteins, K_s, was determined from the slope of the plot of the logarithm of the concentration of proteins partitioned in the salt-rich phase (lnC) versus the concentration of salt in the salt-rich phase (m_s) using the salting-out equation, Eq. (1). The concentration of salt was expressed in the molarity.

Salting-out Constant of Proteins in Salt Solutions

The salting-out constant of proteins in salt solutions (a single phase in the absence of PEG) was determined to compare with that determined in two-phase systems and to evaluate the effects of PEG present in the salt-rich phase. BSA (0.5%, w/v), OA (0.5%, w/v), and lysozyme (0.5%, w/v) in 5 ml of salt solutions were shaken for 10 min with a Thermolyne Speci-Mix, allowed to stand for 30 min, and centrifuged for 10 min at 700 × g. The supernatant was filtered with a 0.22 μm disposable filter unit (Millipore Corporation, Bedford, MA) to remove precipitates and diluted 4 times with deionized water. The concentration of proteins in the diluted samples was measured using the same method described above. The salting-out constant of proteins in the salt solutions was also determined according to the method described above.

RESULTS AND DISCUSSION

Concentration of Salt in Two-phase Systems

A series of PEG8000/potassium phosphate two-phase

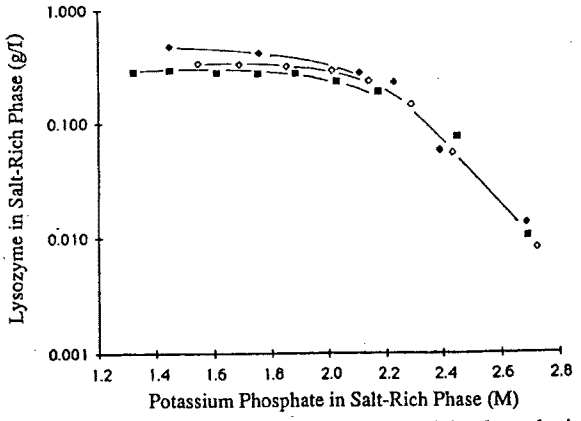

Fig. 1. Concentration of lysozyme partitioned in the salt-rich phase of PEG8000/potassium phosphate aqueous two-phase systems at given concentrations of PEG8000.
—■—, 0.013 M PEG8000; —◇—, 0.019 M PEG8000; —◆—, 0.025 M PEG8000.

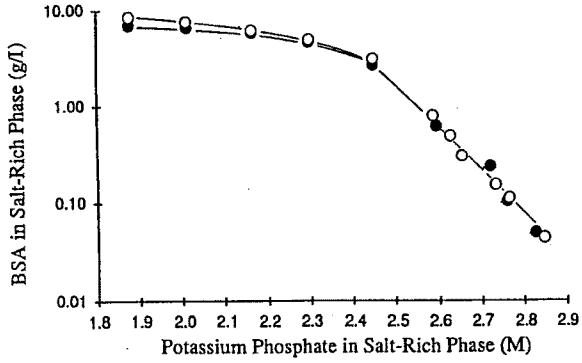

Fig. 2. Concentration of bovine serum albumin partitioned in the salt-rich phase of PEG8000/potassium phosphate aqueous two-phase systems at given concentrations of PEG8000.
—●—, 0.019 M PEG8000; —○—, 0.022 M PEG8000.

systems were constructed. They were composed of various concentrations of potassium phosphate at a given concentration of PEG8000. These two-phase systems demonstrated the effects of the concentration of salt in the aqueous two-phase systems on the partition of proteins in the salt-rich phase. The concentration of lysozyme partitioned was plotted against the concentration of potassium phosphate in the salt-rich phase in Fig. 1. The logarithm of the concentration of lysozyme partitioned in the salt-rich phase decreased linearly with increases in the concentration of potassium phosphate in the salt-rich phase above the salting-out concentration of potassium phosphate. This was true regardless of the total concentrations of potassium phosphate or PEG in the two-phase systems. All points were on the same line when the concentration of salt in the salt-rich phase was above the salting-out concentration. The same results were obtained with BSA (Fig. 2). This indicated that the partition of proteins in the salt-rich phase was limited by the salting-out effects.

The concentrations of proteins in this experiment simulated practical conditions (0.5%, w/v). The concentrations were below the solubility limits of the proteins in some two-phase systems. In such case, the concentration of lysozyme and BSA partitioned in the salt-rich phases decreased to compensate for the volume of salt-rich phase. The volume of the salt-rich phase increased with increases in the total concentration of salt and decreased with increases in the total concentration of PEG8000. In reiteration, the concentration of proteins partitioned in the salt-rich phase decreased with increases in the total concentration of salt and with decreases in the total concentration of PEG in the two-phase systems when the concentration of salt in the salt-rich phase was lower than the salting-out concentration (dilution effects).

Proteins salted-out in the salt-rich phase did not transfer into the PEG-rich phase but remained in between the two aqueous phases or interface. The concentration of lysozyme and BSA partitioned in the PEG-rich phase of all two-phase systems tested remained very low (> 0.03 g/l, > 3% of total protein in the two-phase system). The precipitation of proteins at the interface, therefore, increased with increases in the concentration of salt in the salt-rich phase.

Concentration of PEG in Two-phase Systems

A series of PEG8000/potassium phosphate two-phase systems composed of various concentrations of PEG8000 at a given concentration of potassium phosphate were constructed to determine the effects of the total concentration of PEG on the partition of proteins in the salt-rich phase. The concentration of lysozyme partitioned in the salt-rich phases was plotted against the concentration of potassium phosphate in the salt-rich phase in Fig. 3. The logarithm of the concentration of lysozyme and BSA partitioned decreased linearly with increases in the concentration of potassium phosphate in the salt-rich phase above the salting-out concentration of potassium phosphate. This was true regardless of the total concentration of PEG8000 in the two-phase systems. All points were on the same line again. The same results were obtained with BSA (Fig. 4).

When the concentration of potassium phosphate was lower than the salting-out concentration, the concentration of lysozyme and BSA partitioned in the salt-rich phase increased. This may also be explained with the volume change of the salt-rich phase. The volume of the salt-rich phase decreased with increases in the total concentration of PEG and increased with increases in the total concentration of salt. Therefore, the concentration of BSA and lysozyme partitioned in the salt-rich phase

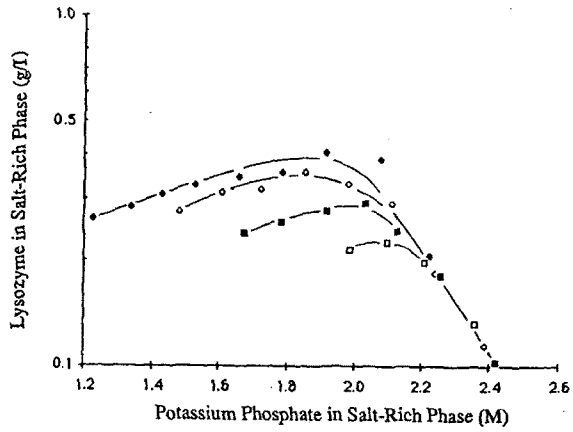

Fig. 3. Concentration of lysozyme partitioned in the salt-rich phase of PEG8000/potassium phosphate aqueous two-phase systems at given concentrations of potassium phosphate.
—◆—, 1.00 M potassium phosphate; —◇—, 1.25 M potassium phosphate; —■—, 1.50 M potassium phosphate; —□—, 1.75 M potassium phosphate.

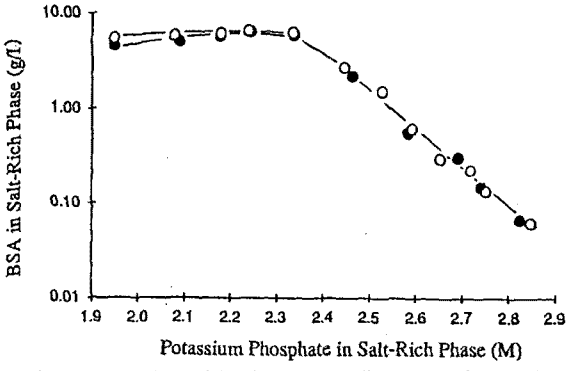

Fig. 4. Concentration of bovine serum albumin partitioned in the salt-rich phase of PEG8000/potassium phosphate aqueous two-phase systems at given concentrations of potassium phosphate.
—○—, 1.75 M potassium phosphate, —●—, 2.00 M potassium phosphate.

increased with increases in the total concentration of PEG and decreases in the total concentration of salt (concentration effects).

The concentration of lysozyme and BSA partitioned in the PEG-rich phase of all two-phase systems tested again remained very low (> 0.03 g/l, > 3% of total protein in the two-phase system). Proteins salted-out in the salt-rich phase did not transfer into the PEG-rich phase. The precipitation of proteins at the interface, therefore, increased with increases in the concentration of PEG8000 in the two-phase systems.

Molecular Weight of PEG

Fig. 5 shows the effects of the molecular weight of PEG on the concentration of BSA partitioned in the salt-rich phase of PEG/potassium phosphate two-phase

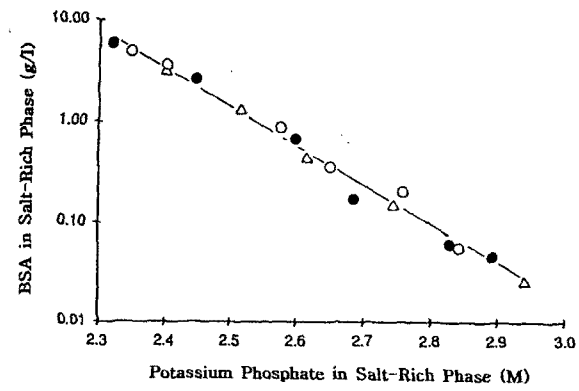

Fig. 5. Concentration of bovine serum albumin partitioned in the salt-rich phase of PEG(20%)/potassium phosphate aqueous two-phase systems constructed with various molecular weights of PEG.
—○—, PEG1450; —●—, PEG3350; —△—, PEG8000.

systems. The partition of BSA in the salt-rich phase was independent of the molecular weight of PEG (1,450 to 8,000 daltons). The concentration of BSA partitioned in the salt-rich phase was the same regardless of the molecular weight of PEG when the concentration of salt in the salt-rich phase was higher than the salting-out concentration. The logarithm of the concentration of BSA partitioned in the salt-rich phase decreased linearly with increases in the concentration of potassium phosphate in the salt-rich phase.

Therefore, when the concentration of salt in the salt-rich phase was higher than the salting-out concentration, the partition of the proteins in the salt-rich phases depended only on the concentration of salt in the salt-rich phase.

Type of Salt

The effect of the type of salt used to construct aqueous two-phase systems on the partition of proteins in the salt-rich phase were studied. The concentration of ovalbumin (OA) and lysozyme partitioned in the salt-rich phase of PEG/salt aqueous two-phase systems (PEG8000/ammonium sulfate, PEG8000/potassium phosphate, PEG 8000/sodium phosphate, PEG8000/potassium citrate, and PEG8000/sodium tartrate) are shown in Fig. 6 and 7, respectively. The logarithm of the concentration of proteins partitioned in the salt-rich phase decreased linearly with increases in the salt concentration in the salt-rich phase. Therefore, the partition of proteins in the salt-rich phases of all PEG/salt two-phase systems tested could be expressed with the salting-out equation.

The ability of salt to increase the surface tension of the solution, the molar surface tension increment of salt (σ), is one of the major parameters governing the salting-out constant (slope of the solubility line) of salt for a given protein in the salt solutions. The surface tension of salt solutions increased linearly with increases in the con-

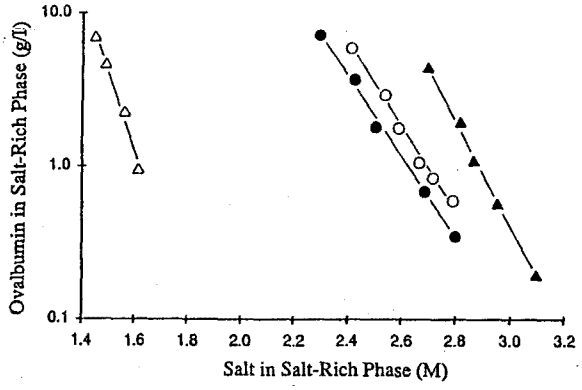

Fig. 6. Concentration of ovalbumin partitioned in the salt-rich phase of PEG8000/salt aqueous two-phase systems constructed with various types of salt.
—▲—, ammonium sulfate; —○—, potassium phosphate; —●—, sodium phosphate; —△—, potassium citrate.

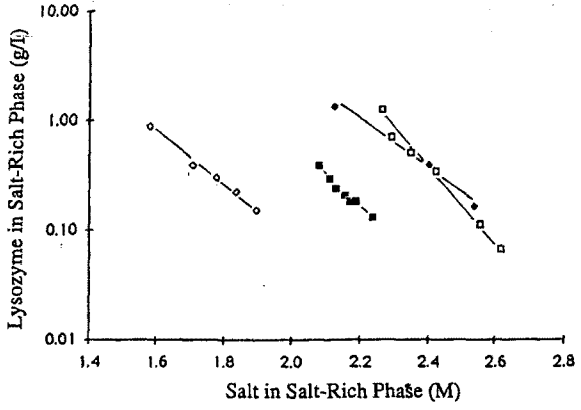

Fig. 7. Concentration of lysozyme partitioned in the salt-rich phase of PEG8000/salt aqueous two-phase systems constructed with various types of salt.
—◆—, ammonium sulfate; —◇—, sodium tartrate; —■—, potassium phosphate; —□—, sodium phosphate.

centration of salt (molarity) and the molar surface tension increment of salt was determined from the slope (Table 1).

From Eqs. (1) and (2), the salting-out constant (K_s) is

$$K_s = \Lambda - \Omega\sigma \quad (3)$$

where Λ is the electrostatic parameter of a protein and Ω is the hydrophobic parameter of a protein. The salting-out constant of salt for proteins generally has a negative value and decreases with increases in the molar surface tension increment of salt. Therefore, the higher the molar surface tension of salt, the greater the decreases in the solubility of proteins in the salt solutions. Fig. 8 shows the salting-out constant of various salts. The salting-out constant decreased linearly with increases in the molar surface tension increment of salt. Therefore, the decreases in the solubility of proteins in the salt-rich phase

Table 1. Molar surface tension increment of salt.

Salt	Molar surface tension increment (dyne/cmM)
Sodium Formate	1.32
Sodium Sulfate	2.44
Sodium Phosphate	2.75
Sodium Tartrate	3.13
Ammonium Sulfate	3.15
Potassium Phosphate	3.21
Potassium Citrate	3.48
Sodium Citrate	3.58

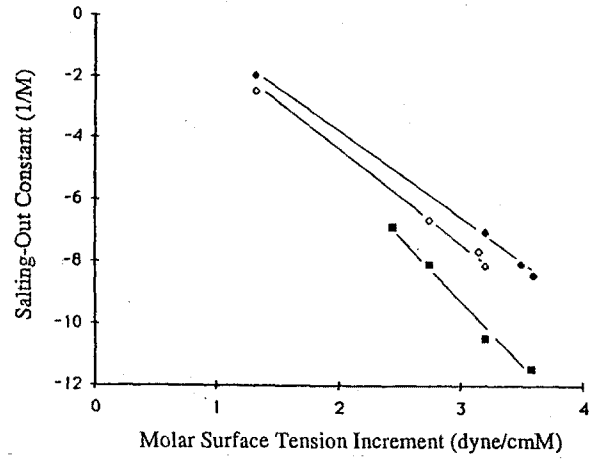

Fig. 8. Salting-out constant of proteins with various molar surface tension increments of salts.
—◆—, lysozyme; —◇—, ovalbumin; —■—, bovine serum albumin.

of two-phase systems is determined by the type of salt used to construct two-phase systems.

As shown in Eq. (3) the salting-out constant is also determined by the electrostatic parameter and the hydrophobic parameter of proteins (4, 11, 14). Therefore, the salting-out constant of a given salt is different with different proteins.

CONCLUSION

The partition of proteins in the salt-rich phase of PEG/salt two-phase systems is limited by the salting-out effects of salt. The concentration of proteins partitioned in the salt-rich phase, therefore, can be controlled by the concentration of salt in the salt-rich phase and the type of salt used to construct the aqueous two-phase systems.

Acknowledgements

This work was supported in part by the Korea University and in part by the research project of the Center for

Advanced Food Science and Technology of Korea University, a center of recommendation for SRC of the Korea Science and Engineering Foundation (CAFST research paper No. 96006).

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(Received January 19, 1996)