Separation Science and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713708471

Comparison of Two Methods to Recover Lysozyme from Reverse Micellar Phases

Youn-Ok Shin[a]; Martin E. Weber[a]; Juan H. Vera[a]
[a] Department of Chemical Engineering, McGill University, Montreal, Quebec, Canada

Online publication date: 04 July 2003

To cite this Article Shin, Youn-Ok , Weber, Martin E. and Vera, Juan H.(2003) 'Comparison of Two Methods to Recover Lysozyme from Reverse Micellar Phases', Separation Science and Technology, 38: 8, 1733 — 1748
To link to this Article: DOI: 10.1081/SS-120019406
URL: http://dx.doi.org/10.1081/SS-120019406

MARCEL DEKKER, INC. • 270 MADISON AVENUE • NEW YORK, NY 10016

SEPARATION SCIENCE AND TECHNOLOGY
Vol. 38, No. 8, pp. 1733–1748, 2003

Comparison of Two Methods to Recover Lysozyme from Reverse Micellar Phases

Youn-Ok Shin, Martin E. Weber, and Juan H. Vera*

Department of Chemical Engineering, McGill University, Montreal, Quebec, Canada

ABSTRACT

Two methods were tested to recover lysozyme from reverse micellar phases formed either with an anionic surfactant or with a cationic surfactant. The conventional back extraction method of contacting the protein-containing reverse micellar phase with a fresh aqueous phase, with pH and salt concentration adjustment, did not recover lysozyme from either reverse micellar phase. The lysozyme removed from the reverse micellar phase precipitated at the aqueous–organic interface. A solvent precipitation method using a polar organic solvent added to either lysozyme-containing reverse micellar phase, precipitated lysozyme as a solid, while the surfactant was solubilized in the polar solvent. Of the seven solvents tested, acetone recovered 70% of the original lysozyme from the anionic reverse micellar phase without loss of activity. No active lysozyme could be recovered from the cationic reverse micellar system.

*Correspondence: Professor Juan H. Vera, Department of Chemical Engineering, McGill University, 3610 University Street, Montreal, Quebec, H3A 2B2, Canada; Fax: 514 398 6678; E-mail: Juan.Vera@mcgill.ca.

DOI: 10.1081/SS-120019406 0149-6395 (Print); 1520-5754 (Online)

MARCEL DEKKER, INC. • 270 MADISON AVENUE • NEW YORK, NY 10016

In this case, the lysozyme was denatured by the high pH required for the initial extraction into the reverse micellar phase.

Key Words: Reverse micelles; Lysozyme; Protein recovery; Polar solvent; Enzyme activity.

INTRODUCTION

Reverse micelles, often referred to as water-in-oil microemulsions, are aggregates of surfactants whose inner core contains a water pool in an apolar solvent. Proteins and other hydrophilic species can be solubilized in the water pool of the reverse micelles, maintaining the aqueous environment in the organic phase.[1] Proteins, such as lipase,[2–5] α-amylase,[6–9] cytochrome c[10–14] and lysozyme,[14–17] were successfully solubilized in reverse micellar systems. Once extracted, the proteins must be recovered from the reverse micellar phase.

The conventional method for protein recovery is to contact the protein-loaded reverse micellar phase with a fresh aqueous phase. This process is often referred to as *back extraction*.[6,12] Usually the pH and the ionic strength of the receiving aqueous phase are set to favor the electrostatic repulsion between the protein and the surfactant head group.[18] However, back extraction is a slow process,[12,17,19] and a simple adjustment of pH and/or ionic strength often results in a low recovery of protein in the aqueous phase.[20] Ethanol,[2] isopropanol,[21] ethyl acetate,[22] or counter surfactants[14,23] have been added to the receiving aqueous phase in an attempt to increase the recovery. The addition of cosolvent to the reverse micellar phase decreases the water uptake, causing the protein to be expelled from the reverse micellar phase.[2,21]

Instead of using a fresh aqueous phase, with pH and salt concentration adjustment, the addition of acetone directly to the protein-containing reverse micellar phase can break the reverse micellar structure. The surfactant dissolves in the acetone phase while the protein precipitates as a solid due to its low solubility in the organic solvent.[24] We refer to this process as *solvent precipitation*. Using acetone, γ-glutamyltransferase[25] and ribonuclease-A[26] were recovered from a reverse micellar phase with their original enzymatic activity.

One limitation of the conventional reverse micellar extraction method is the back extraction step, which is slow and has poor yields. Due to this limitation, reverse micellar extraction has not been considered as an alternative to the existing industrial purification processes such as

MARCEL DEKKER, INC. • 270 MADISON AVENUE • NEW YORK, NY 10016

chromatography and membrane technology. Thus, the aim of this study was to compare two methods of protein recovery from a reverse micellar phase: back extraction and solvent precipitation. Two reverse micellar systems were used: one formed with the anionic surfactant, sodium di(2-ethylhexyl) sulfosuccinate (AOT), and the other formed with the cationic surfactant, dioctyldimethyl ammonium chloride (DODMAC). Lysozyme was chosen to prepare a protein-loaded reverse micellar solution. To increase the recovery efficiency in the back extraction step, we attempted to break the reverse micellar structure by controlling the salt concentration or by adding decanol, ethanol, or ethyl acetate as cosolvents. As another alternative to the conventional back extraction method, we tested acetone and seven other polar organic solvents for solvent precipitation of the lysozyme. The results presented here provide new information towards improving protein recovery in the reverse micellar extraction process.

MATERIALS AND METHODS

Materials

The commercial surfactant, BARDAC LF-80, containing 80 wt % DODMAC, 10 wt % ethanol, and 10 wt % water, was obtained from Lonza Inc. (Fair Lawn, NJ). This surfactant was used directly in the experiments, without any further purification, since the presence of ethanol had a negligible effect on the extraction process.[27] Sodium di(2-ethylhexyl) sulfosuccinate (AOT), lysozyme (chicken egg white, pI 11.0, 14.3 kDa) and *Micrococcus lysodeikticus* were obtained from Sigma (Oakville, Canada). The lysozyme obtained from the supplier contained 5 to 10 wt % sodium acetate/sodium chloride/acetic acid buffer. The surfactant AOT, received with 99% purity, was used without further purification. The phosphate buffer (pH 6.2) was purchased from Anachemia (Oakville, Canada). All of the other chemicals were obtained from Fisher Scientific (Montreal, Canada).

Loading of Lysozyme into the Reverse Micellar Phase

Initial lysozyme solutions containing from 0.1 to 1 g/L lysozyme were contacted with an equal volume (10 mL) of an organic phase containing either 30 mM AOT or 100 mM DODMAC. Isooctane was used as the organic solvent, and the DODMAC reverse micellar phase contained 250 mM decanol as a cosurfactant. For the AOT reverse micellar phase, no pH adjustment was

made to the aqueous phase, since the pH of the lysozyme solution was between 4.5 and 5.5. The low pH of the lysozyme solution is due to the presence of the acid in the bulk lysozyme powder. At this pH range, the extraction of lysozyme into the AOT reverse micellar phase is 100%.[18,19] For the DODMAC reverse micellar extraction, the pH of the initial lysozyme solution was adjusted to 12.0 using 1 N NaOH. Complete extraction of lysozyme into the DODMAC reverse micellar system was obtained at this pH.[16] The salt concentration in the initial lysozyme solution was set at 0.1 M NaCl. The lysozyme-containing aqueous phase and the reverse micellar solutions were vortexed for 5 seconds, and left for phase separation at room temperature (approximately 21–23 °C) for 12 hours.

Recovery of Lysozyme from the Reverse Micellar Phase

For the back extraction method, 5 mL of a reverse micellar phase containing lysozyme were contacted with an equal volume of a fresh aqueous solution with fixed pH and salt concentration. The pH of the aqueous solution was fixed using 1 N HCl or NaOH solutions. The change in pH after the aqueous phase was contacted with the reverse micellar phase was less than 0.2 pH units. The salt concentration was set using NaCl, and no other buffer salts were added to the aqueous solution. The mixture was vortexed for 5 seconds and left for phase separation for 12 hours at room temperature. The use of cosolvent, claimed to induce a size exclusion of protein[2,21] in the reverse micellar phase, was attempted by adding ethanol or ethyl acetate to yield 400 mM cosolvent in the reverse micellar phases.

For the solvent precipitation method, eight polar organic solvents were tested: acetone, methyl acetate, methyl ethyl ketone, 30% aqueous formaldehyde, acetonitrile, methanol, isopropanol, and pentanol. Five mL of the lysozyme-containing reverse micellar phase were contacted with 5–20 mL of polar organic solvent. The mixture was vortexed for 5 seconds at room temperature, and left for lysozyme precipitation for 5 minutes. The precipitated lysozyme was collected by centrifugation and washed twice with the polar solvent to remove any surfactant residue.

Analytical Techniques

The concentrations of lysozyme and of surfactant in the aqueous phase were measured using an Agilent High Performance Liquid Chromatograph (HPLC)-1100. The mobile phase was prepared using HPLC grade water and

MARCEL DEKKER, INC. • 270 MADISON AVENUE • NEW YORK, NY 10016

acetonitrile. Solvent A contained 5% acetonitrile and 0.1% triflouric acid in water, and solvent B contained 5% water and 0.085% triflouric acid in acetonitrile. The pH of the solvent A was 1.9. The initial composition of the mobile phase was 90% solvent A and 10% solvent B. The solvent gradient was 40% solvent A and 60% solvent B in 20 minutes. All samples were filtered before injection. The flow rate through the column was 1.0 mL/min, and the temperature of the column was 25 °C. The sample volume was 5 μL, and the wavelength of the detector was 210 nm. The enzyme activity of lysozyme was measured following Davis et al.[28] A substrate solution of 0.3 g/L *Micrococcus lysodeikticus* was prepared in 50 mM phosphate buffer solution set at pH 6.2. The temperature of the UV cells was 25 ± 0.1 °C, and the wavelength was 450 nm. A substrate solution volume of 3.0 mL was pipetted into both the reference and the sample cuvettes, which were held in the spectrophotometer for about 2 minutes to allow them to reach the set temperature. The instrument was zeroed, and 100 μL of phosphate buffer solution was pipetted into the reference cuvette. Then, 100 μL of lysozyme solution was added to the sample cuvette, and the decrease in the turbidity of the substrate solution was monitored as a function of time for 3 minutes. The initial activity of lysozyme was measured before contact with the reverse micellar phase. The lysozyme recovered from the reverse micellar phase was measured and compared with the initial values.

All experiments were carried out using two to six replicates. The results presented here are the average values and the sample standard deviations.

RESULTS AND DISCUSSION

Back Extraction from an AOT Reverse Micellar Phase

A reverse micellar solution, containing 30 mM AOT and a measured lysozyme concentration of 1.0 ± 0.02 g/L, was contacted with an equal volume of a receiving aqueous phase, with pH adjusted to 9.8, 12, or 13.5 ± 0.1 using NaOH. The salt concentration in the receiving aqueous phase was 0.1 or 0.3 M NaCl. The change in pH, after the contact with a reverse micellar phase and phase separation, was less than 0.2 pH units. Figure 1 presents the percent of lysozyme remaining in the reverse micellar phase and the percent of lysozyme solubilized into the receiving aqueous phase, with three replicates at each condition, as a function of pH. When the pH of the receiving aqueous solution was 9.8, about 1.2 units lower than the pI of lysozyme, over 90% of the lysozyme remained in the reverse micellar phase both at 0.1 and 0.3 M NaCl. An increase in the pH to 12.0 showed little change in the percentage of

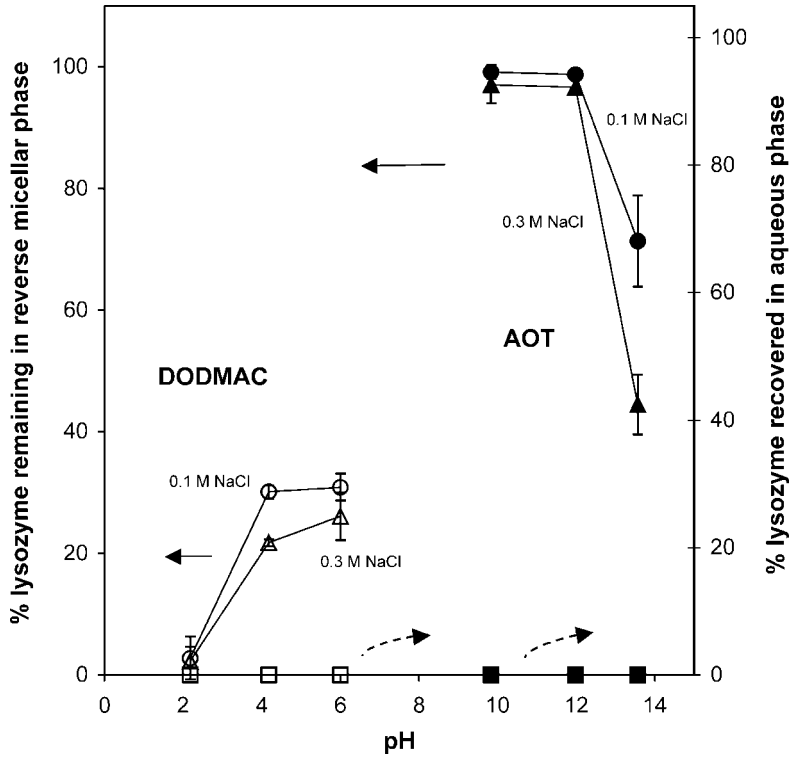

Figure 1. Percent of lysozyme in the reverse micellar phase and in the receiving aqueous phase after back extraction at room temperature. Initial reverse micellar phase: 100 mM DODMAC (open symbols) or 30 mM AOT (filled symbols) containing 1 g/L lysozyme.

lysozyme remaining in the reverse micellar phase. When the pH was 13.6, the percent lysozyme remaining in the reverse micellar phase decreased to 71% at 0.1 M NaCl and 44% at 0.3 M NaCl. These results confirm the electrostatic repulsion between the negatively charged lysozyme and the AOT surfactant head group,[18,29] and also the salting out effect.[17,30] The increase in the salt concentration results in a "dewatering effect"[31] of the reverse micellar phase, and thus, the proteins are removed from the reverse micelles due to size exclusion.[2,21,32]

However, the concentration of lysozyme in the receiving aqueous phase in all cases was below the detection limit of the HPLC, indicating that no lysozyme was solubilized into the receiving aqueous phase;

instead, the lysozyme formed a white precipitate at the aqueous–organic interface. As the pH and the salt concentration increased, an increasing amount of lysozyme was removed from the reverse micellar phase. This lysozyme was not solubilized into the receiving aqueous phase, but it precipitated at the aqueous–organic interface. This phenomenon suggests that the lysozyme removed from the reverse micellar phase is an insoluble complex with the surfactant. The pH and the ionic strength adjustments were not sufficient to separate the AOT from the lysozyme. In the literature, about 77% recovery of lysozyme was obtained from an AOT reverse micellar phase using a fresh aqueous phase at 2 M KCl and pH 11 and mixing time of 30 minutes.[33] Similar results were reported when the mixing time of 45 minutes was used.[30] However, lysozyme becomes inactive at salt concentrations higher than 0.16 M,[28] and the use of such high ionic strength in the aqueous phase should be avoided. The results shown in Fig. 1, compared with the literature data, confirm that the back extraction of lysozyme from an AOT reverse micellar phase requires high salt concentration and extensive mixing, which cannot be applied to most proteins with less conformational stability than lysozyme. No results of recovery of lysozyme from a DODMAC reverse micellar phase have been previously reported.

In an attempt to increase the recovery of lysozyme into the receiving aqueous phase, ethanol or ethyl acetate were added to the reverse micellar phase as a cosolvent. Having 400 mM ethanol in the 30 mM AOT reverse micellar phase, while the receiving aqueous phase was set at pH 12.0 and 0.1 M NaCl, only 3.2 ± 0.2% of the lysozyme remained in the reverse micellar phase. However, the concentration of lysozyme in the aqueous phase was below the HPLC detection limit. Although the percent of lysozyme remaining in the reverse micellar phase decreased from 99% to 3.2% with the addition of ethanol, no lysozyme was recovered in the aqueous phase. The behavior was similar for ethyl acetate.

An attempt was also made to break the reverse micelles by reducing the salt concentration below 0.05 M NaCl in the receiving aqueous phase. At this salt concentration, no reverse micelles are formed in the organic phase.[34] The pH of the receiving aqueous solution was 13.6. Upon contact, both lysozyme and AOT were solubilized in the aqueous phase, which became milky. The salt concentration of the aqueous phase was then increased to induce the formation of reverse micelles. If the electrostatic repulsion theory were correct, the lysozyme should remain in the aqueous phase while AOT should migrate into the organic phase, forming reverse micelles. When the salt concentration was increased to 0.3 M NaCl, the aqueous and organic phases

became clear, but a white precipitate formed at the aqueous–organic interface, and the lysozyme concentration in both the aqueous and the organic phases was below the detection limit.

The formation of precipitate at the interface is due to the hydrophobicity of the protein-surfactant complex.[10,13,19,35] Upon mixing of a protein containing aqueous phase and a surfactant containing organic phase, the charged surfactant head groups form an ion-paired complex with the oppositely charged protein. This protein–surfactant complex requires additional surfactant molecules to wrap around the protein and yield a hydrophobic surface that solubilizes the protein into the organic phase.[35] In contrast, the protein has to be dissociated from the surfactant to be solubilized into an aqueous phase during the back extraction process. The formation of a protein-surfactant precipitate was found to depend largely on interfacial tension[10,12] and mass transfer kinetics.[35] The effect of interfacial tension on the extraction of lysozyme was reported using an AOT reverse micellar phase formed in different organic solvents[36] and using an aqueous two-phase micellar system.[37] However, the fact that the addition of cosolvent or that the breakage of the reverse micellar structure did not improve the lysozyme recovery, as reported in this study, indicates that the conventional back extraction method is not able to dissociate lysozyme from the surfactant AOT, and thus lysozyme is insoluble in the receiving aqueous phase.

Back Extraction from a DODMAC Reverse Micellar Phase

A 100 mM DODMAC reverse micellar phase containing 250 mM decanol and 1 g/L lysozyme was contacted with an equal volume of a fresh aqueous phase with pH adjusted to 2.2, 4.2, or 6.0 using HCl. The salt concentration in the receiving aqueous phase was at 0.1 or 0.3 M NaCl. Similar to the results obtained from the AOT reverse micellar phase, Fig. 1 shows that there was no recovery of lysozyme from a DODMAC reverse micellar phase into the fresh aqueous phase. When the pH of the aqueous solution was 2.2, at 0.1 or 0.3 M NaCl, only 2% of the lysozyme remained in the reverse micellar phase. At this pH, lysozyme has an overall positive surface charge resulting in an electrostatic repulsion between lysozyme and the positively charged DODMAC surfactant head group. At a pH of 4.2, more lysozyme remained in the reverse micellar phase. When the pH was 6.0, the lysozyme remaining in the reverse micellar phase at 0.1 M and 0.3 M NaCl was about 30 and 26%, respectively. This result supports the dewatering hypothesis. However, the concentration of lysozyme in the receiving aqueous phase was below the detection limit. As more lysozyme was removed from the reverse micellar

phase, an increasing amount of white precipitate was observed at the aqueous–organic interface. Ethanol or ethyl acetate was added to the DODMAC reverse micellar phase, in order to increase the percent recovery. However, similar to the AOT reverse micellar system, although the percent of lysozyme remaining in the reverse micellar phase decreased upon the addition of ethanol, lysozyme was not solubilized in the fresh aqueous phase.

Unlike AOT, DODMAC requires a cosurfactant such as decanol, to form a reverse micellar phase. A previous study using DODMAC reported that the molar ratio of 4 between decanol and DODMAC resulted in a significant decrease in the solubilization of lysozyme in the DODMAC reverse micellar phase[38]. Based on this finding, upon the contact with a fresh aqueous phase with pH 6.0 and 0.1 M NaCl, decanol was added to the 100 mM DODMAC reverse micellar phase to increase its concentration from 250 mM to 400 mM. Again, the concentration of lysozyme in the receiving aqueous phase was below the detection limit, and a white precipitate was observed at the aqueous–organic interface.

For both the AOT and the DODMAC reverse micellar systems, the manipulation of pH and the salt concentration in the receiving aqueous phase or the addition of cosolvent to the reverse micellar phase did not produce solubilization of lysozyme into a fresh aqueous phase. The fact that no lysozyme was recovered using the conventional back extraction method suggests that the lysozyme removed from either reverse micellar phase is in the form of a water–insoluble lysozyme-surfactant complex.

Solvent Precipitation from an AOT Reverse Micellar Phase

Five mL of acetone were added to an equal volume of a 30 mM AOT reverse micellar phase containing 1 g/L lysozyme. The mixture was stirred for 5 seconds at room temperature. Upon the addition of acetone, lysozyme precipitated as a white solid. In the acetone phase, the lysozyme concentration was below the detection limit, and the water content was measured to be less than 0.5 mass %. The precipitated lysozyme was then dissolved in three different aqueous phases: 25 mM phosphate buffer solution[26], an aqueous NaOH solution at pH = 11, the pI of lysozyme[1], and distilled water. Figure 2 presents the percent recovery of lysozyme in the final aqueous phases vs. the ratio of the volume of polar organic solvent, V_{ps}, to the volume of initial reverse micellar phase, V_o. Using a fixed micellar phase volume of 5 mL, a volume ratio below unity was insufficient to induce precipitation of lysozyme; instead, the acetone and the organic phase containing AOT formed two liquid phases, a milky bottom phase and a clear top phase. The highest recovery of

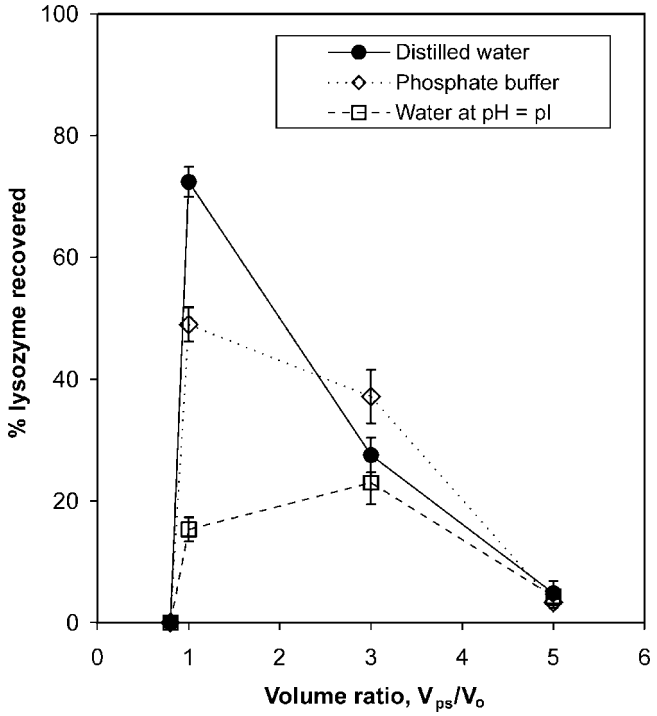

Figure 2. Effect of ratio of volume of acetone (V_{ps}) to volume of lysozyme-containing reverse micellar phase (V_o) on the percent lysozyme recovered. Initial reverse micellar phase: 30 mM AOT containing 1 g/L lysozyme.

70% was obtained with distilled water at a volume ratio of unity. At most, 50% of the lysozyme was recovered in a 25 mM phosphate buffer solution, while an aqueous phase at a pH of 11 recovered 20% of the lysozyme. Equal volumes of the polar organic solvent and reverse micellar phase were used in subsequent experiments.

Table 1 summarizes the effect of different polar solvents on the lysozyme recovery. All solvents tested provided complete removal of lysozyme from the reverse micellar phase. The reverse micellar phase formed one clear phase with acetone, methyl acetate, and methyl ethyl ketone, and the lysozyme precipitated at the bottom of the test tube. The use of formaldehyde and acetonitrile gave two clear liquid phases, and a white precipitate formed at the solvent-organic interface. For the alcohols, methanol gave two clear liquid phases and a white precipitate formed at the solvent-organic interface, while

Table 1. Effect of polar solvents on the percent lysozyme precipitated and subsequently recovered: initial reverse micellar phase, 30 mM AOT with 1 g/L lysozyme; $V_{ps}/V_o = 1$; final aqueous phase, distilled water.

Polar solvents	% Lysozyme precipitated	% Lysozyme recovered	Activity unit/mg protein
Acetone	100%	72 ± 2%	48,000 ± 3,000
Methyl acetate	100%	3 ± 1%	18,000 ± 3,000
Methyl ethyl ketone	100%	0%	–
Formaldehyde	100%[a]	0%	–
Acetonitrile	100%[a]	0%	–
Methanol	100%[a]	0%	–
Isopropanol	100%	18 ± 1%	35,000 ± 5,000
Pentanol	0%	0%	–

[a] The lysozyme precipitated at the solvent-reverse micellar phase interface.

isopropanol and pentanol formed homogeneous clear solutions, and the lysozyme precipitated at the bottom of the test tubes. The largest recovery was 72% using acetone as the polar solvent and distilled water as the final aqueous phase. An increase in the hydrophobicity of the solvent from acetone to methyl acetate and methyl ethyl acetate significantly reduced the recovery of lysozyme. The precipitate recovered using formaldehyde and acetonitrile was insoluble in water at any pH from 1 to 14, as well as in isooctane. For the alcohols, the precipitate obtained from methanol was water insoluble. The use of isopropanol gave a lysozyme recovery of 18%. No precipitation was found when pentanol was used, and the concentration of lysozyme in the final aqueous phase was below the detection limit. Before it was solubilized into the AOT reverse micellar phase, the lysozyme activity was $48,000 \pm 3000$ units/mg protein. As shown in Table 1, the lysozyme precipitated by acetone retained its original activity. According to Luisi et al.[39], lysozyme was found to be denatured in AOT reverse micelles. However, when recovered from the reverse micelles by solvent precipitation with acetone, lysozyme recovered its original activity. When precipitated by methyl acetate, less than 40% of the original activity remained. The use of isopropanol resulted in a lysozyme activity of 35,000 units/mg, about 75% of its original value. The AOT concentration in the aqueous phase was below the HPLC detection limit for all cases. Since the lysozyme recovered using acetone showed the same activity as the initial lysozyme sample, it was concluded that the recovered lysozyme was free of surfactant. Successful recovery of γ-glutamyltransferase[25] and ribonuclease-A[26] from an AOT reverse micellar phase was reported using

Table 2. Comparison of the effect of washing with acetone on the percent recovery of lysozyme dissolved in distilled water.

Polar solvents	Without acetone wash	With acetone wash
Methyl acetate	3 ± 1%	55 ± 5%
Methyl ethyl ketone	0%	24 ± 5%
Isopropanol	18 ± 1%	65 ± 5%

acetone. The decreased activity of the lysozyme recovered by methyl acetate or isopropanol may be due to the AOT being associated with the protein and thus not appearing in the HPLC analysis. To test this possibility, the precipitate obtained with these solvents was washed in acetone several times, dried, and then solubilized in distilled water. Table 2 gives the percent of lysozyme recovered after being washed with acetone and the values obtained without acetone washing. The percent recovery increased significantly after washing with acetone. Acetone dissociated the surfactant–lysozyme complex precipitated with the other polar solvents, and produced a surfactant-free lysozyme with its original activity.

Solvent Precipitation from a DODMAC Reverse Micellar Phase

Five mL of a 100 mM DODMAC reverse micellar phase containing 1 g/L lysozyme were contacted with an equal volume of acetone. The mixture formed a clear liquid phase, and lysozyme precipitated as a white solid. Unlike the AOT reverse micellar system, the lysozyme recovered with acetone from a DODMAC reverse micellar phase was not soluble in distilled water, i.e., the concentration of lysozyme was below the HPLC detection limit. To investigate further, an aqueous solution containing 1 g/L lysozyme and 0.1 M NaCl was prepared with a pH of 12, the same condition used for the extraction of lysozyme into the DODMAC reverse micellar phase. This solution was contacted with an equal volume of acetone. No DODMAC, decanol, or isooctane, was present in this experiment. The lysozyme, which precipitated from the aqueous phase, was not soluble in water. In contrast, when no NaOH was added to the initial lysozyme solution, i.e., pH $\cong$ 5, the recovery of lysozyme into water after precipitation with acetone was 75 ± 10%. In this case, the denaturing of lysozyme is due to the extreme pH required for a DODMAC reverse micellar system to extract lysozyme.

CONCLUSIONS

The recovery of lysozyme from the reverse micellar phases created with an anionic surfactant (AOT) and with a cationic surfactant (DODMAC) was studied using back extraction and solvent precipitation. Back extraction using a fresh aqueous phase with pH and salt concentration adjustment reduced the lysozyme concentration in the reverse micellar phase, but no lysozyme was recovered into the aqueous phase, from either the AOT or the DODMAC reverse micellar phase. Solvent precipitation with acetone recovered lysozyme from the AOT reverse micellar phase with its original activity. Active lysozyme could not be recovered from the DOMAC reverse micellar phase, because the high pH needed to extract lysozyme into the reverse micellar phase denatured the protein.

ACKNOWLEDGMENT

The authors are grateful to the Natural Sciences and Engineering Research Council of Canada for financial support.

REFERENCES

1. Sadana, A. *Bioseparation of Proteins: Unfolding/Folding and Validation*; Ahuja, S., Ed.; Academic Press: New York, 1998.
2. Aires-Barros, M.R.; Cabral, J.M.S. Selective separation and purification of two lipases from *Chromobacterium viscosum using* AOT reversed micelles. Biotechnol. Bioeng. **1991**, *38*, 1302–1307.
3. Arcos, J.A.; Robledo, L.; Otero, C. Stability of a *Pseudomonas sp.* lipase: Comparison between solubilized enzyme in reverse micelles and suspended lipase in dry solvents. Biocatal. Biotrans. **1997**, *14*, 251–267.
4. Chiang, C.L. Activity and stability of lipase in Aerosol-OT/isooctane reverse micelles. Biotechnol. Tech. **1999**, *13*, 453–457.
5. Fletcher, P.D.I.; Robinson, B.H.; Freedman, R.B.; Oldfield, C. Activity of lipase in water-in-oil microemulsions. J. Chem. Soc., Faraday Trans. **1985**, *81* (1), 2667–2679.
6. Dekker, M.; Van't Riet, K.; Weijers, S.R.; Baltussen, J.W.A.; Laane, C.; Bijsterbosch, B.H. Enzyme recovery by liquid-liquid extraction using reversed micelles. Chem. Eng. J. **1986**, *33*, B27–B33.
7. Krei, G.A.; Hustedt, H. Extraction of enzymes by reverse micelles. Chem. Eng. Sci. **1992**, *47* (1), 99–111.

8. Lazarova, Z.; Tonova, K. Integrated reversed micellar extraction and stripping of α-amylase. Biotechnol. Bioeng. **1999**, *63* (5), 583–592.
9. Shah, C.; Sellappan, S.; Madamwar, D. Role of environment on the activity and stability of α-amylase incorporated in reverse micelles. Appl. Biochem. Biotechnol. **1997**, *62*, 183–189.
10. Adachi, M.; Harada, M. Solubilization mechanism of cytochrome c in sodium bis(2-ethylhexyl) sulfosuccinate water/oil microemulsion. J. Phys. Chem. **1993**, *97*, 3631–3640.
11. Cassin, G.; Illy, S.; Pileni, M.P. Chemically modified proteins solubilized in AOT reverse micelles: Influence of proteins charges on intermicellar interactions. Chem. Phys. Lett. **1994**, *221*, 205–212.
12. Dungan, S.R.; Bausch, T.; Hatton, T.A.; Plucinski, P.; Nitsch, W. Interfacial transport processes in the reversed micellar extraction of proteins. J. Colloid Interface Sci. **1991**, *145* (1), 335–350.
13. Imai, M.; Natsume, T.; Naoe, K.; Shimizu, M.; Ichikawa, S.; Furusaki, S. Hydrophilic surroundings requisite for the solubilization of proteins related with their hydrophobicity in the AOT reversed micellar extraction. Bioseparation **1997**, *6*, 325–333.
14. Jarudilokkul, S.; Poppenborg, L.H.; Stuckey, D.C. Backward extraction of reverse micellar encapsulated proteins using a counterionic surfactant. Biotechnol. Bioeng. **1999**, *62* (5), 593–601.
15. Lye, G.J.; Asenjo, J.A.; Pyle, D.L. Extraction of lysozyme and ribonuclease-a using reverse micelles: Limits to protein solubilization. Biotechnol. Bioeng. **1995**, *47*, 509–519.
16. Rabie, H.R.; Suyyagh, T.; Vera, J.H. Reverse micellar extraction of proteins using dioctyldimethylammonium chloride. Sep. Sci. Technol. **1998**, *33* (2), 241–257.
17. Sun, Y.; Ichikawa, S.; Sugiura, S.; Furusaki, S. Affinity extraction of proteins with a reversed micellar system composed of cibacron blue-modified lecithin. Biotechnol. Bioeng. **1998**, *58* (1), 58–64.
18. Göklen, K.E.; Hatton, T.A. Liquid-liquid extraction of low molecular-weight proteins by selective solubilization in reversed micelles. Sep. Sci. Technol. **1987**, *22* (2-3), 831–841.
19. Lye, G.J.; Asenjo, J.A.; Pyle, D.L. Protein extraction using reverse micelles: Kinetics of protein partitioning. Chem. Eng. Sci. **1994**, *49* (19), 3195–3204.
20. Rahaman, R.S.; Chee, J.Y.; Cabral, J.M.S.; Hatton, T.A. Recovery of an extracellular alkaline protease from whole fermentation broth using reversed micelles. Biotechnol. Prog. **1988**, *4* (4), 218–224.
21. Chen, J.P.; Jen, J.T. Extraction of concanavalin A with affinity reversed micellar systems. Sep. Sci. Technol. **1994**, *29* (9), 1115–1132.

22. Hagen, A.J.; Hatton, T.A.; Wang, D.I.C. Protein refolding in reversed micelles. Biotechnol. Bioeng. **1990**, *35*, 955–965.
23. Jarudilokkul, S.; Stuckey, D.C. Continuous forward and back extraction of lysozyme from egg white using reverse micelles. Sep. Sci. Technol. **2001**, *36* (4), 657–669.
24. Fox, S.W.; Foster, J.F. *Introduction to Protein Chemistry*; John Wiley & Sons: New York, 1957; 250–252.
25. Kabanov, A.V.; Nametkin, S.N.; Evtushenko, G.N.; Chernov, N.N.; Klyachko, N.L.; Levashov, A.V.; Martinek, K. A new strategy for the study of oligomeric enzymes: γ-glutamyltransferase in reversed micelles of surfactants in organic solvents. Biochim. Biophys. Acta **1989**, *996*, 147–152.
26. Goto, M.; Hashimoto, Y.; Fujita, T.; Ono, T.; Furusaki, S. Important parameters affecting efficiency of protein refolding by reversed micelles. Biotechnol. Prog. **2000**, *16*, 1079–1085.
27. Rabie, H.R.; Weber, M.E.; Vera, J.H. Effects of surfactant purity and concentration, of surfactant counterion, and of different ions on the water uptake of dioctyldimethylammonium salt-decanol-isooctane reverse micellar systems. J. Colloid Interface Sci. **1995**, *174*, 1–9.
28. Davies, R.C.; Neuberger, A.; Wilson, B.M. The dependence of lysozyme activity on pH and ionic strength. Biochim. Biophys. Acta **1969**, *178*, 294–305.
29. Choe, J.; VanderNoot, V.A.; Linhardt, R.J.; Dordick, J.S. Resolution of glycoproteins by affinity–based reversed micellar extraction and separation. AIChE J. **1998**, *44* (11), 2542–2548.
30. Chou, S.-T.; Chiang, B.-H. Reversed micellar extraction of hen egg lysozyme. J. Food Sci. **1998**, *63* (3), 399–402.
31. Paradkar, V.M.; Dordick, J.S. Affinity-based reverse micellar extraction and separation (ARMES): A facile technique for the purification of peroxidase from soybean hulls. Biotechnol. Prog. **1993**, *9*, 199–203.
32. Kelly, B.D.; Wang, D.I.C.; Hatton, T.A. Affinity-based reversed micellar protein extraction: I. Principles and protein-ligand systems. Biotechnol. Bioeng. **1993**, *42*, 1199–1208.
33. Jarudilokkul, S.; Poppenborg, L.H.; Stuckey, D.C. Selective reverse micellar extraction of three proteins from filtered fermentation broth using response surface methodology. Sep. Sci. Technol. **2000**, *35* (4), 503–517.
34. Rabie, H.R.; Helou, D.; Weber, M.E.; Vera, J.H. Comparison of the titration and contact methods for the water solubilization capacity of AOT reverse micelles in the presence of a cosurfactant. J. Colloid Interface Sci. **1997**, *189*, 208–215.

35. Paradkar, V.M.; Dordick, J.S. Mechanism of extraction of chymotrypsin into isooctane at very low concentrations of Aerosol OT in the absence of reverse micelles. Biotechnol. Bioeng. **1994**, *43*, 529–540.
36. Tong, J.; Furusaki, S. Extraction behavior of proteins using AOT reversed micellar systems formed by kerosene and silicone oil. Sep. Sci. Technol. **1998**, *33* (6), 899–907.
37. Kamei, D.T.; Wang, D.I.C.; Blankschtein, D. Fundamental investigation of protein partitioning in two-phase aqueous mixed (nonionic/ionic) micellar systems. Langmuir **2002**, *18*, 3047–3057.
38. Shin, Y.-O.; Vera, J.H. Solubilization limit of lysozyme into DODMAC reverse micelles. Biotechnol. Bioeng. **2002**, *80* (5), 537–543.
39. Luisi, P.L.; Giomini, M.; Pileni, M.P.; Robinson, B.H. Reverse micelles as hosts for proteins and small molecules. Biochim. Biophys. Acta **1988**, *947*, 209–246.

Received June 2002
Revised December 2002