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Effect of the Type of Cosolvent on the Extraction Process for Separation and Purification of Two Enzymes from *Bacillus subtilis* Using Aliquat 336 Reversed Micelles

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ABSTRACT

Seven kinds of alkyl alcohol (*n*-butanol, *n*-pentanol, *n*-hexanol, *n*-heptanol, *n*-octanol, *n*-nonanol, and *n*-decanol) were chosen as the cosolvent for Aliquat 336/isooctane reversed micelles. By performing liquid–liquid extraction with Aliquat 336 reversed micelles as the extractant, the separation and purification of two enzymes (α-amylase and neutral protease) from *Bacillus subtilis* were investigated. Experiments revealed that when *n*-butanol is used as the cosolvent, the two enzymes from *Bacillus subtilis* can be effectively separated and purified after a full forward and backward extraction cycle. α-Amylase was separated in the stripping solution with about 85% of its total activity recovered and purified about 1.6-fold. Neutral protease was separated in the raffinate with about 80% of its total activity recovered and purified about 3.5-fold. For the other six alcohols used as the cosolvent for Aliquat 336/isooctane reversed micelles, separation was not achieved.

Key Words. Reversed micelles; Cosolvent; Separation; α-Amylase; Neutral protease

INTRODUCTION

The microbial α-amylase and neutral protease are two kinds of important enzymes produced in large amounts. They have been shown to be

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of wide use in food, cosmetics, medicine, and other related production processes (1). The main method to produce α-amylase is by using the strain *Bacillus subtilis* to culture in a fermenter. It is usually directly recovered from the fermentation broth by precipitation and drying, and often with a lot of impurities included. Among the impurities, neutral protease, which is secreted by the strain *Bacillus subtilis* at the same time of the secretion of α-amylase, is the most important since it can hydrolyze and denature α-amylase slowly. The refinement of this crude enzyme preparation generally involves the usage of chromatography or electrophoresis, which is rather time-consuming and costly.

Reversed micellar extraction represents a novel and effective approach to separate and purify protein because of its high selectivity for protein and the ease of scaling-up (2). The process is essentially a double step cycle in which protein is transferred from an aqueous phase into a reversed micellar phase during the forward extraction, and recovered in a second aqueous phase during the backward extraction. The efficiency of this process depends on the manipulation of the parameters in both aqueous phase and reversed micellar solution.

Several kinds of reversed micellar system have been used to extract proteins (3). The most common is the reversed micelles of the anionic surfactant sodium bis-(2-ethylhexyl) sulfosuccinate (AOT) in isooctane, because AOT can easily dissolve in an apolar solvent and form a reversed micellar system without the help of any cosolvent (or so-called cosurfactant) (4–7). Some other reversed micellar systems, such as a cationic trioctylmethylammonium (TOMAC) in an apolar solvent, are also available. Only a cosolvent is usually required in this system to help the surfactant to dissolve in the apolar solvent and form reversed micelles. The ability of this kind of reversed micelle to extract and separate protein has been demonstrated by some researchers. For example, Dekker and coworkers used TOMAC as the surfactant, octanol as the cosolvent, and isooctane as the solvent to recover and concentrate α-amylase by continuous extraction (8); Jolivalt and coworkers successfully solubilized α-chymotrypsin in reversed micelles by dissolving another cationic surfactant, Aliquat 336, in isooctane, with the addition of isotridecanol as the cosolvent (9).

The precise role played by cosolvent in the formation of reversed micelles is still unclear. It is believed that the cosolvent molecules are inserted between the surfactant molecules, thereby resulting in two important effects. First, the interaction between the hydrophiles of the surfactant is changed. Second, the arrangement of surfactant molecules in the solvent is loosened and the steric difficulty for arranging the big surfactant molecules in a loose manner may be overcome, so that the

surfactant can be readily dissolved in the solvent. It is then quite possible that the cosolvent will affect the transfer of protein between the reversed micellar solution and the aqueous phase. However, it seems that no systematic work has been done to date to examine this effect.

In this paper, by using different kinds of alkyl alcohols as the cosolvent for the reversed micelles of Aliquat 336/isooctane, the effect of cosolvent type on the separation and purification of the two enzymes from *Bacillus subtilis* with liquid–liquid extraction is investigated. The purpose of this research is to selectively recover the two enzymes while removing other undesirable impurities from the crude enzyme preparation.

MATERIALS AND METHODS

Chemicals

The crude enzyme preparation from *Bacillus subtilis* was obtained from Wuxi Enzyme Reagent Factory, China. It contained α-amylase (640 U/mg, pI 5.4) and neutral protease (20 U/mg, pI 7.5), with a total protein of 0.17 mg/mg (crude enzyme). Aliquat 336 (purchased from Fluka) is a kind of trialkylmethyl ammonium chloride with the number of carbon atoms from 8 to 11 in alkyl groups. Isooctane, *n*-butanol, *n*-pentanol, *n*-hexanol, *n*-heptanol, *n*-octanol, *n*-nonanol, and *n*-decanol 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 an apolar solvent with the aid of a cosolvent (or cosurfactant) [9]. In our experiments, seven kinds of straight chain alkyl alcohol were chosen as the cosolvent, and the reversed micellar solution contained 50 mM Aliquat 336 and 1.0% (v/v) cosolvent in isooctane (the concentration of cosolvent is given by referring the volume of cosolvent to the total volume of the reversed micellar system). The aqueous phase was 30 mM citric acid–dibasic potassium phosphate buffered solution for pH 7–9 or 30 mM borax–sodium hydroxide buffered solution for pH 9–11. The crude enzyme preparation (about 1.0 mg total protein/mL aqueous solution) was dissolved in the buffered solution.

Experiments were carried out in tightly stoppered 50 mL 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 minute. In the backward extraction, the enzyme-loaded micellar solution from the forward extraction was mixed with an equal volume of aqueous stripping

solution at 250 rpm for 1 minute. In each case the mixtures were centrifuged at 3500 rpm for 5 minutes to separate the two phases. The protein content and enzyme activity were assayed for each phase.

Analytical Methods

Protein concentration was determined by measuring absorbance of the aqueous and reversed micellar solution at 280 nm on a model 751G UV/Vis spectrophotometer. The method of Lowry (10) was used to confirm the protein content in the aqueous phase. 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 minute at pH 6.0. Neutral protease activity was determined by using casein as substrate. One unit was defined as the amount of enzyme that liberated 1 μg of tyrosine at 37°C in 1 minute at pH 7.2. The purification factor of enzyme was calculated as the ratio of the specific activity in the stripping solution or raffinate to that in the initial aqueous solution. The separation factor is defined as the ratio of the purification factors of two enzymes, namely α-amylase to neutral protease in the stripping solution, or neutral protease to α-amylase in the raffinate. The percentage of enzyme activity recovery is calculated by referring the enzyme activity in the stripping solution or raffinate to that in the initial aqueous phase.

RESULTS AND DISCUSSION

Phase Transfer of Protein between Aqueous and Reversed Micellar Solution

Effect of pH on Protein Solubilization

The influence of initial aqueous pH on the solubilization of protein (the percentage of protein transfer from the initial aqueous phase to the reversed micellar solution) is demonstrated in Fig. 1. At low pH values, little protein is solubilized in the reversed micellar solution. By increasing the pH, a rapid increase in the solubilization of protein occurs, such that more than 90% of protein in the initial aqueous phase can be extracted into the micellar solution at pH above 9.5. Furthermore, the solubilization of protein changes very little with different alcohols as the cosolvent, which suggests that the kind of alcohol has no significant effect on the percentage of protein transfer in the forward extraction process.

Protein solubilization in the reversed micelles appears to be dominated by electrostatic interaction between the charged protein and the surfactant head layer forming the walls of the micellar polar core. Only when the two are of opposite polarity can solubilization be expected (5, 11). With

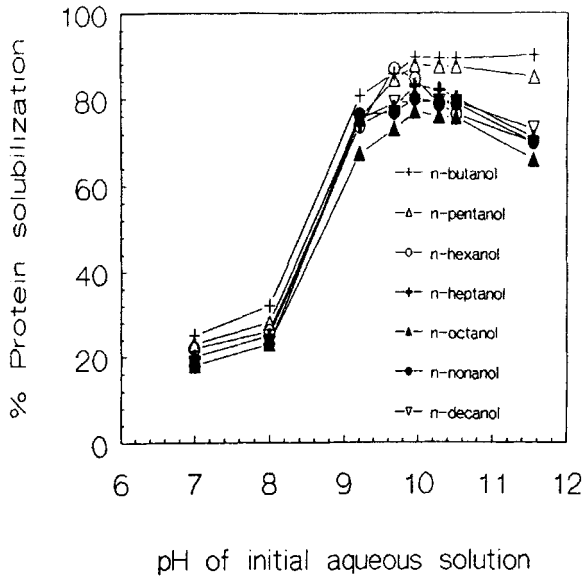

FIG. 1 Influence of the initial aqueous pH on the solubilization of protein. Organic phase: 50 mM Aliquat 336/isooctane + 1% (v/v) of different cosolvents. Aqueous phase: 30 mM buffer + 6.0 mg crude α-amylase/mL.

the cationic surfactant Aliquat 336, the interface of reversed micelles has a positive charge. Thus, the solubilization of protein should be observed for a pH higher than the isoelectric point of the protein (5.4 for α-amylase and 7.5 for neutral protease) (3). This is evidently supported by the data shown in Fig. 1.

Backextraction of Protein

Although backextraction is an important step for recovering protein and enzyme activity from the reversed micellar solution, it is little studied in the literature (12). Figure 2 presents the percentage of protein backward transfer with different KCl concentrations in the stripping solution when two different alcohols (*n*-butanol and *n*-octanol) are used as the cosolvent. It reveals that the extent of protein backward transfer depends upon the cosolvent used. In the case of *n*-butanol, it seems quite easy to strip protein from the reversed micellar solution even at low KCl concentration. In the case of *n*-octanol, however, a high level of protein backtransfer can only be achieved at higher KCl concentration.

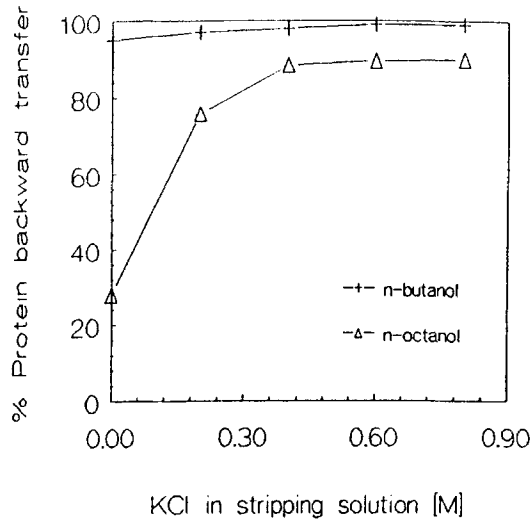

FIG. 2 Influence of KCl concentration in the stripping aqueous solution on the percentage of protein backward transfer. Forward extraction conditions—Organic phase: 50 mM Aliquat 336/isooctane + 1% (v/v) *n*-butanol or *n*-octanol. Aqueous phase: 30 mM buffer at pH 10 + 6.0 mg crude α-amylase/mL. Backward extraction conditions—Stripping aqueous phase: 30 mM buffer at pH 6 + different concentrations of KCl.

The different properties of the two alcohols may contribute to this phenomenon. Because of its high polarity and higher solubilization in water, *n*-butanol can form looser reversed micelles with Aliquat 336 in isooctane, which can be easily destroyed during the backward extraction process and make protein release back into the aqueous solution. *n*-Octanol with its lower polarity and lower solubilization in water, however, forms more compact reversed micelles with Aliquat 336 in isooctane, which can not be destroyed unless a more intense experimental condition, such as a higher ionic concentration, is applied. Thus, to recover as much α-amylase as possible, the stripping solution with the higher KCl concentration of 0.4 M KCl at pH 6 was chosen.

Separation and Purification of Two Enzymes from *Bacillus subtilis*

Separation and purification of the two enzymes could be achieved by performing a full forward and backward extraction cycle. Data from the preliminary experiment indicated that one of the two enzymes, α-amylase, was mostly transferred into the stripping solution via reversed micellar

solution after an extraction cycle while the other enzyme, neutral protease, remained in the raffinate.

The feasibility of the process was tested by following the separation factor and recovery activity of the two enzymes throughout the extraction procedure. Extraction conditions were selected according to the aforementioned experiments only by the criterion of the largest retention of activity of the two enzymes. The main parameters examined included the initial aqueous pH and the type of cosolvent used for Aliquat 336/isooctane reversed micelles.

Recovery of α-Amylase in the Stripping Solution

To study the effect of cosolvent type, the cosolvent concentration was fixed at 1% (v/v) throughout the experiments. With different alkyl alcohols as the cosolvent and at various initial aqueous pH values, the activity recoveries of α-amylase and neutral protease in the stripping solution were determined, as shown in Figs. 3A and 3B.

Concerning the activity recovery of α-amylase, the seven cosolvent curves in Fig. 3A have similar shapes, with their maximum at pH 10. Two important influences contribute to this behavior: first, the enzyme can be easily denatured at high pH; second, a special electrostatic interaction between protein and surfactant which may occur at a certain pH value, such as at pH 10, also plays an important role on protein transfer and activity recovery (7, 11).

Notice also that the data reveal a significant cosolvent specificity on the recovery of enzyme activity. For example, about 85% of total α-amylase activity can be recovered at pH 10 with *n*-butanol as the cosolvent, and about 62% of the total activity is recovered in the case of *n*-pentanol. In the cases of the other five alcohols, however, only about 30% of total activity can be recovered at pH 10. This may be useful in the selection of a cosolvent for Aliquat 336 reversed micelles used to separate and purify enzymes.

The activity recovery of neutral protease in the stripping solution is presented in Fig. 3B, which indicates that only about 10–15% of its total activity can be recovered in all cases, meaning that the selectivity of Aliquat 336 reversed micelles toward neutral protease is poor and most of this enzyme cannot be transferred by liquid–liquid reversed micellar extraction.

The purification factors of α-amylase and neutral protease in the stripping solution are presented in Figs. 3C and 3D. The maximum purification factor of α-amylase is found around pH 10, which also shows a cosolvent specificity. For example, α-amylase can be purified about 1.7-fold at pH

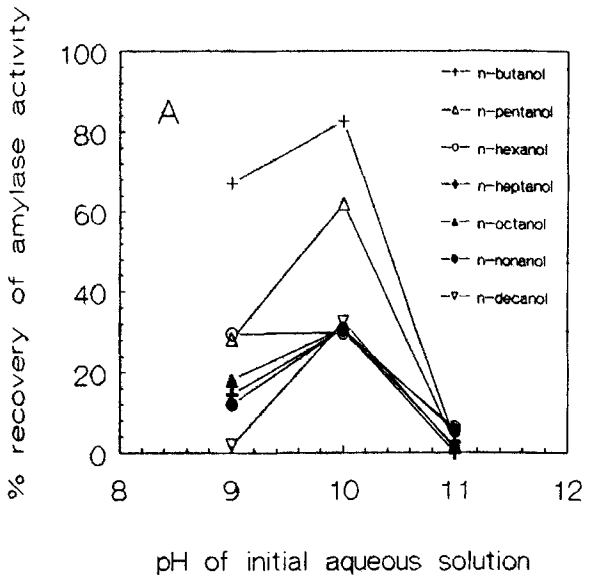

FIG. 3 The influence of the initial aqueous pH (A) on the activity recovery of α-amylase in the stripping solution, (B) on the activity recovery of neutral protease in the stripping solution, (C) on the purification factor of α-amylase in the stripping solution, (D) on the purification factor of neutral protease in the stripping solution, and (E) on the separation factor of α-amylase to neutral protease in the stripping solution. Forward extraction conditions—Organic phase: 50 mM Aliquat 336/isooctane/1% (v/v) different cosolvents. Aqueous phase: 30 mM buffer + 6.0 mg crude enzyme/mL aqueous phase. Backward extraction conditions: 30 mM buffer at pH 6 + 0.4 M KCl.

10 with *n*-butanol as the cosolvent, and can be purified 1.2-fold in the case of *n*-pentanol. But if other five alcohols are used as the cosolvent, the specific activity of α-amylase in the stripping solution will be decreased. As expected, the purification factors of neutral protease in the stripping solution were low (<1).

The phenomenon of the high activity recovery of α-amylase and the low recovery of neutral protease suggests that α-amylase may be separated and recovered from the two enzymes into the stripping solution by Aliquat 336 reversed micelles. To quantatively determine the extent of separation, the separation factors of α-amylase to neutral protease in the stripping were calculated and are presented in Fig. 3E. For all curves in the figure, the maximum appears around pH 10, showing a obvious cosolvent specificity. The figure indicates that the value of the separation factor can

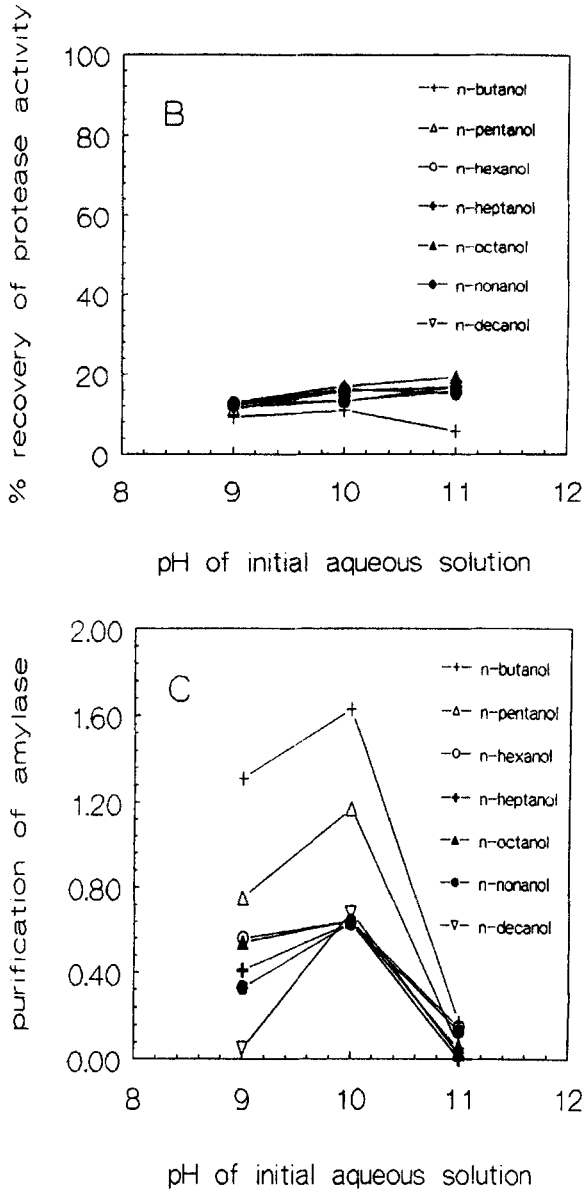

FIG. 3 Continued

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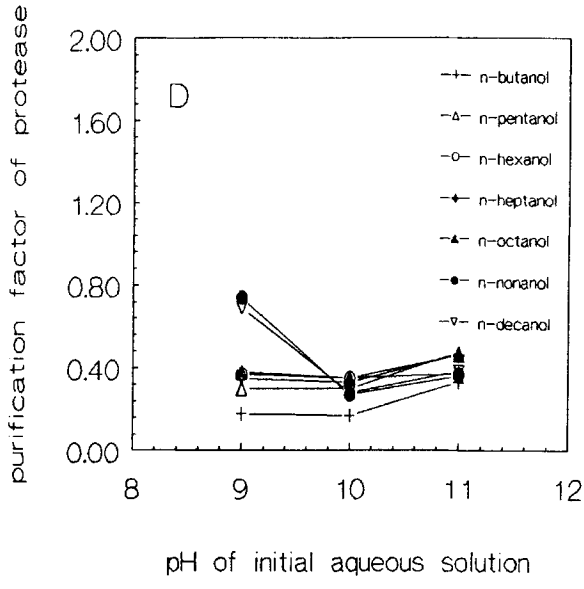

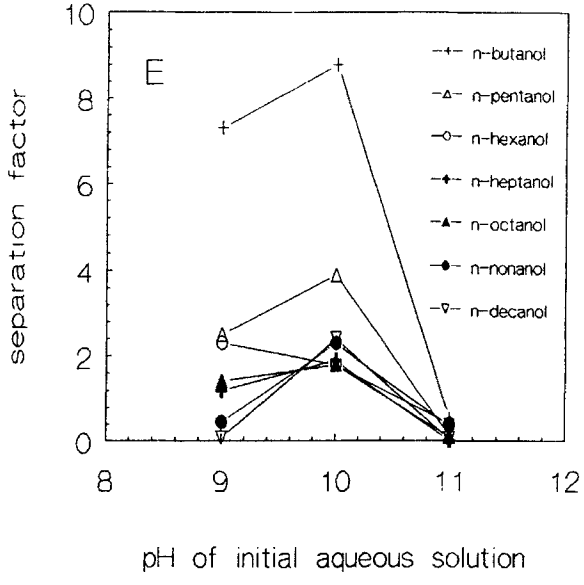

FIG. 3 Continued

reach about 9 at pH 10 with *n*-butanol as the cosolvent, and is about 4 in the case of *n*-pentanol. In the cases of the other five alcohols, the purification factors at pH 10 are around 2.0. This implies that among these alkyl alcohols, *n*-butanol is the best cosolvent with which α-amylase can be effectively recovered from the crude enzyme preparation.

Recovery of Neutral Protease in the Raffinate

Another concern is the enzyme activity remaining in the raffinate. Figure 4A presents the activity recovery of neutral protease in the raffinate as a function of the initial aqueous pH with different alcohols as the cosolvent. The maximum is again shown around pH 10, at which about 80% of its total activity can be recovered but there no significant cosolvent effect is exerted. The activity recovery of α-amylase in the raffinate as

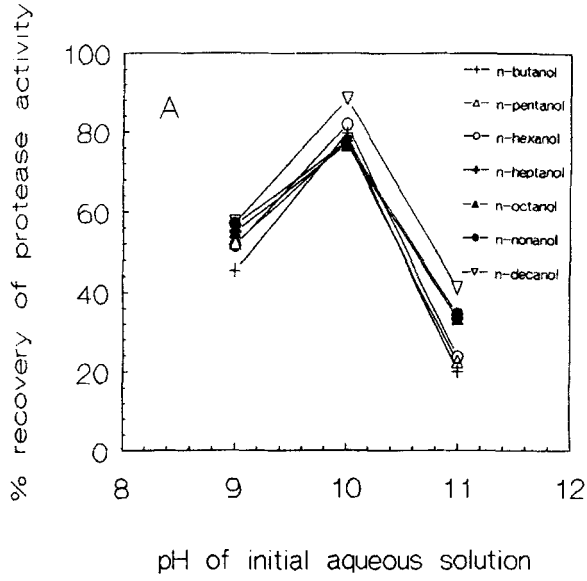

FIG. 4 The influence of the initial aqueous pH (A) on the activity recovery of neutral protease in the raffinate, (B) on the activity recovery of α-amylase in the raffinate, (C) on the purification factor of neutral protease in the raffinate, (D) on the purification factor of α-amylase in the raffinate, and (E) on the separation factor of neutral protease to α-amylase in the raffinate. Forward extraction conditions—Organic phase: 50 mM Aliquat 336/isooctane/1% (v/v) different cosolvents. Aqueous phase: 30 mM buffer + 6.0 mg crude enzyme/mL aqueous phase. Backward extraction conditions: 30 mM buffer at pH 6 + 0.4 M KCl.

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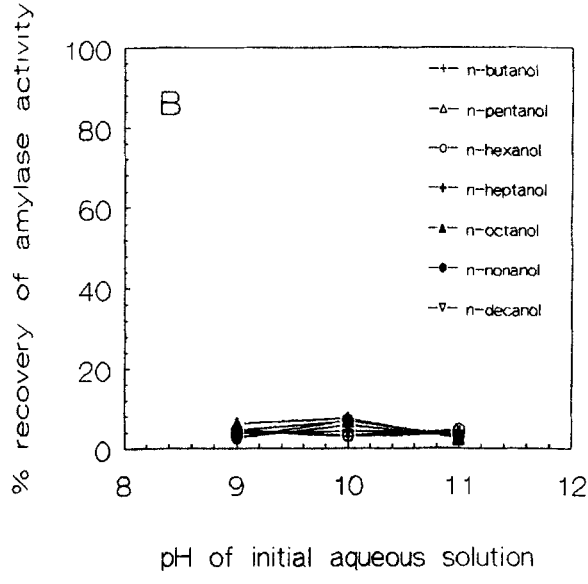


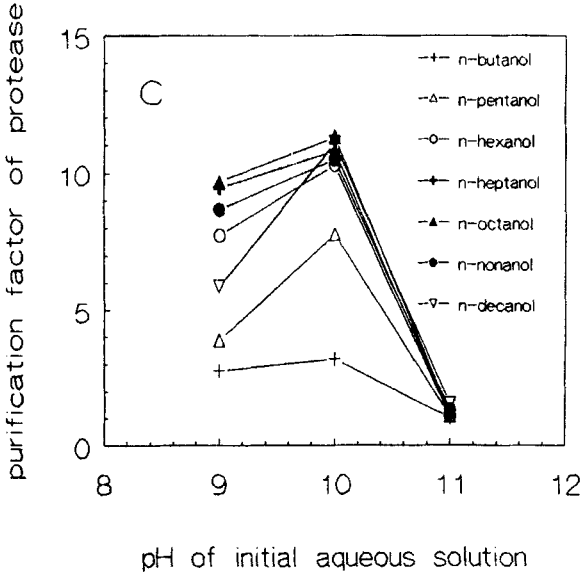
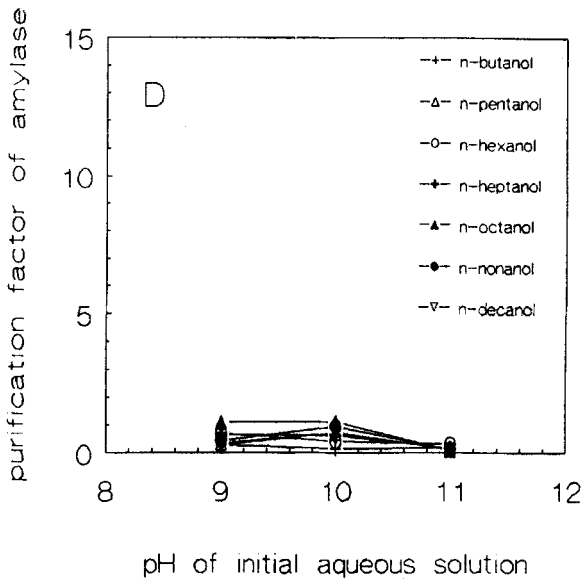


FIG. 4 Continued



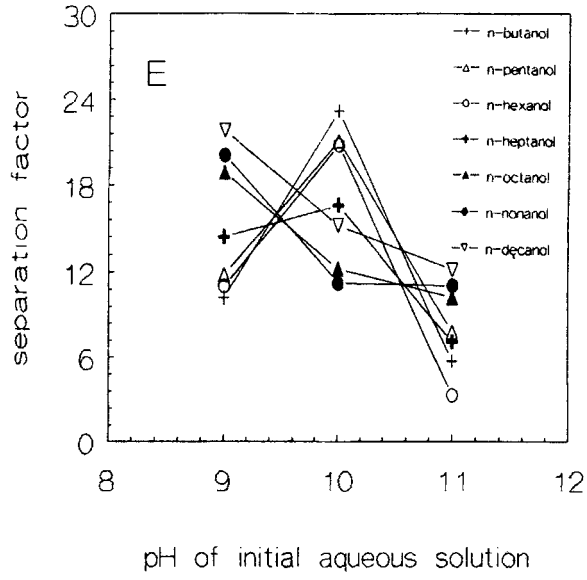


FIG. 4 Continued

given in Fig. 4B is, however very low. This further demonstrates that the selectivity of Aliquat 336 reversed micelles toward neutral protease is poor, and most neutral protease in the crude enzyme preparation still remains in the aqueous phase after liquid–liquid extraction. It may be because of this poor solubilization selectivity that it is possible to separate and purify the two enzymes from the crude enzyme preparation.

The purification factors of neutral protease in the raffinate are shown in Fig. 4C: they are pH-dependent and also have a maximum around pH 10. At pH 10, neutral protease can be purified about 4- to 12-fold when different alcohols are used, and a obvious cosolvent effect is exerted. The purification factor of α-amylase is rather low, as shown in Fig. 4D. and its specific activity is decreased by a factor of 2- to 7-fold in the raffinate.

The quantitative extent of the separation of the two enzymes in the raffinate is estimated by following the separation factor of neutral protease to α-amylase, as presented in Fig. 4E. The first interesting observation is that some curves have a maximum around pH 10, as in the cases of alcohols with fewer carbon atoms; some curves decrease with increasing pH values, as in the cases of alcohols with more carbon atoms. Many reasons can contribute to this behavior, but the different protein losses during the extraction process are believed to be the most important.

More in general, the optimal purification factors are all higher than 10, especially in the case of *n*-butanol, where the separation factor can reach about 23, suggesting that neutral protease in the mixture can be effectively separated and recovered in the raffinate.

To summarize the effect of the type of cosolvent, it might be concluded that when *n*-butanol is used as the cosolvent for Aliquat 336 reversed micelles to extract the crude enzyme preparation from *Bacillus subtilis*, the two enzymes in the crude preparation can be effectively separated and purified. α-Amylase can be separated and purified in the stripping solution with about 85% of its activity recovered and concentrated about 1.6-fold, and most of neutral protease is removed at the same time. Neutral protease can be recovered and purified in the raffinate with about 80% of its activity recovered and concentrated about 4-fold; meanwhile, most of the α-amylase is removed. For the other six alcohols, although they can extract neutral protease from the crude enzyme preparation into the raffinate, they cannot effectively recover and concentrate α-amylase into the stripping solution. Thus, they are not suitable cosolvents for Aliquat 336 reversed micelles used to separate and purify enzymes.

CONCLUSIONS

By using *n*-butanol as the cosolvent for Aliquat 336/isooctane reversed micelles, two enzymes from *Bacillus subtilis* (α-amylase and neutral pro-

tease) can be selectively separated and purified by liquid–liquid reversed micellar extraction. α-Amylase can be effectively recovered and purified in the stripping solution, and neutral protease can be recovered and concentrated in the raffinate. For six other alcohols used as the cosolvent, the separation efficiency was less. This investigation offers a new method to separate and purify enzyme from crude product or fermentation broth.

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