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Effect of Nucleic Acid Contamination on Partitioning of Proteins in Two-Phase PEG-Dextran System

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ABSTRACT

Downstream processing of bioproducts results in considerable losses of compounds of interest in a large number of cases. For the intracellular enzyme tartrate dehydrogenase, an analysis of the laboratory process for enzyme recovery revealed that maximum losses occur in the initial stages of purification when the enzyme is separated from nucleic acids and other undesirable enzymes. Hence, aqueous two-phase extraction was studied to investigate the separation of several enzymes from nucleic acids. Single-component and binary equilibria for three commercially available enzymes (bovine serum albumin, trypsin, chymotrypsin) and yeast RNA were studied in a two-phase system consisting of dextran and polyethylene glycol (PEG). The effects of pH and concentrations of the components and salts (NaCl) were investigated.

Index Entries: Nucleic acid contamination; protein partitioning; two-phase PEG-Dextran system; two-phase aqueous partitioning; partition coefficient.

INTRODUCTION

Several industrially important biomolecules are formed as intracellular proteins. Their recovery involves disruption of cells, as a result of which these products must be purified from a mixture of proteins and nucleic acids. A typical recovery process for an intracellular enzyme is presented

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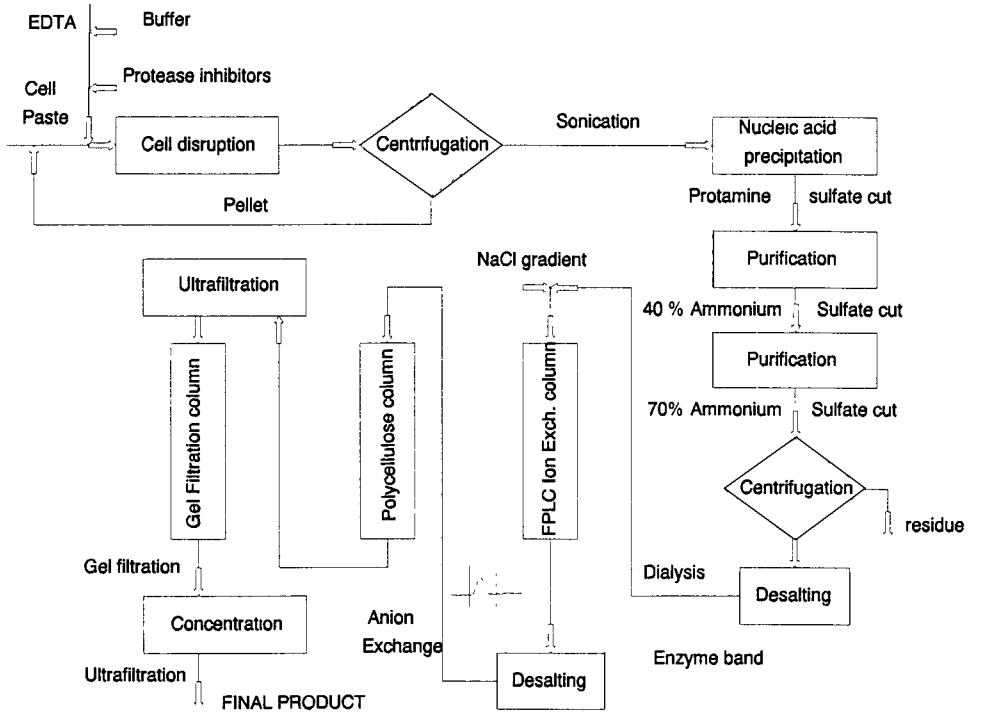

Fig. 1. Schematic of downstream process for recovering intracellular tartrate dehydrogenase from *P. putida* cells.

in Fig. 1. Similar schemes are commonly used for other intracellular products. A number of published reports suggest that significant losses of protein products occur during their separation from other proteins and nucleic acids (*1–5*). Activities of an intracellular enzyme tartrate dehydrogenase, produced by *Pseudomonas putida,* at different stages of its recovery process (Fig. 1) are listed in Table 1. Clearly, the maximum losses of the enzyme occur during precipitation of nucleic acids and during preliminary purification using ammonium sulfate fractionation. Therefore, efficient methods for separation of proteins from each other and from nucleic acids are needed for improvement of the economics of production of protein bioproducts. It is also desirable if the engineering principles for design and scale-up of the methods are well established.

Liquid–liquid extraction is a well-established unit operation for separating compounds on the basis of their affinities in two immiscible phases (*6*). Aqueous solutions of polyethylene glycol (PEG) in the presence of salts or another water-soluble polymer (dextran) have been known to form a two-phase system under a wide range of concentrations (*7,8*). These have been suggested to be an effective tool for separating proteins not only from each other, but also from nucleic acids (*9*). The polymers have been found to stabilize the tertiary structures and the biological activities (*10*). The extraction process is also amenable to easy scale-up. As a

Table 1
Purification of Tartrate Dehydrogenase Using the Classical Scheme of Fig. 1

Stage, as shown in Fig. 1	Volume, mL	Total activity, U[a]	Total protein, mg	Specific activity, U/mg protein	Activity yield, %	Purification factor
Sonication	60	40.2 ± 8	1379	0.029	100	1
Protamine sulfate cut	75	31.2 ± 2.7	1489	0.021	77	0.72
40% Ammonium sulfate cut	64	9.45 ± 0.68[b]	754	0.0125[b]	23.5	0.43
70% Ammonium sulfate cut	12	4.6 ± 0.15[b]	642	0.0072[b]	11.5	0.24
Dialysis	—	15.2 ± 2.5	345.6	0.044	37.8	1.5
Anion exchange	7	15.1 ± 0.7	34.5	0.44	37.7	1.5
Gel filtration	54	11.0 ± 0.3	23.9	0.46	29.3	15.7
Ultrafiltration	0.9	6.86 ± 0.5	14.5	0.47	16.2	16.2

[a] μmol/L.
[b] The activity measurements are low owing to interference by salts.

result, aqueous two-phase extractions have been explored for purifications of several protein products from fermentation broths (*11,12*).

Johansson (*13*) reported that aqueous two-phase extraction can be used for obtaining virtually nucleic acid-free proteins. On the other hand, Cascone et al. (*14*) found problems in separating their target protein in the presence of cell homogenate, and suggested that system parameters, such as pH, salt concentration, polymer types, and concentrations, need to be modified in order to achieve adequate separation using the aqueous two-phase extraction method. Interactions between proteins and nucleic acids when present together in cell homogenate are likely to be the causes of these problems. Hence, this study focused on the effect of nucleic acid contaminants on the partitioning of proteins in aqueous two-phase systems. The objectives of this study were:

1. To establish the partition coefficients of three commercially available proteins (bovine serum albumin, trypsin, and chymotrypsin) and yeast RNA in a two-phase aqueous system consisting of PEG and dextran.
2. To determine the interactive effects when a protein and yeast RNA are present in a solution together; and
3. To determine the effects of salts and pH of solution on partition coefficients of the proteins when present alone and in mixtures with yeast RNA.

MATERIALS AND METHODS

Chymotrypsin, bovine serum albumin, yeast ribonucleic acids (RNA), PEG 8000, and dextran (average mol wt ~70,000) were purchased from Sigma Chemical Company, St. Louis, MO. Trypsin was purchased from ICN Biomedicals Inc., Irvine, CA. Cibacron FGF dye was obtained from Ciba Geigy, Greensborough, NC. Tartrate dehydrogenase was produced in our laboratories by growing *P. putida* ATCC 17642 on (+)tartrate and *m*-tartrate according to a method described by Tipton and Peisach (*15*). Salts and other reagents used were of analytical grade. Affinity-ligated PEG was prepared from PEG and Cibacron blue FGF using a method described by Kopperschläger and Johansson (*16*).

Analyses

The concentrations of dextran were measured with a Perkin Elmer polarimeter using the specific rotation for dextran to be 1.99°/(dm × g/100). The refractive index of PEG and dextran was measured by a Reichert Abbe Mark-II digital refractometer using a specific refractive index of 0.00139 for PEG and 0.00153 for dextran using a protocol described by Bamberger et al. (*17*).

Protein and RNA concentrations were measured with a Hewlett Packard HP8452A general-purpose UV/visible spectrophotometer. Proteins were analyzed at 280 nm and the nucleic acids at 260 nm. Calibration curves were prepared using solutions of single components. When protein and nucleic acid were present together, Warburg and Christian's method (*18*) was used for analysis. Appropriate dilutions were made to ensure linearity of calibration.

TDH activity was measured as the rate of TDH-assisted tartrate oxidation by monitoring the formation of $NADH{\cdot}H^+$ at 340 nm (ϵ = 6220 M^{-1}/cm) in a reaction mixture containing the enzyme, 10 m*M* $K_2(+)$ tartrate, 1.5 m*M* NAD^+, 0.4 m*M* $Mn(OAc)_2$, and 1 m*M* DTT in 100 m*M* HEPES, pH 8.0. All the assays were carried out at 25°C in a quartz cuvet (total volume 1 mL, path length 1 cm). Activity of the enzyme was represented as U/mL with 1 U being the amount of enzyme that catalyzes the formation of 1 μmol of oxaloglycolate/min under the conditions of assay as described above.

Experiments

All the experiments were conducted at 4°C with a fixed composition of the PEG-dextran two-phase system. A tie line was established by mixing 4.8 g dextran solution (21.78% w/w) and 1.33 g PEG (45% w/w) solutions with 3.87 g of 20 m*M* phosphate buffer to produce a mixture having 10.0% dextran and 5.1% PEG. The mixture was allowed to equilibrate for 24 h and on separation, it formed PEG (light) and dextran (heavy) phases in a volume ratio of 3:7. The equilibrium composition (w/w) of the PEG phase was 9.25% PEG and 0.52% dextran. The corresponding composition of the dextran phase was 0.54% PEG and 21.1% dextran. This tie line was not affected significantly by the presence of up to 5*M* NaCl. All the solutions were made in 20 m*M* phosphate buffer (pH 7.2).

The different concentrations of the proteins and nucleic acids in the two-phase system were obtained by replacing part or all of the phosphate buffer by the stock solutions. The protein stock solutions were 4.53% (w/w), and those for nucleic acid were 0.793% by weight. The four different concentration levels used in this research were achieved by using 0.5, 1.0, 2.0 or 3.87 g of stock solutions in making the two-phase system. The effect of pH was studied at five different values (5.5, 6.5, 7.2, 7.7, and 8.2). pH of the system was changed by making all the solutions in 20 m*M* phosphate buffer after adjusting its pH by addition of 2*N* HCl or 2*N* KOH. Similarly, the effect of salt concentration was studied by making all the solutions in 20 m*M* phosphate buffer (pH 7.2) containing 0 and 5*M* sodium chloride. For studies with nucleic acid and protein mixtures, the stock solutions were mixed in a ratio of 23:77. Equilibrium studies were carried out by thoroughly shaking the mixtures and then letting them equilibrate statically for 24 h at 4°C.

Preparation of P. putida *ATCC 17642 Homogenate*

The cell paste was suspended in an equal volume of 20 m*M* phosphate buffer, pH 7.2, containing 1 m*M* DTT. Immediately prior to cell disruption, the protease inhibitors PMSF in acetone and TLCK were added to final concentration of 0.5 m*M*. The suspended cells were disrupted with a Branson sonifier. The cell suspension was sonicated three times for 2 min with cooling between each sonication and then centrifuged for 30 min at 10°C at 25,000*g*.

RESULTS AND DISCUSSION

Partition Coefficients of Single Components

Equilibrium partitioning experiments were conducted for each compound (trypsin, chymotrypsin, bovine albumin, and yeast RNA) individually, and their concentrations in the PEG and dextran phases were analyzed. Typical equilibrium isotherms for trypsin and bovine serum albumin are presented in Fig. 2. All the three proteins studied here partitioned preferentially in the PEG phase, whereas the yeast RNA favored dextran phase. In all the cases studied, the isotherms were nonlinear in nature. Starting with low concentrations, the partition coefficient (ratio of concentration in the PEG phase to that in dextran phase) of proteins increased and then decreased with increase in concentrations; yeast RNA behaved in an exactly opposite manner. These effects of concentration of solute on its partition coefficient is in contrast with the observation of Albertsson (*19*), who found the partition coefficient for human serum albumin in a PEG6000/dextran 48 system to be constant at 20°C and pH 6.8. On the other hand, the effect of concentration of proteins on their partition coefficients has not often been reported in published literature (*19*). Macromolecules may undergo conformational changes when their concentration in the solution changes (*20*). The commonly used spectrophotometric method of protein analysis, also used in this work, does not distinguish between the different macromolcular structures. The influence of any degradative process on the results observed here is minimal owing to the low temperature (4°C) in the experiments.

Average values of partition coefficients were calculated assuming linear equilibrium isotherms in the whole range of concentrations used. These are presented in Table 2. The partition coefficients observed in this work are generally higher than those published by Johansson (*21*), who used different dextran and PEG polymers, and conducted all the experiments at 20°C. At a pH of 7.2, trypsin (pI = 10.8) and chymotrypsin (pI = 8.6) possess a net positive charge, whereas bovine serum albumin (pI = 4.71) possesses a net negative charge. RNA molecules are also negatively charged. However, all the proteins partitioned preferentially in the PEG phase, whereas RNA favored the dextran phase. This observa-

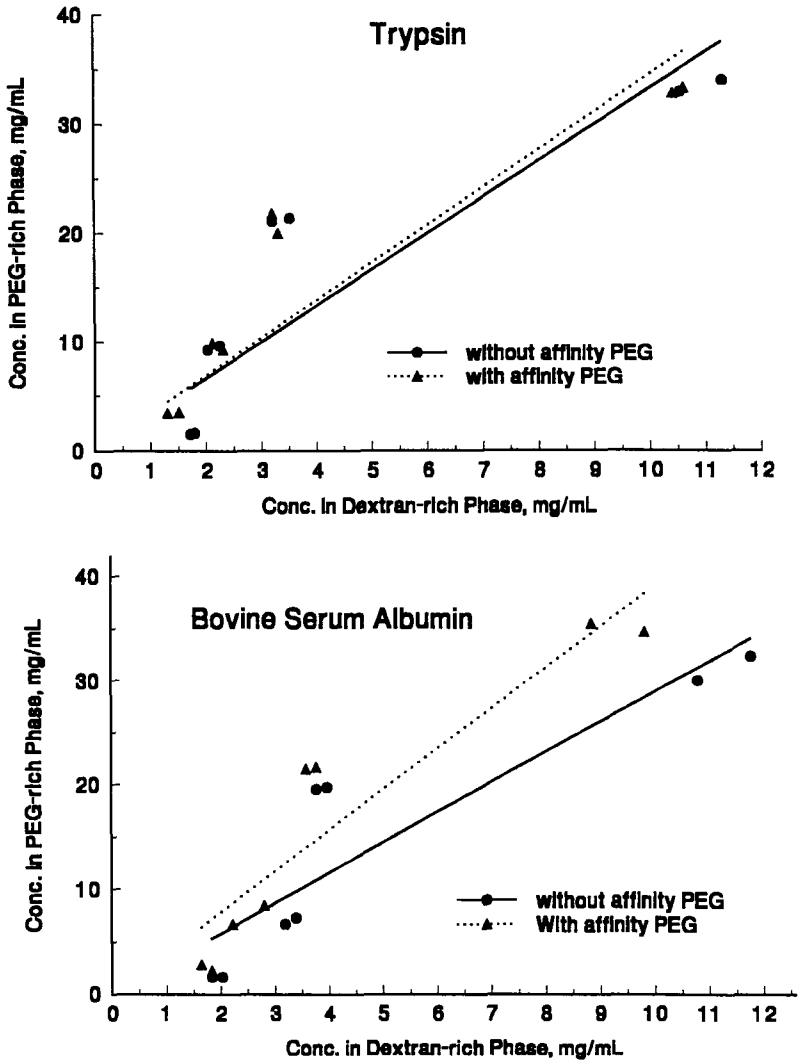

Fig. 2. Equilibrium isotherms for partitioning of trypsin and bovine serum albumin in PEG8000-dextran (70000) system. pH = 7.2, temperature = 4°C, no NaCl.

tion is in agreement with that of Albertsson (*8*) that hydrophobic–hydrophilic interactions play an important role in aqueous two-phase partitioning. The PEG-rich phase is more hydrophobic than the dextran-rich phase (*8*), and hydrophobic segments in protein interact preferentially with the PEG-rich phase. On the other hand, the polyanionic and hydrophilic RNA molecules prefer association with the dextran-rich phase. Charge interactions have also been suggested to play an important role in aqueous two-phase partitioning (*14*). These apparently were not significant in the present studies owing to the low ionic concentration of the buffer.

Table 2
Partition Coefficients of Proteins
and Yeast RNA in PEG-Dextran System, pH 7.2, No Salt

Components	$C_{PEG}/C_{Dextran}$	Comments
Trypsin	3.21 ± 0.85	
	3.45 ± 0.83	In presence of affinity PEG
	0.89 ± 0.37	In presence of yeast RNA
	1.31 ± 0.39	In presence of yeast RNA and affinity PEG
Chymotrypsin	2.78 ± 0.61	
	2.98 ± 0.63	In presence of affinity PEG
	0.56 ± 0.34	In presence of yeast RNA
	0.74 ± 0.38	In presence of yeast RNA and affinity PEG
Bovine albumin	2.90 ± 0.7	
	3.91 ± 0.78	In presence of affinity PEG
	0.94 ± 0.31	In presence of yeast RNA
	1.521 ± 0.31	In presence of yeast RNA and affinity PEG
Nucleic acid	0.46 ± 0.11	
	0.36 ± 0.08	In presence of affinity PEG
	0.89 ± 0.37	In presence of yeast RNA
	0.31 ± 0.39	In presence of yeast RNA and affinity PEG

Partition coefficients in the presence of affinity-ligated PEG are also presented in Table 2. Here a triazine dye, Cibacron blue FGF, was used to form affinity-PEG, because this ligand has been reported to increase selectively the partitioning of dehydrogenases (22). The partition coefficients of the serine proteases, trypsin and chymotrypsin, change only marginally in the presence of the affinity ligand, whereas that of more hydrophobic bovine serum albumin (22) changes significantly. It has been shown by sorption and elution calculations (22) that the triazine dye enhances hydrophobic interactions. For the same reason, only a small change is seen in the partition coefficient of the yeast RNA.

The data showing the effect of salt (NaCl) concentration on partitioning of proteins and nucleic acids are presented in Table 3. These experiments were conducted at a pH of 7.2 in duplicate at intermediate loadings of the proteins and yeast RNA (2.0 g stock solution/10 g two-phase mixture). Hence, the partition coefficients were higher than the average values reported in Table 2 for zero salt concentration. For the three proteins, small increases in the partition coefficients were seen as NaCl concentration was changed from 0 to 5*M*. The partition coefficient of the yeast RNA decreased with increase in salt concentration. The effects were stronger in systems with affinity-ligated PEG. The presence of salts causes a reduction in the electric double layer of protein molecules and,

Table 3
Effect of Salt Concentration on the Partition Coefficients, pH 7.2

Compound	NaCl concentration, *M*	Conditions			
		–	+NA[a]	+A[b]	+NAA
Trypsin	0	5.61 ± 0.51	2.24 ± .05	6.04 ± .03	2.51 ± .05
	5	7.0 ± 0.22	2.7 ± 0.41	10.7 ± 0.49	3.77 ± 0.53
Chymotrypsin	0	4.8 ± 1.0	1.78 ± 0.14	5.75 ± 0.48	2.15 ± 0.16
	5	6.42 ± 0.08	2.67	9.15	3.87 ± 0.27
Bovine serum albumin	0	4.37 ± 0.18	1.67 ± 0.08	5.03 ± 0.21	2.0 ± 0.16
	5	6.56 ± 0.14	2.51 ± 0.29	8.76 ± 0.40	3.37 ± 0.30
Yeast RNA	0	0.23 ± 0.02		0.27 ± 0.005	
	5	0.11 ± 0.006		0.18 ± 0.01	

[a]NA = nucleic acid.
[b]A = affinity PEG.

thus, causes a reduction in net polarity. As a result, hydrophobic interactions play an even more important role in these systems. This is very likely the reason for the increased effect of salt on the partitioning of bovine serum albumin. Cascone et al. (*14*) indicate that the charge effects are different for positively and negatively charged proteins. This, however, was not found in this work, because of the low concentration (0.02*M*) of the partitioning ion (phosphate).

The effect of pH on the partition coefficients is shown in Fig. 3. These experiments were also conducted at intermediate loadings. Hence, the partition coefficients are different from the average values reported in Table 2 for pH 7.2. As the pH was lowered, the partition coefficients of all the proteins increased. This is in agreement with the expected contributions of the effect of hydrophobicity and protein charge. Reduction of pH results in increasingly higher positive charges on trypsin and chymotrypsin molecules. This should cause an increasing role of charge interactions between the positively charged protein molecules and the more negatively charged PEG-rich phase (*14*). This is supported by the trends shown in Fig. 3. On the other hand, the molecules of bovine serum albumin will tend to be more electroneutral as pH decreases, thus enhancing the effect of hydrophobic interactions. The net result is an increase in their partition coefficient with decreasing pH. Hence, these observations also support the assertion made earlier that hydrophobic interactions between the proteins and PEG molecules dominated the partitioning of proteins in the present system. The partition coefficient of yeast RNA in the PEG/dextran system also increased with decreasing pH of the mixture.

Effect of Presence of Nucleic Acids

The results of experiments involving partitioning of the three proteins when present along with yeast RNA in the solution have been presented in Tables 2 and 3 and in Fig. 3. It is clear from the data that the presence of nucleic acids in the solution had a profound effect on the partitioning of proteins in the two-phase system. As an example, the partition coefficient of bovine serum albumin changed from 2.90 $\pm$ 0.7 in the absence of yeast RNA to 0.94 $\pm$ 0.31 in presence of yeast RNA. Similar results were observed for chymotrypsin and trypsin as well. However, the partition coefficient of RNA itself remained unaffected by the presence of proteins (Table 4). A possible reason for this phenomenon appears to be binding between the proteins and yeast RNA in solution.

Similar drops in the partition coefficients were also observed when the partitioning was carried out in the presence of affinity-ligated PEG. As expected, the partition coefficients in the presence of affinity-ligated PEG were higher than those obtained with PEG only. This coupled with the fact that the presence of affinity PEG lowers the partition coefficient of nucleic acids (Table 2) suggests that affinity ligands are desirable in

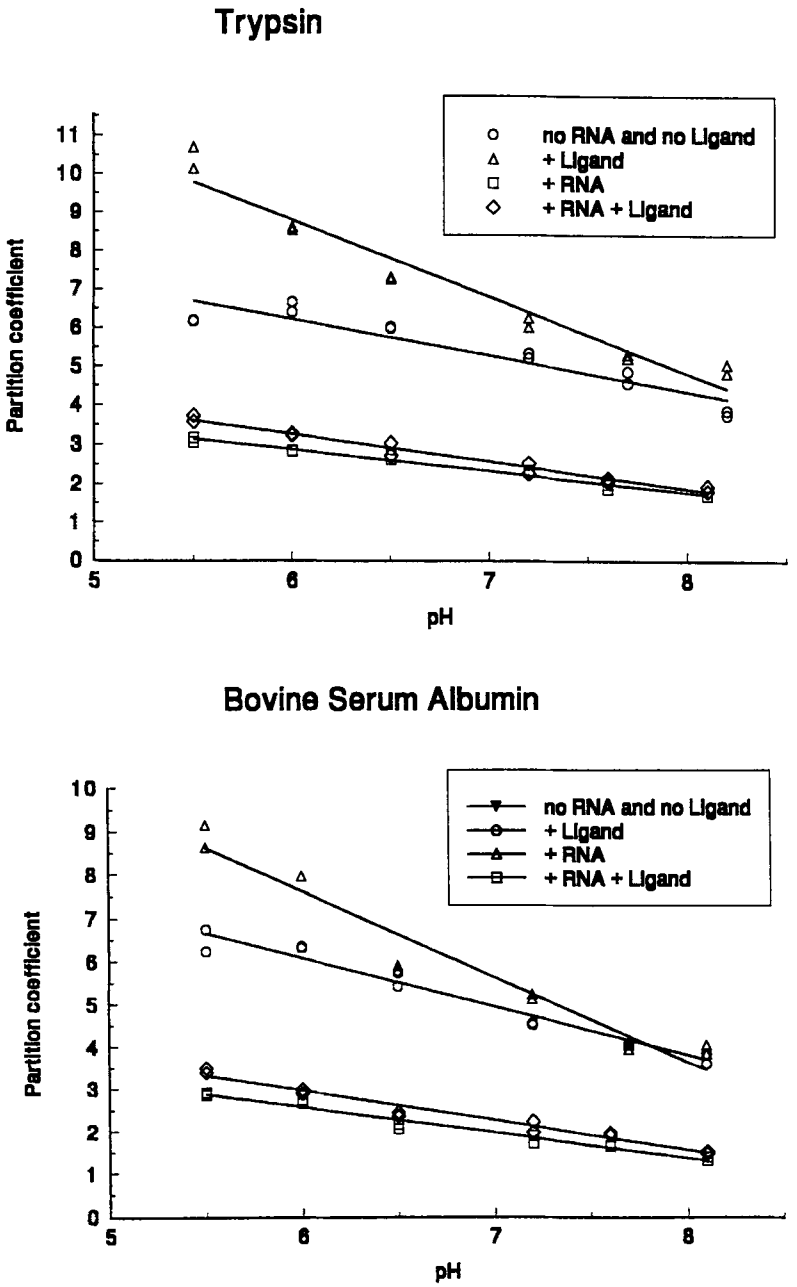

Fig. 3. Effect of pH on partition coefficients on trypsin and bovine serum albumin in PEG8000-dextran (70000) system. Temperature = 4°C, no NaCl.

order to improve the separation of proteins from nucleic acids using aqueous two-phase extraction. Comparatively speaking, large improvements in partition coefficients were observed by manipulations of pH (Fig. 3), provided the pH manipulations are permitted by the chemistry of the desired molecules.

Table 4
Effect of Proteins on the Partitioning of Yeast RNA in the Aqueous Two-Phase System, pH 7.2, No Salt

	$C_{PEG}/C_{dextran}$ for nucleic acid
Nucleic acid only	0.44 ± 0.1
Nucleic acid with trypsin	0.55
Nucleic acid with trypsin and affinity PEG	0.56
Nucleic acid with chymotrypsin	0.34 ± 0.056
Nucleic acid with bovine serum albumin and affinity PEG	0.53 0.48

CONCLUSIONS

Based on the results presented in this work, it can be concluded that aqueous two-phase extraction has a potential to separate effectively the proteins from each other and from nucleic acids. However, the operating conditions developed with pure proteins are likely to be altered drastically in realistic fermentation broths where nucleic acids are present as contaminants. Manipulations of pH and salt concentrations can result in large improvements in the separation of proteins from nucleic acids. Affinity ligands can partially offset the effect of nucleic acid contaminants. A major-cost factor for aqueous two-phase extraction is the cost of dextran. Hence, methods for recovery and recycling of dextran and PEG need to be developed in order to make this method economical. There is a need to search for effective and cheap affinity-ligands and polymers for the extraction process.

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