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Selective Reverse Micellar Extraction of Three Proteins from Filtered Fermentation Broth Using Response Surface Methodology

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ABSTRACT

The influence of three system parameters [bis(2-ethylhexyl)sulfosuccinate (AOT) concentration, pH, and temperature] on the selective separation of cytochrome *c*, lysozyme, and ribonuclease A from buffer solution and a filtered fermentation broth was examined. It was found that a minimal AOT concentration exists for ≥90% extraction from a buffer solution, and this concentration depends on the pH of solution and hydrophobicity of the protein. Extraction from filtered broth resulted in a reduction of the minimal concentration for both cytochrome *c* and lysozyme, while for ribonuclease A it was unchanged. It appears that certain broth constituents act as cosurfactants and reduce the charge repulsion between the surfactant headgroups leading to smaller micelles. This is turn led to a reduction in the pH range, although extraction yields were unaltered. The water content (W_0) was measured in a reverse micellar phase in contact with buffer, filtered fermentation broth, and molecular weight fractionated or extracted broth. This data led to the conclusion that the broth constituents responsible for changes in the minimal AOT concentration and W_0 were low molecular weight positively charged amino acids and phospholipids. Finally, response surface methodology was used to optimize key system parameters in order to maximize protein extraction, and this technique minimizes the experiments required. By using these optimized conditions, a mixture of the three proteins could be recovered from a filtered broth with high yields (70–97%) and high purity. Furthermore, the initial protein concentration was found not to influence protein recovery.

Key Words. Reverse micelles; Extraction; Selectivity; Proteins; Fermentation broth

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INTRODUCTION

In biotechnology the desired products are usually present at very low concentrations in a complex biomedium such as a fermentation broth. This makes product separation difficult, and since downstream separation is usually the dominant cost for most products, improving separation has considerable economic benefits. Many techniques have been developed in biotechnology to achieve a highly efficient and economical separation process. One novel separation technique with the ability to be scaled up easily, to be operated continuously, and to be highly selective is liquid–liquid extraction using reverse micelles.

The ability of reverse micelle (RM) systems to selectively extract a target protein from a mixture is not fully understand yet, but is known to be influenced by a number of factors, particularly pH, salt type and concentration, solvent type, temperature, surfactant type and concentration, and the incorporation of bioaffinity ligands (3, 9). Understanding the role of these system properties and using this knowledge to enhance selectivity is crucial if this technique is to be used at an industrial scale. Selective solubilization of proteins into a reverse micellar phase has only been addressed by a few researchers. Göklen and Hatton (3) demonstrated that the separation of a single protein from a simple synthetic protein mixture can be accomplished relatively easily. Woll and Hatton (22) separated ribonuclease A from concanvalin A, while Aires-Barros and Cabral (1) were able to separate a crude mixture of two lipases in buffer solution. Furthermore, Rahaman et al. (18) showed that a reverse micellar phase can selectively extract alkaline protease from whole fermentation broth. Recently, Krei and Hustedt (11) extracted α-amylase from a complex fermentation medium. However, there were many problems encountered in these studies, in particular low extraction yields and severe emulsion formation, and a number of key system variables have not been investigated in much depth. In addition, in all this work there was little or no attempt to compare the selective separation of proteins from model buffer solutions and real fermentation broths to determine if broth constituents had a significant influence on extraction behavior and yield, and if so, which component, and why did it influence the extraction. This information is vital for the scale-up of RM systems. Therefore, in order to improve protein selectivity and RM scale-up, a number of important system parameters, such as surfactant concentration and temperature, still need to be studied.

Due to the number of variables involved in these experiments, factorial experimental design would lead to an unworkably large number of experiments. Hence, a more suitable experimental design is required, and response surface methodology is a suitable technique (10). This technique has been used for the development of optimized media composition, or operating conditions to maximize the bioproduct such as in the production of lipid (17) and the growth

of yeast (21). In the case of reverse micelles, the method has been used to optimize the enzymatic synthesis of peptides (8) and the recovery of horseradish peroxidase (19).

In this study, factors influencing the selective solubilization of cytochrome *c*, lysozyme, and ribonuclease A from a buffer solution and a filtered fermentation broth were studied and compared. These proteins were chosen due to their similar molecular weights (MW), thus resulting in a challenging separation. The specific effects of surfactant concentration, pH, temperature and initial protein concentration on selectivity were investigated. A response surface methodology (RSM) was used to optimize the variation in system parameters to maximize protein recovery. In addition, the effect of fermentation broth constituents on the water content of reverse micelles was also investigated in order to gain greater insights into the effect of broth on reverse micelle extraction.

MATERIALS AND METHODS

Bis(2-ethylhexyl)sulfosuccinate sodium salt (AOT), 2,2,4-trimethylpentane (isooctane), lysozyme (pI 11; 14,300 Da), ribonuclease A type IAS (pI 7.8; 13,700 Da) and cytochrome *c* (pI 10.6; 12,348 Da) were purchased from Sigma (Poole, UK). Sodium chloride and sodium phosphate (dibasic and monobasic) were Anal-R grade from Merck (Dorset, UK), while trioctylmethylammonium chloride (TOMAC) was obtained from Aldrich (Dorset, UK). Filtered fermentation broth (kindly provided by N. Beaumont, Imperial College, London, UK) was taken from an *E. coli* batch culture which was grown aerobically in LB medium supplemented with 50 mg/L ampicillin at 37°C for 24 hours. The broth before fermentation had the following main constituents (% w/w): lipid < 0.01, total N 1.79, amino N 0.61, glutamic acid 2.03, proline 0.80, leucine 0.82, arginine 0.13, and phenylalanine 0.44 (23). The broth had a salt content of 0.1 M NaCl, a pH of 9.0, and was filtered through a hollow fiber membrane (MW cutoff = 100 kDa). All other chemicals were purchased from Sigma and were of analytical grade.

Extraction Procedures

Forward Extraction

All forward extractions were performed by contacting equal volumes (typically 750 μL) of aqueous and organic solutions in a 1.5-mL test tube. The organic solution always consisted of 2.5–50 mM anionic surfactant AOT in isooctane. Extraction equilibrium was achieved by rotary inversion at 50 rpm for 30 minutes. Samples were then centrifuged at 3000 rpm for 5 minutes to separate the phases, and the organic phase was then analyzed spectrophotometrically. All data from these experiments are based on duplicate

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samples. The coefficient of variation for the total forward extraction process was ±2%.

Forward Extraction of a Single Protein in Buffer. In all cases, 1 g/L cytochrome *c*, 1 g/L lysozyme, or 2.5 g/L ribonuclease A solutions were dissolved in a mixture of 80% 0.1 M NaCl and 20% of 0.1 M buffer solution, resulting in a solution with 0.1 M Na^+-ions. The buffer solutions were acetate buffer (pH 3–5), phosphate buffer (pH 6–8), and carbonate buffer (pH 9–12).

Forward Extraction of a Single Protein Dissolved in Filtered Fermentation Broth. One type of protein (cytochrome *c*, lysozyme, or ribonuclease A) was dissolved in the broth at the same concentration as in the buffer system. Then the pH of the protein solution was adjusted to the required pH (pH 3–12) using 0.1 M HCl or 0.1 M NaOH. Filtered broth without additional protein was also contacted with the organic phase, and the organic phase remaining after equilibrium was used as a blank.

Forward Extraction of Binary and Ternary Protein Mixtures: Effect of Initial Concentration. For the binary protein mixture consisting of cytochrome *c* and ribonuclease A, the total protein concentration was kept constant at 4 g/L while the concentration of cytochrome *c* was varied (0, 1, 2, 3, and 4 g/L). The optimum extraction conditions for cytochrome *c* were obtained from the response surface methodology (see later) and were 25 mM AOT, pH 9, and 15°C.

The ternary protein mixture consisted of lysozyme, cytochrome *c*, and ribonuclease A, and the total concentration was kept constant at 5 g/L. The concentration of ribonuclease A was kept constant at 2.5 g/L, while the concentrations of cytochrome *c* and lysozyme were varied (0, 0.5, 1.0, 1.5, 2.0, and 2.5 g/L) such that their total amounted to 2.5 g/L also.

Molecular Weight Fractionation and Solvent Extraction of the Low Molecular Weight Components

In order to investigate the effect of broth components on the water content in a reverse micellar phase (W_0), the broth was separated into three fractions using Amicon ultrafiltration disc membranes, YM 30 and YM 1, which were purchased from Millipore. The fractions contained high molecular weight components (HMW: >30 kDa), medium molecular weight components (MMW: 1–30 kDa), and low molecular weight components (LMW: <1 kDa). After fractionation the salt concentration in the fractions was adjusted by adding 0.1 M NaCl (the salt concentration in the broth) to regain the ionic strength of the initial filtered fermentation broth. One type of protein was then added to all the fractions which were then adjusted to the required pH of 9 for cytochrome *c* and lysozyme, and 5 for ribonuclease A.

Hydrophobic compounds such as lipids, or negatively charged amino acid were removed from the LMW fraction by solvent extraction with decanol, or reactive extraction with 0.5 M TOMAC in octanol, respectively.

Backward Extraction

Lysozyme and cytochrome *c* were backextracted using the conventional method with high ionic strength and a pH above the pI of the particular protein. 750 μL of the protein containing reverse micellar phase was rotary mixed at 50 rpm with an equal volume of aqueous solution consisting of 90% KCl solution and 10% potassium phosphate buffer at the required concentration and pH in a 1.5 mL test tube for 30 minutes. The conditions used for lysozyme were 2 M KCl/pH 11, and for cytochrome *c* were 1 M KCl/pH 10. Phase separation was achieved by centrifugation for 2 minutes at 12,000 rpm. The coefficient of variation for the entire process was ±2%.

Ribonuclease A was backextracted by adding a counterionic surfactant (TOMAC) (7). 650 μL of the protein containing reverse micellar phase was contacted with 50 μL of 850 mM TOMAC in isooctane, and 700 μL of 0.1 M phosphate buffer at pH 8, in a 1.5 mL test tube, and the vial was then mixed by rotary inversion at 50 rpm for 5 minutes. The mixture was centrifuged at 12,000 rpm for 2 minutes to achieve phase separation. The coefficient of variation for the entire process was ±2%.

Experimental Design

In order to optimize the reverse micellar extraction of a target protein from the protein mixture, the response surface methodology (RSM) was used to maximize extraction yield. The experiments were designed as a central composite design within RSM. Table 1 shows the range of each treatment for lysozyme, cytochrome *c*, and ribonuclease A separation. The data analysis was carried out using Statistical Analysis System software (SAS Institute Inc.,

TABLE 1
System Parameters of Protein Separation from Filtered Fermentation Broth

	Forward extraction parameter		
Protein	Surfactant concentration (mM)	pH	Temperature (°C)
Lysozyme	10.8–19.2	10.66–12.34	3.2–36.8
Cytochrome *c*	16.7–33.4	8.16–9.84	3.2–36.8
Ribonuclease A	43.2–72.8	4.16–5.84	3.2–36.8

Cary, NC), while Mathematica software was used to plot the experimental data.

Protein Assay

The concentration of protein in the aqueous and organic phases was determined by UV absorption scanning and peak area integration using a Shimadzu UV2101 spectrophotometer; for lysozyme and ribonuclease A at 250–300 nm, and cytochrome *c* at 350–450 nm. Protein concentrations were calculated using appropriate peak areas for cytochrome *c* ($A_{350–450\,nm} = 72.2$ arbitrary units corresponded to the concentration 1 g/L), for lysozyme ($A_{250–300\,nm} = 29.4$ arbitrary units corresponded to the concentration 1 g/L), and for ribonuclease A ($A_{250–300\,nm} = 4.5$ arbitrary units corresponded to 1 g/L). The standard deviation of the measurements was ±5%. Quantitative analysis of each protein in the aqueous solution was performed by HPLC. A Zorbax 300 SB C8 column was fitted to a Shimadzu A-6 chromatography system, and UV detection was used at 280 nm. The protein was eluted from the column with a mobile phase consisting of 0.1% trifluoroacetic acid and acetonitrile (gradient 0–70%v/v) at a flow rate of 1.5 mL/min.

Water Detection in Solvent Phase

The water content of the reverse micellar phase was determined by Karl-Fischer titration using a Mettler DC-37 coulometer. The reagents used were Hydranal Coulomat A (anode reagent) and Hydranal Coulomat B (cathode reagent) purchased from Riedel-de Haën (Seelze, Germany). 10 μL of solution was injected into the titrator at a time using a microsyringe, and the coefficient of variation was ±5%.

RESULTS AND DISCUSSION

Effect of AOT Concentration and pH

Single Protein Dissolved in Buffer System

The extraction yields of the three proteins, cytochrome *c*, lysozyme, and ribonuclease A, were studied as a function of pH and AOT concentration, and these data are shown in Fig. 1(A–C). At pH > pI, where the charge of the proteins was the same as the surfactant, none of the proteins could be extracted. At a pH < pl and high AOT concentrations (≥25 mM), 90–100% of all the proteins could be extracted into the reverse micellar phase. However, at very low pH (pH 3–4), denaturation of the protein reduced the extraction yield, and this has been noted in past work (4). With increasing AOT concentration the yield of all proteins increased, and this agreed with the previous work by

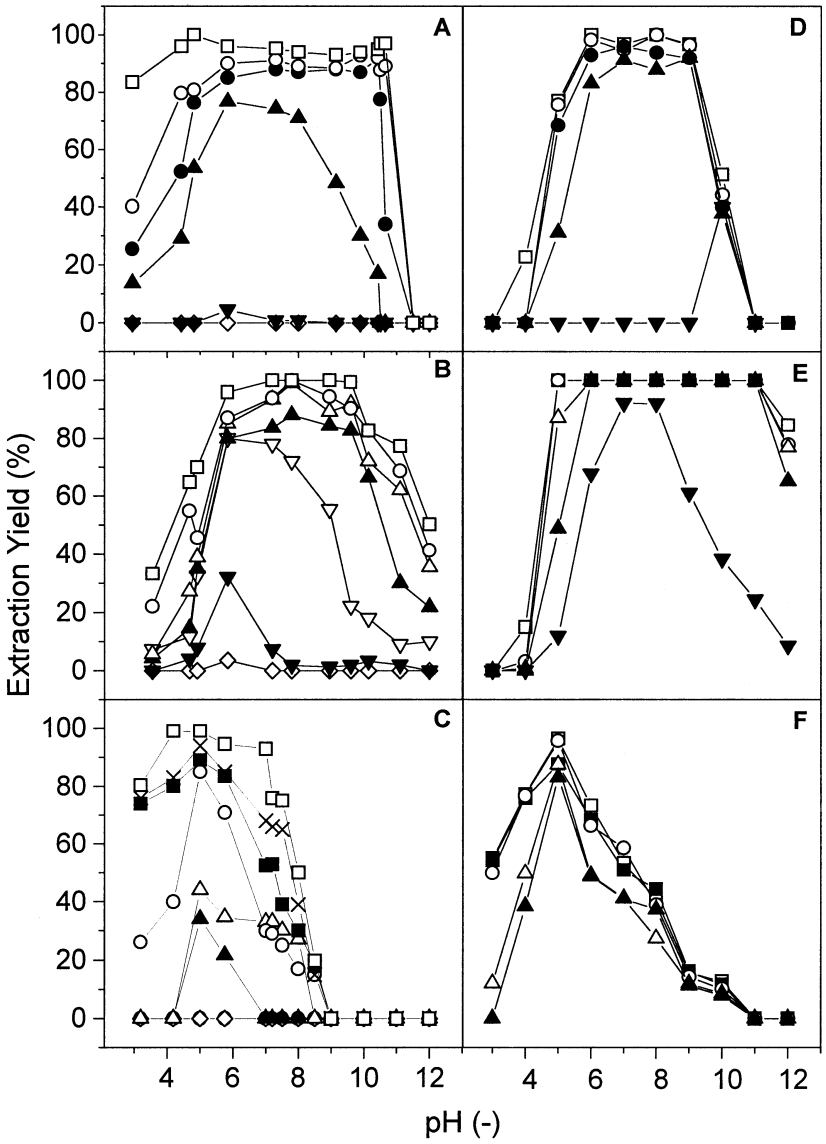

FIG. 1 Protein extraction from a buffer solution and filtered fermentation broth. (A and D) 1 g/L cytochrome *c*, (B and E) 1 g/L lysozyme, (C and F) 2.5 g/L ribonuclease A with various AOT concentrations: (□) 50 mM, (×) 45 mM, (■) 30 mM, (○) 25 mM, (●) 16 mM, (△) 12.5 mM (▲) 10 mM, (▽) 7 mM, (▼) 5 mM, and (◊) 2.5 mM.

Ichikawa et al. (5). The AOT concentration at which 90% extraction was attained was defined as the minimal AOT concentration, and from experimental data this was found to be 16, 12.5, and 18 mM/g protein for cytochrome *c*, lysozyme, and ribonuclease A, respectively. These concentrations were found to depend on the pH, and the minimal concentration given is the one measured at the optimum pH for extraction of each protein, which was pH 10, pH 8, and pH 5 for cytochrome *c*, lysozyme, and ribonuclease A, respectively. Under the

optimum conditions of pH and AOT concentration, a protein molecule is surrounded by a certain amount of solubilizing water in a reverse micelle, and this is the minimum water required for protein solubilization (5). This in turn defines the minimal AOT concentration required for >90% removal. The difference in minimum AOT concentration required for specific proteins can be explained by the hydrophilicity of each protein. The order of elution from a hydrophobic HPLC column showed that ribonuclease A was more hydrophilic than cytochrome *c* and lysozyme (data not shown), hence for more hydrophilic proteins there is a greater amount of water surrounding them and hence higher minimal surfactant concentrations are required for substantial protein solubilization. Imai et al. (6) also found a similar trend for the three proteins.

Single Protein Dissolved in Filtered Fermentation Broth

Figure 1(D–F) shows the extraction yield of the three proteins added to filtered broth. The minimum AOT concentrations for the extraction of cytochrome *c*, lysozyme, and ribonuclease A were 10 mM, 10 mM, and 12 mM/g protein, which were less than the minimum concentration required in the buffer system. This lower requirement for surfactant may be due to compounds likely to be found in the broth, such as hydrophobic amino acids and/or phospholipids, acting as cosurfactants. These substances seem to reduce the strong repulsive ion–ion interaction between the surfactant headgroups (11), and can result in decreases in the size, water content, and aggregation number of reverse micelles (2, 12). However, the decreases observed in the minimum AOT concentration seem to indicate that these compounds are more influential with hydrophobic proteins (cytochrome *c* and lysozyme) than with hydrophilic ones (ribonuclease A). The reasons for this are not clear at present, but may be related to the strength of electrostatic interactions between the protein and the charged internal surfactant interface of the reverse micelle. The broth constituents also influenced the pH range of extraction, and this was narrower than from the buffer. The explanation for this may also be due to certain broth constituents physically altering the reverse micelles. In order to obtain more detailed information about reverse micelles in equilibrium with broth, measurements of the water content in the organic phase in contact with a variety of aqueous phases were carried out.

Water Content of the Reverse Micellar Phase

The water content, W_0, of a reverse micellar phase is defined as the molar ratio of water to surfactant in a reverse micellar phase. Figure 2 shows W_0 values of the reverse micellar phase, with and without proteins, in equilibrium with filtered and fractionated/extracted broth compared with buffer solution. It appears that the presence of molecules acting as cosurfactants may have

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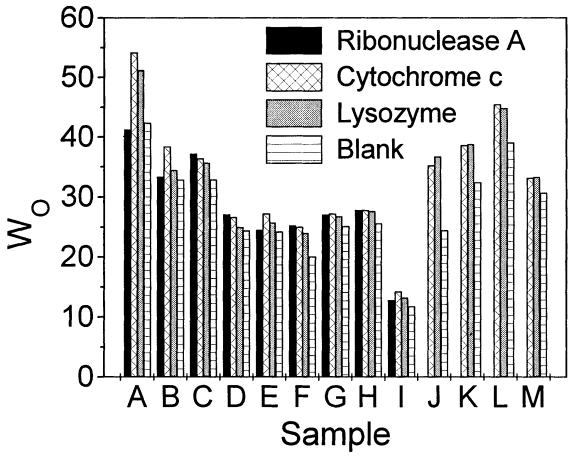

FIG. 2 Effect of a variety of aqueous phases and fermentation broths on the water content of reverse micelles with and without proteins. (A) 0.1 M Sodium salt buffer solution, (B) HMW, (C) MMW, (D) LMW, (E) fermentation broth, (F) LMW fraction extracted by TOMAC in octanol, (G) LMW fraction extracted by AOT in isooctane, (H) LMW fraction extracted by decanol, (I) 0.1 M potassium salt buffer solution, (J) 1.5 g/L phosphatidylcholine added to buffer solution, (K) 1.5 g/L phosphatidylcholine added to buffer solution extracted by decanol, (L) 1 g/L arginine added to pH 10 buffer solution, and (M) 1 g/L arginine added to pH 5 buffer solution.

caused the substantial decrease in water content in the organic phase when contacted with the broth or certain broth fractions. However, these values were higher than the W_0 value of the reverse micelles in contact with a potassium salt. The effect of many cosurfactants is to increase the curvature of the micellar shell (16), and hence reduce its size and W_0.

In order to better understand the effect of certain broth constituents on reverse micelle formation, the broth was fractionated into three samples. The W_0 value of the reverse micellar phase equilibrated with the HMW or MMW (RM_{HMW}, RM_{MMW}) fraction was found to be higher than the LMW (RM_{LMW}) fraction, which had the same W_0 as the reverse micellar phase equilibrated with the whole broth, RM_{broth} (Fig. 2-E). This implies that the reduction in W_0 when in contact with the RM_{broth} may have been caused by LMW substances such as phospholipids, positively charged amino acids, and simple sugars (20). To identify the effect of each class of substances, solvent extraction of the LMW fraction using decanol was then carried out to remove any nonpolar constituents (H). Figure 2 shows that the absence of most of the lipids caused a slight increase in W_0 in relation to the unextracted LMW fraction. Furthermore, a substantial decrease in W_0 was also noted when phospholipid was added to the buffer solution (J and K). Moreover, the absence of negatively charged amino acids from the LMW fraction, which were extracted into a TOMAC solution (F), resulted in a slight decrease in W_0. Finally, the addition

of a negatively charged amino acid (pH 10, arginine, L) to the buffer resulted in a small decrease in W_0, while at pH 5 (positively charged, M) resulted in a considerably larger drop in W_0. Therefore, it appears that the reduction of W_0 in the organic phase when contacted with the broth was caused by the combined effect of both phospholipids and positively charged amino acids.

With regard to protein selectivity, these results show that the pH of the aqueous broth, and the AOT concentration, can potentially be manipulated in order to extract a single target protein from a mixture into a reverse micellar phase.

Optimizing System Conditions for Selective Separation from a Protein Mixture in Filtered Fermentation Broth Using RSM

Table 2 shows the optimal system conditions for each protein separation from the statistical analysis. Figure 3(A) shows the response surface of the predicted purity yield of lysozyme ($PY_{L\text{-}C}$) as a function of the three parameters at ambient temperature (20°C), 10°C, and 30°C. These results indicate that the surface response is very sensitive to pH and temperature, which are very significant, while the effect of AOT concentration is much less significant. It can also be seen from these figures that the maximum $PY_{L\text{-}C}$ increases with decreasing temperature. This increase in the yield may have been caused by a reduction in the hydrophobic interaction of the two proteins (lysozyme and cytochrome *c*) at low temperatures (15). Moreover, the predicted results of cytochrome *c* separation, $PY_{C\text{-}R}$ (Fig. 3B), show the same trend as lysozyme separation in which the effect of pH and temperature were found to be stronger than AOT concentration. Figure 3(C) shows the response surface of the predicted ribonuclease A separation (PY_R) as a function of the three parameters at 10°C, ambient temperature, and 30°C, respectively. According to these results, the temperature did not significantly affect the estimated response, whereas AOT concentration and pH effects were significant.

TABLE 2
Optimal Conditions of Protein Separation from Filtered Fermentation Broth

Protein	Surfactant concentration (mM)	pH	Temperature (°C)
Lysozyme	15	11.5	12
Cytochrome *c*	25	8.8	15
Ribonuclease A	62	5.1	20

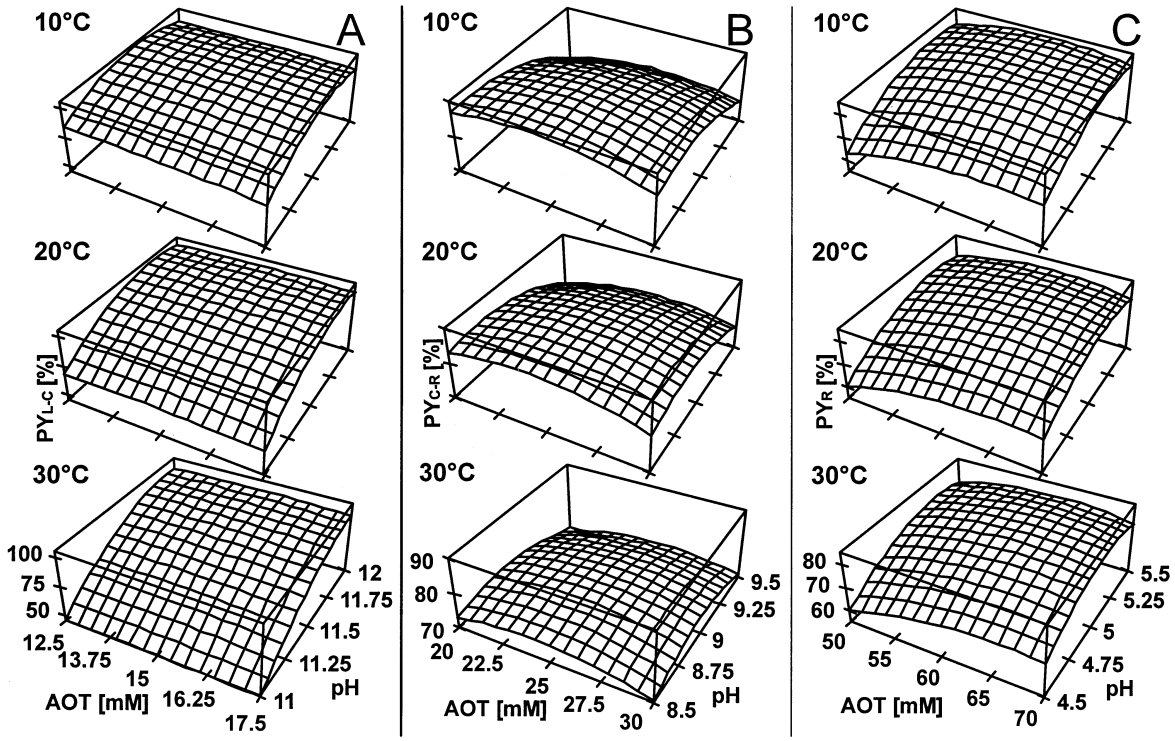

FIG. 3 Lysozyme, cytochrome *c*, and ribonuclease A separation at 10°C, ambient temperature (20°C), and 30°C.

Selective Separation of a Ternary Protein Mixture

Forward Extraction

The results of the RSM study were used to select the optimum solubilization parameters for each protein. Firstly, lysozyme was extracted from the protein mixture in filtered broth, followed by cytochrome *c*, and finally ribonuclease A. The initial feed solution and each resulting aqueous solution were analyzed by HPLC, and the chromatograms are shown in Fig. 4. The forward extraction yield of each protein is shown in Table 3, and the results indicate that protein separation from a filtered fermentation broth by a sequence of three forward extractions was possible, and that high yields are obtainable. Under the optimum conditions, AOT reverse micellar extraction can selectively separate the target protein without being affected by the composition of the fermentation broth, and this observation agrees with that of Krei and Hustedt (11) for α-amylase extraction by cetyl-trimethyl ammonium bromide. Furthermore, the use of an AOT reverse micellar phase led to a clear interface between the two phases, in contrast to extraction from a buffer, and this is one advantage of using an anionic as opposed to a cationic surfactant as noted by Krei and Hustedt (11).

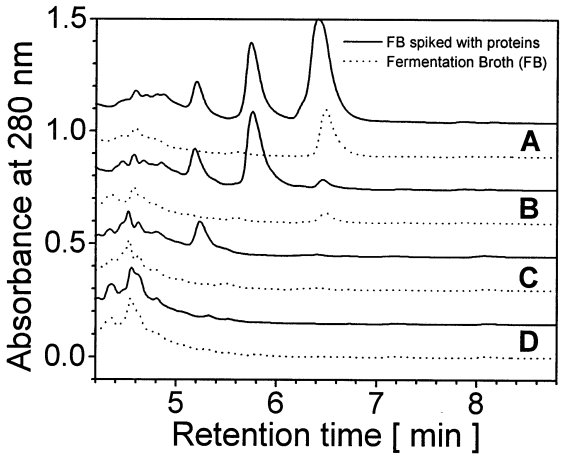

FIG. 4 Sequential separation of the three protein mixture. (A) Initial feed solution, (B) lysozyme separation, (C) cytochrome *c* separation, and (D) ribonuclease A separation.

Backward Extraction

If the protein forward extraction was carried out using a low concentration of AOT (15 mM and 25 mM for lysozyme and cytochrome *c*, respectively), then the two proteins could be backextracted easily at a pH close to the pI of the relevant protein, and at low salt concentration, because the protein-filled reverse micelles are not stable under these conditions (14). However, ribonuclease A was extracted using a high AOT concentration (60 mM), hence the backextraction was carried out by adding counterionic surfactant (7). The chromatograms of the recovered proteins are shown in Fig. 5, and it can be seen that there are very few contaminants from the broth in the aqueous phase of the recovered proteins. Hence, in addition to being selective, reverse micellar extraction from broths usually results in relatively pure protein solutions.

Effect of Initial Concentration

Different concentrations of proteins, lysozyme and cytochrome *c* in a ternary protein mixture with a constant concentration of ribonuclease A (2.5

TABLE 3
Sequential Fermentation Broth Extraction

Forward extraction	Ribonuclease A	Cytochrome *c*	Lysozyme
First	0	0.5	98
Second	0	97	0
Third	70	2	0
Backextraction (% of initial concentration)	70	97	77

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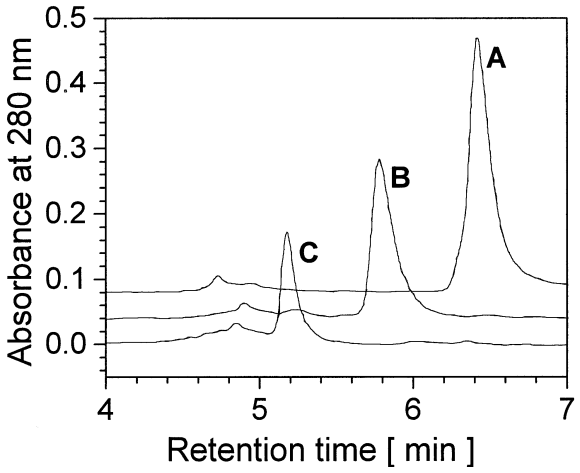

FIG. 5 Backextraction of the three protein mixture; (A) lysozyme, (B) cytochrome *c*, and (C) ribonuclease A.

g/L), and cytochrome *c* and ribonuclease A in a binary protein mixture, were used to study the effect of initial protein concentration on the selective solubilization of lysozyme and cytochrome *c*. Under the optimum extraction conditions, and within the range of initial protein concentrations investigated, the target protein was not affected by the contribution of the other protein present, as shown in Fig. 6. Lye et al. (13) reported that the concen-

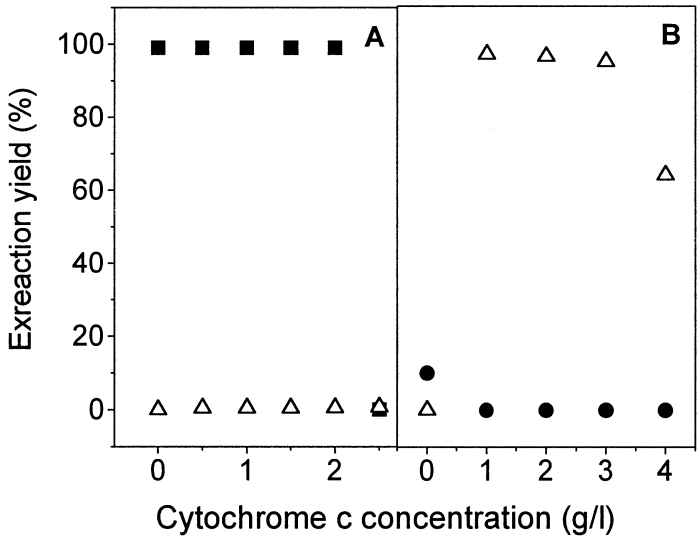

FIG. 6 Effect of initial protein concentration. (A) Ternary protein mixture (constant ribonuclease A concentration): (■) lysozyme and (△) cytochrome *c*. (B) Binary protein mixture: (△) cytochrome *c* and (●) ribonuclease A.

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tration of lysozyme in reverse micelles decreased at high initial concentrations (>2.8 mg/mL), and this was accompanied by the appearance of an increasing amount of precipitate being formed at the interface. However, for the binary protein mixture, the decline in solubilization of cytochrome *c* at 4 g/L appeared to be due to the low surfactant concentration used (50 mM) which determines the maximum protein concentration that can be extracted into a reverse micelle phase (13).

CONCLUSIONS

- A minimal AOT concentration exists, defined as the surfactant concentration required for ≥90% protein extraction, which is dependent on pH and the hydrophobicity of the protein. For cytochrome *c*, lysozyme, and ribonuclease A in buffer these values were 16, 12.5, and 18 mM/g at pH 10, 8, and 5, respectively.
- The minimal AOT concentration when extracting the same proteins from filtered broth was found to be 10, 10, and 12 mM/g. Hence, in two out of three cases the concentration was reduced, and it appears that this was due to certain broth constituents acting as cosurfactants and altering the size, water content, and aggregation number of the reverse micelles. This effect was manifest in narrowing the pH range of extraction, although the extent of extraction was not altered.
- Measurement of W_0 in a reverse micellar phase in contact with buffer, filtered broth, and fractionated/extracted broth revealed that the W_0 decreases by 50% when contacted with filtered broth in comparison with buffer, and this was due to both positively charged amino acids and phospholipids reducing the size of the micelles by reducing the repulsion between the surfactant headgroups.
- Response surface methodology was used to optimize the extraction of the three proteins, and it was found that in all cases two out of the three system variables (AOT concentration, pH, and temperature) had a significant effect on protein extraction.
- Using the optimized extraction parameters, a mixture of three proteins in filtered fermentation broth was separated and backextracted with high yields (70–97%) and high purity. Initial protein concentrations up to 3 g/L were found not to influence the extraction yield.

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REFERENCES

1. M. R. Aires-Barros and J. M. S. Cabral, *Biotechnol. Bioeng.*, *38*, 1302 (1991).
2. E. Caponetti, A. Lizzio, and R. Triolo, *Langmuir*, *8*, 1554 (1992).
3. K. E. Göklen and T. A. Hatton, *Sep. Sci. Technol.*, *22*, 831 (1987).
4. S. Ichikawa and S. Furusaki, *Trans. Inst. Chem. Eng.*, *Part C*, *73*, 33 (1995).
5. S. Ichikawa, M. Imai, and M. Shimizu, *Biotechnol. Bioeng.*, *39*, 20 (1992).
6. M. Imai, T. Natsume, K. Naoe, M. Shimizu, S. Ichikawa, and S. Furusaki, *Bioseparation*, *6*, 325 (1997).
7. S. Jarudilokkul, L. H. Poppenborg, and D. C. Stuckey, *Biotechnol. Bioeng.*, *62*, 593 (1998).
8. X. Jorba, P. Clapes, N. Xaus, S. Calvet, J. L. Torres, G. Valencia, and J. Mata, *Enzyme Microbiol. Technol.*, *14*, 117 (1992).
9. B. D. Kelly, D. I. C. Wang, and T. A. Hatton, *Biotechnol. Bioeng.*, *42*, 1199 (1993).
10. A. I. Khuri and J. A. Cornell, *Response Surfaces Designs and Analysis*, 2nd ed., Dekker, New York, NY, 1996.
11. G. A. Krei and H. Hustedt, *Chem. Eng. Sci.*, *47*, 99 (1992).
12. J. Lang, N. Lalem, and R. Zana, *J. Phys. Chem.*, *95*, 9533 (1991).
13. G. L. Lye, J. A. Asenjo, and D. L. Pyle, *Biotechnol. Bioeng.*, *97*, 509 (1995).
14. K. Naoe, M. Imai, and M. Shimizu, *Trans. Inst. Chem. Eng.*, *Part C*, *74*, 163 (1996).
15. M. J. Pires and J. M. S. Cabral, *Biotechnol. Prog.*, *9*, 647 (1993).
16. P. Plucinski and J. Reitmeir, *Colloids Surf. A*, *97*, 157 (1995).
17. S. G. Prapulla, Z. Jacob, N. Chand, D. Rajalakshmi, and N. G. Karanth, *Biotechnol. Bioeng.*, *40*, 965 (1992).
18. R. S. Rahaman, J. Y. Chee, J. M. S. Cabral, and T. A. Hatton, *Biotechnol. Prog.*, *4*, 218 (1988).
19. C. Regalado, J. A. Asenjo, D. L. Pyle, L. A. Trinca, and S. Gilmour, *Trans. Inst. Chem. Eng.*, *Part C*, *72*, 123 (1994).
20. G. Spirovska and J. B. Chaudhuri, *Biotechnol. Bioeng.*, *58*, 374 (1998).
21. M. Vazque and A. M. Martin, *Ibid.*, *57*, 314 (1998).
22. J. M. Woll and T. A. Hatton, *Bioprocess Eng.*, *4*, 193 (1989).
23. J. M. Rechcigl, *CRC Handbook Series in Nutrition and Food*, *Section G*: *Diets*, *Culture Media*, *Food Supplements*, *Vol. III*, CRC Press, Cleveland, OH, 1978.

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