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Separation of α-Amylase by Reversed Micellar Extraction

Effect of Solvent Type and Cosolvent Concentration on the Transfer Process

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ABSTRACT

The recovery of α-amylase from the crude enzyme preparation by the reversed micellar liquid-liquid extraction was investigated. The reversed micellar solution was formed by dissolving a cationic surfactant Aliquat 336 in six different alkanes (cyclohexane, n-hexane, isooctane, n-octane, n-decane, and n-dodecane) respectively with addition of a cosolvent n-octanol. It was found that a minimal quantity of n-octanol was needed for Aliquat 336 to dissolve in apolar solvent and form reversed micelles. Furthermore, this minimal amount of n-octanol needed was found to be different when Aliquat 336 was dissolved in different alkanes. It tended to increase with the number of carbon atoms in alkane and also depended on the solvent structure. During the forward extraction process, it was revealed that a high value of solubilization of protein in Aliquat 336 reversed micelles could be achieved when four out of the six alkanes (cyclohexane, n-hexane, isooctane, n-octane) were used as the solvent for Aliquat 336. After a full forward and backward extraction cycle, however, a high recovery of both the protein mass and α-amylase activity in the stripping solution could be obtained only when two out of the six alkanes (n-hexane and isooctane) were used as the solvent for Aliquat 336. When n-hexane and isooctane were used as the solvent for Aliquat 336, up to 80% of the total α-amylase activity in the crude enzyme preparation could be recovered at the end of extraction cycle, meanwhile α-amylase could be concentrated about 1.4-fold. In the cases of other four alkanes (cyclohexane, n-

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octane, n-decane, and n-dodecane) as solvent, most of the α-amylase activity in the crude enzyme preparation would be denatured after an extraction cycle.

Index Entries: Reversed micelles; solvent; cosolvent; separation; α-amylase.

INTRODUCTION

With the rapid progress in biotechnology, more attention has been paid to the development of efficient methods for separation and purification of proteins and other bioproducts from fermentation broth and cell culture media. Liquid–liquid extraction by using reversed micellar solution as the extractant might serve this purpose *(1,2)*. In reversed micelles, the proteins are solubilized inside the polar core of the surfactant aggregate and prevented from contacting with the hostile organic solvent *(3,4)*.

During the liquid-liquid reversed micellar extraction process, the transfer of protein is controlled by various parameters of both phases, such as pH, ionic strength of the aqueous phase, type and concentration of surfactant, solvent, and cosolvent of the reversed micellar solution. In recent years, extensive research has been concentrated on the parameters in the aqueous phase *(5)*, but no systematic investigation has been carried out on the effect of the solvent and cosolvent type on protein partitioning and enzyme activity changing during the extraction process. It has been reported that the solvent has significant effect on the aggregate behavior of the surfactant, its structure and properties such as the size and shape of the reversed micelles, and the solubilization capacity of the system *(6)*. In order to obtain better understanding and further develop a practical solvent selection criterion, it is then necessary to examine the effect of solvent type on the transfer of protein in the reversed micellar system.

Cosolvent is a kind of solvent which can help surfactant to dissolve in the solvent and form reversed micelles if possible. A cationic surfactant, especially the quaternary ammonium salt, often needs a cosolvent to aid it in forming a reversed micellar solution in the apolar solvent. The study of extraction of protein using this kind of reversed micelles has been reported by some researchers, such as continuous extraction of α-amylase by reversed micelles with trioctylmethylammonium chloride (TOMAC) as surfactant, octanol as cosolvent and isooctane as solvent *(7)*; the transfer of protein by reversed micelles of cetyltrimethylammonium bromide (CTAB) in isooctane with addition of a cosolvent *n*-hexanol *(8)*; the solubilization of α-chymotrypsin by reversed micelles of a mixture of quaternary ammonium salt Aliquat 336 in isooctane with addition of isotridecanol as the cosolvent *(9)* and so on. Despite these works, the effect of cosolvent on extraction process has never been systematically described.

In this paper, by using the reversed micelles of Aliquat 336 in different solvent (with addition of *n*-octanol as the cosolvent) to extract α-amy-

lase from the crude enzyme preparation, we will investigate the effect of solvent type and cosolvent concentration on the recovery of α-amylase activity. The purpose of this study is to develop a practical selection criterion for solvent and cosolvent in the reversed micellar extraction processes.

MATERIALS AND METHODS

Chemicals

Industrial grade α-amylase from *Bacillus subtilis*, containing α-amylase (640 U/mg, pI = 5.4) with a total protein of 0.17 mg/mg (crude enzyme), was obtained from Wuxi Enzyme Reagent Factory, Wuxi, Jiangshu Province, China. Aliquat 336, a kind of trialkylmethyl ammonium chloride with the number of carbon atoms from 8 to 11 in alkyl groups, was purchased from Fluka. Cyclohexane, *n*-hexane, *n*-octane, *n*-decane, *n*-dodecane, isooctane and *n*-octanol were supplied by Beijing Chemical Reagent Plant (reagent grade). All other chemicals were purchased from a local market and were of analytic grade. Experiments were performed at room temperature.

Forward and Backward Extraction

Aliquat 336 can form reversed micelles in apolar solvent when a cosolvent (or cosurfactant) is added *(9)*. In our experiments, *n*-octanol was chosen as the cosolvent, and the reversed micellar solution was formed by dissolving 50m*M* Aliquat 336 in six alkanes (cyclohexane, *n*-hexane, *n*-octane, isooctane, *n*-decane, *n*-dodecane) respectively with addition of *n*-octanol. The aqueous phase was 30 m*M* citric acid-dibasic potassium phosphate buffered solution for pH 7–9 and borax-sodium hydroxide buffered solution for pH 9–11. The crude α-amylase preparation was dissolved in buffered solution to form the initial aqueous phase.

Experiments were carried out in tightly-stoppered 50mL-glass flasks. In the forward extraction, equal volumes (usually 5 mL) of reversed micellar solution and aqueous phase were mixed at 250 rpm for 1 min. Then, the mixtures were centrifuged at 3500 rpm for 5 min to separate the two phases. In the backward extraction, the enzyme-loaded reversed micellar solution from the forward extraction was mixed with equal volume of the aqueous stripping solution at 250 rpm for 3 min. Then, the mixtures were centrifuged at 3500 rpm for 5 min to separate the two phases. The protein content and enzyme activity were assayed for each phase.

Analytical Methods

The protein concentration in the aqueous and reversed micellar solution was determined by measuring the absorbance at 280 nm on a model 751G UV/Vis spectrophotometer. The method of Lowry *(10)* was used to

confirm the protein content in the initial aqueous phase. The α-amylase activity was determined by using soluble starch as the substrate. One unit was defined as the amount of enzyme that liberated 1 mg of maltose from starch at 37°C in 1 min at pH 6.0. The purification factor was calculated as the ratio of the specific activity in the stripping solution to that in the initial aqueous solution. The recovery percentages of enzyme activity and protein mass were evaluated by the ratio of the enzyme activity or protein concentration in the stripping solution to those in the initial aqueous phase.

RESULTS AND DISCUSSION

Effect of Cosolvent

Firstly, we carried out the experiment to examine how Aliquat 336 can be dissolved in these six different kinds of alkane. In the experiment, it was found the a minimal quantity of n-octanol was needed for Aliquat 336 to dissolve in these alkanes and build up a reversed micellar system. Moreover, this minimal quantity of *n*-octanol was found to be different for Aliquat 336 dissolving in different kinds of alkane, as listed in Table 1, column 3. From the data in Table 1, it is also shown that no *n*-octanol is needed for Aliquat 336 to dissolve in the alkanes with six carbon atoms (cyclohexane and *n*-hexane). For Aliquat 336 dissolving in alkanes with more than six carbon atoms, however, a certain amount of *n*-octanol is needed. This minimal amount of *n*-octanol seems to increase with the

Table 1
The Amount of *n*-octanol Required for Aliquat 336 Dissolving in Different Alkanes

Solvent	Number of carbon atoms	Minimal[a] amount (% v/v)	Secondary minimal[b] amount (% v/v)	Practical[c] amount (% v/v)
cyclohexane	6	0	0.16	0.32
n-hexane	6	0	0.32	0.4
isooctane	8	0.08	0.4	0.56
n-octane	8	0.24	1.04	1.22
n-decane	10	0.48	1.44	1.6
n-dodecane	12	0.96	1.09	1.12

[a]The minimal amount of *n*-octanol required for dissolving Aliquat 336 in the alkane (% v/v).

[b]The minimal amount of *n*-octanol required for Aliquat 336 reversed micellar system to obtain a clean boundary during the liquid-liquid extraction process (% v/v).

[c]The amount of *n*-octanol required for Aliquat 336 reversed micellar system which can be used for a practically liquid-liquid extraction process (% v/v).

increase of the carbon atom number in alkanes and the solvent structure also affects this minimal amount of cosolvent, such as the cases of Aliquat 336 in *n*-octane and isooctane.

The reason for above behavior may be related to the function of cosolvent. It has been proposed that cosolvent molecules are inserted between the molecules of surfactant *(9)*, thereby resulting in two important effects. First, the interaction between hydrophiles of the surfactant is changed. Second, the arrangement of surfactant molecules in the solvent is loosened and it will be possible to overcome the steric difficulty and arrange the big surfactant molecules in a looser manner *(11)*. The result of these two effects will be the collapse of the cohesive force of surfactant, which is followed by the dissolution of surfactant in solvent. But, if the interaction between surfactant and solvent is strong enough to overcome the cohesive force of surfactant, the surfactant will be able to dissolve in solvent without addition of cosolvent, just as the cases of Aliquat 336 dissolving in n-hexane or cyclohexane. When the carbon chain of alkane becomes long, the interaction between Aliquat 336 and solvent will become weak and not be able to destroy the close arrangement of Aliquat 336 molecules. Under such a case, a cosolvent will be needed to help Aliquat 336 dissolve in solvent. Moreover, the longer the carbon chain, the weaker the interaction will be and the more the amount of cosolvent will be required. Besides, the solvent structure can also influence this interaction and makes the amount of *n*-octanol required for Aliquat 336 to be different.

However, if only this minimal quantity of n-octanol as used for Aliquat 336 reversed micelles during the liquid–liquid extraction process, the mixed solutions would become turbid after the extraction and could not be separated into two clean phases by centrifugation. In order to obtain a clean boundary, therefore, a greater amount of *n*-octanol is required for Aliquat 336 reversed micelles during the extraction process. Such amount of *n*-octanol is small, larger than that minimal amount of *n*-octanol mentioned before and we call it the secondary minimal amount of *n*-octanol, as listed in Table 1, column 4. It is apparently dependent on the solvent structure and number of carbon atoms in alkane. For example, the secondary minimal amount of *n*-octanol increases with the increase of carbon atom number in alkane from C6 to C10. For C12, however, the secondary minimal amount of *n*-octanol is decreased. In the cases of cyclohexane and *n*-hexane, the secondary minimal amount of *n*-octanol is also different for each as are the cases of isooctane and *n*-octane. This phenomenon may arise from some more complicate mechanisms and needs to be examined in the future.

On the basis of above data, the amounts of *n*-octanol which will be practically used for Aliquat 336 reversed micellar extraction can be determined, as listed in Table 1, column 5. This amount of *n*-octanol was determined according to the secondary minimal amount of *n*-octanol (Table 1, column 4) but was a little higher than that. It can ensure the formation of

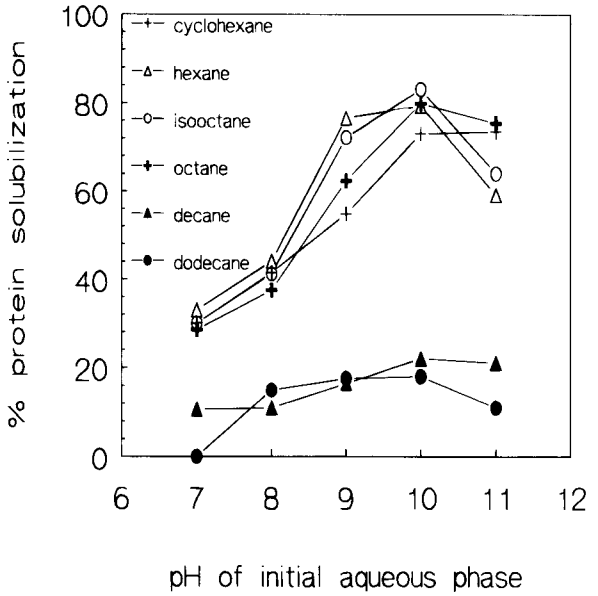

Fig. 1. Influence of initial aqueous pH on the solubilization of protein in Aliquat 336 reversed micelles. Organic phase: 50 m*M* Aliquat 336 reversed micelles. Aqueous phase: 30 m*M* buffered solution + 6.0 mg crude α-amylase/mL.

clean boundary between two phases under all extraction conditions. Without the special statement, all the following reversed micellar solution of Aliquat 336 in different alkanes will contain the amount of n-octanol listed in Table 1, column 5.

Effect of pH

Figure 1 shows the effect of initial aqueous pH on the solubilization of protein into Aliquat 336 reversed micelles during the forward extraction process. All curves in the figure have a similar shape with a maximal transfer efficiency around pH 10.0.

Aliquat 336 is a cationic surfactant and can make the interface of reversed micelles positively charged. Thus, solubilization of the protein in reversed micelles is favored at pH above the isoelectric point, pI, of protein *(11)*, such as the case of extraction of α—amylase (pI around 5.4) in this experiment. The maximal transfer efficiency around pH 10.0 may imply that not only the sign of protein charge but also the surface charge density is an important factor for protein solubilization in reversed micelles *(3,12)*. The denaturation of enzyme at high pH values may also be attributed to this phenomenon.

Notice also in Fig. 1 that the solvent type shows a specificity on the protein solubilization. For example, the solubilization of protein at pH 10.0 is around 80% in the cases of cyclohexane, *n*-hexane, *n*-octane and isooctane as the solvent. When using *n*-decane and *n*-dodecane as the solvent

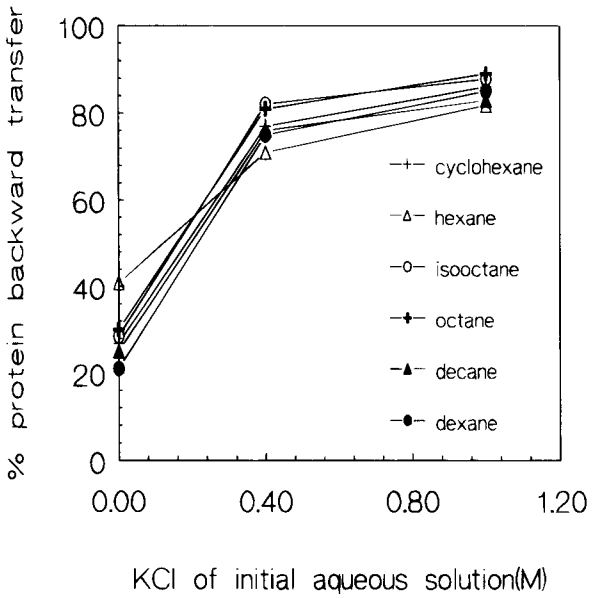

Fig. 2. Influence of KCl concentration in the stripping aqueous solution on the percentages of protein backward transfer. Forward extraction conditions—Organic phase: 50 m*M* Aliquat 336 reversed micelles. Aqueous phase: 30 m*M* buffer at pH 10 + 6.0 mg crude α-amylase/mL. Backward extraction conditions—Stripping aqueous phase: 30 m*M* buffered solution at pH6 + KCl.

for Aliquat 336, however, the maximal protein transfer at pH 10.0 during the forward extraction does not exceed 30%. This might be of importance for the solvent selection.

Protein Backward Transfer

The purpose of backward extraction is to recover protein from the reversed micellar solution *(13)*. Our experimental work is focused on the effect of KCl concentration in the stripping solution on the backward transfer of protein, as shown in Fig. 2. It indicates that the extent of protein backward transfer increases with the increase of KCl concentration. No obvious difference was observed among the six different cases, in which six alkanes were used as the solvent for Aliquat 336. This phenomenon implies that the volume exclusive effect which can act on the protein forward extraction *(3)*, can also be applied to the protein backward transfer process. It is believed that the high ionic strength in the stripping solution can compress the size of the enzyme-loaded reversed micelles, and can exclude the enzyme out of the enzyme-loaded reversed micelles and then back into the aqueous phase. The higher the KCl concentration, the higher the extent of protein backward transfer. Therefore, in order to achieve a high protein back-transfer, the stripping solution with 1*M* KCl will be chosen to perform the backward extraction in following sections.

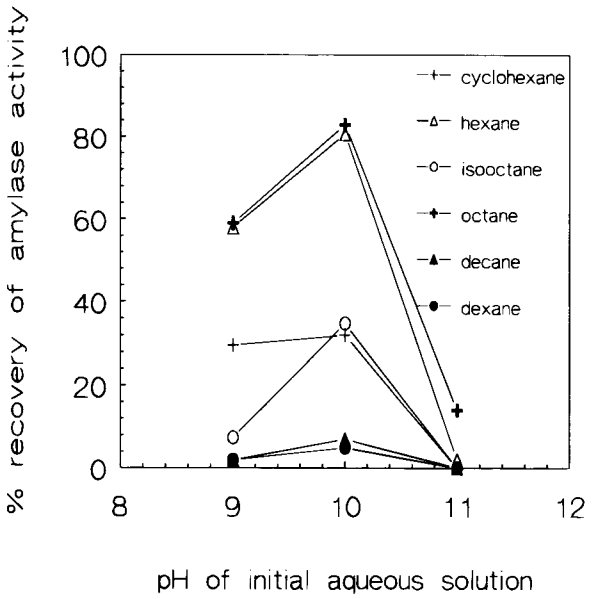

Fig. 3. Influence of initial aqueous pH on the activity recovery of α-amylase at the end of the extraction cycle. Forward extraction conditions—Organic phase: 50 m*M* Aliquat 336 reversed micelles. Aqueous phase: 30 m*M* buffered solution + 6.0 mg crude α-amylase/mL. Backward extraction conditions: 30 m*M* buffered solution at pH6 + 1.0*M* KCl.

Recovery of α—Amylase Activity

After performing a full forward and backward extraction cycle, α—amylase is transferred from the initial aqueous solution into the stripping solution via Aliquat 336 reversed micelles. Figure 3 presents the influence of the initial aqueous pH on the recovery of α—amylase activity at the end of an extraction cycle. It is found that all curves in the figure have a similar shape with a maximal value around pH 10. Notice also that the solvent type also has an obvious specificity on the recovery of enzyme activity. For example, at pH 10.0 almost no α—amylase activity can be recovered in the stripping solution when n-decane and *n*-dodecane were used as the solvent for Aliquat 336. In the cases of cyclohexane and isooctane, about 30% of the total α—amylase activity in the crude enzyme preparation can be recovered. In the cases of *n*-hexane and *n*-octane as the solvent, however, as high as 80% of the total α—amylase activity can be recovered at the end of an extraction cycle.

In order to examine the effect of the solvent type more fully, the recovery of protein in the stripping solution was measured, as presented in Fig. 4. It indicates that all curves have a similar behavior with a maximum at pH 10.0. The solvent specificity on protein recovery is also shown. For example, the recovery of protein mass is nearly the same at pH 10.0

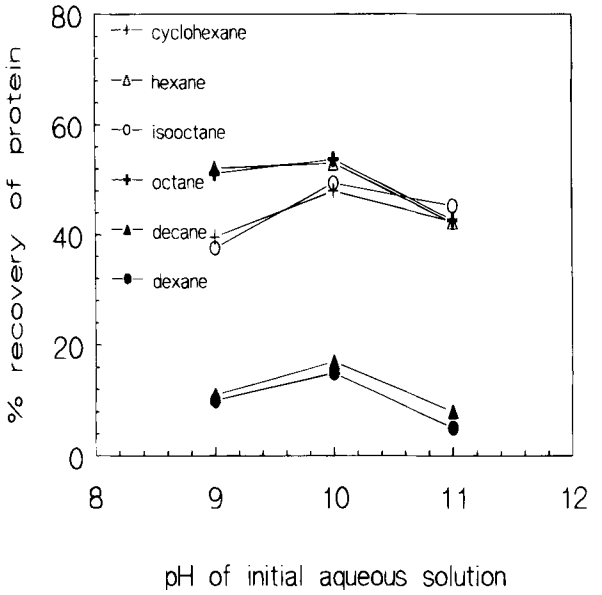

Fig. 4. Influence of initial aqueous pH on the protein recovery at the end of the extraction cycle. Experimental details are given in **Fig. 3**.

when cyclohexane, *n*-hexane, *n*-octane, and isooctane were used as the solvent for Aliquat 336. In the cases of *n*-decane and *n*-dodecane, however, the efficiency will be lower.

Making a comparison between Fig. 3 and 4, it is then evident that some solvents (such as *n*-hexane and isooctane) can favor not only the protein transfer via reversed micellar extraction but also the recover of α—amylase activity from the crude enzyme preparation at the end of an extraction cycle. Some solvents (such as cyclohexane and *n*-octane) can only favor the transfer of protein mass by reversed micellar solution but will denature the enzyme during the extraction process. In the cases of *n*-decane and *n*-dodecane as the solvent for Aliquat 336, however, both the recovery of α—amylase activity and protein mass at the end of an extraction cycle are low.

Furthermore, the purification factor of α—amylase at the initial aqueous pH of 10.0 is calculated and listed in Table 2. It is found that in the cases of n-hexane and isooctane as the solvent for Aliquat 336, α—amylase in the crude enzyme preparation can be concentrated about 1.4-fold by the reversed micellar extraction. In the cases of other four kinds of solvent, the purification factors are all less than 1.0, which means that the specific activity of α—amylase at the end of an extraction cycle is decreased. Therefore, it may be concluded that n-hexane and isooctane are the suitable solvents for Aliquat 336 reversed micelles to separate α—amylase with liquid-liquid extraction.

Table 2
The Purification Factor of α-Amylase in the Stripping Solution, the Initial Aqueous pH Value is 10.0[a]

Solvent	cyclohexane	*n*-hexane	isooctane	*n*-octane	*n*-decane	*n*-dodecane
Purification factor	0.74	1.40	1.42	0.76	0.4	0.41

[a]Experimental details are given in Fig. 3.

CONCLUSION

A cosolvent (*n*-octanol in this experiment) is found to be necessary for Aliquat 336 dissolving in alkanes and forming the reversed micellar solution. But the minimal quantity of *n*-octanol required for Aliquat 336 dissolving in different alkanes is found to be different. During the forward extraction, four out of six alkanes are found to have nearly the same effect on the solubilization of protein in Aliquat 336 reversed micelles. After a full forward and backward extraction cycle, however, only two kinds of solvent (*n*-hexane and isooctane) can achieve the high recovery of both protein mass and α—amylase activity in the stripping solution. Other four solvents (cyclohexane, *n*-octane, *n*-decane, and *n*-dodecane) have shown a low efficiency. *N*-hexane and isooctane may be considered as the suitable solvents for Aliquat 336 reversed micelles to recover α-amylase with liquid–liquid extraction.

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REFERENCES

1. Kadam, K. L. (1986), Reversed micelles as a bioseparation tool. *Enzyme Microb. Technol.* **8,** 266–273.
2. Castro, M. J. M. and Cabral, J. M. S. (1988), Reversed micelles in biotechnology processes. *Biotech. Adv.* **6,** 151–167.
3. Dekker, M., Hilhorst, R., and Laane, C. (1989), Isolating enzyme by reversed micelles. *Anal. Biochem.* **178,** 217–226.
4. Luisi, P. L. and Laane, C. (1986), Solubilization of enzymes in apolar solvents via reversed micelles. *Trends in Biotechnol.* **4,** 153–161.
5. Hatton, T. A. (1989), in: Scamehorn, J. F. and Harwell, J. H. eds. *Surfactant—Based Separation.* Marcel Dekker, New York. pp. 55–90.
6. Mat, H. B. and Stuckey, D. C. (1993), The effect of solvents on water solubilization and protein partitioning in reverse micellar systems. in *Solvent Extraction in the Process Industries.* vol.2. Logsdail, D. H. and Slater, M. J., eds. Published for SCI, Elsevier Applied Science, London and New York. pp. 933–938.

7. Dekker, M., Van't Riet, K., Weijers, S. R., Baltussen, J. W. A., and Laane, C. (1986), Enzyme recovery by liquid-liquid extraction using reversed micelles. *Chem. Eng. J.* **33,** B27–33.
8. Meier, P., Imre, E., Fleschar, M., and Luisi, P. L. (1984), Further investigations of the micellar solubilization of biopolymers in apolar solvents. in *Surfactant in Solution.* vol.2. Mittal, K. L. and Lindam, B. eds. Plenum, New York, pp. 999–1012.
9. Jolivalt, C., Minier, M., and Renon, H. (1990), Extraction of α-chymotrypsin using reversed micelles. *J. Colloid Interf. Sci.* **135,** 85–96.
10. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. (1951), Protein measurement with the Folin Phenol reagent. *J. Biol. Chem.* **193,** 265–275.
11. Bourrel, M. and Schechter, R. S. (1988), *Microemulsions and Related System.* Marcel Dekker, New York and Basel.
12. Marcozzi, G., Correa, N., Luisi, P. L., and Caselli, M. (1991), Protein extraction by reverse micelles: A study of the factors affecting the forward and backward transfer of α-chymotrypsin and its activity. *Biotech. Bioeng.* **38,** 1239–1246.
13. Goklen, K. E. and Hatton, T. A. (1987), Liquid-liquid extraction of low molecular weight protein by selective solubilization in reversed micelles. *Sep. Sci. Technol.* **22,** 831–841.