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Selective Recovery of Two Enzymes from *Bacillus subtilis* Using Aliquat 336 Reversed Micelles

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ABSTRACT

Selective separation and purification of two enzymes from *Bacillus subtilis* (α-amylase and neutral protease) by liquid–liquid extraction using Aliquat 336/isooctane reversed micelles were investigated. After a full forward and backward extraction cycle, α-amylase was separated and purified in the stripping solution, and neutral protease was recovered in the raffinate.

Index Entries: Reversed micelles; separation; α-amylase; neutral protease.

INTRODUCTION

The microbial α-amylase is an important enzyme that is produced in large amounts. It has been shown to be of wide use in food, cosmetics, medicine, and other related production processes (*1*).

Using the strain *Bacillus subtilis* to culture in a fermentor is the most common method to produce α-amylase. Usually, the microbial α-amylase is 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 *B. subtilis* at the same time as the secretion of α-amylase, is the most important. The neutral protease can hydrolyze α-amylase slowly and denature the enzyme activity. In order to utilize α-amylase more effectively, neutral protease must be removed from the microbial α-amylase.

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The ordinary refinement of this crude enzyme generally involves the usage of chromatography or electrophoresis, which is rather time-consuming and costly. The liquid-liquid extraction technique with reversed micellar solution as the extractant represents a new and effective approach to separate and concentrate proteins. The principle of this technique is based on the ability of reversed micelles to selectively solubilize protein inside the polar core of surfactant aggregate and protect protein from denaturation against the harmful organic phase (*2*). Some researchers have demonstrated the bright prospects of protein isolation and purification by reversed micellar solution, such as the separation of a protein mixture (*3*), extraction of extracellular enzyme from whole fermentation broth (*4*), and the separation and purification of two lipases from crude preparation (*5*).

In this article, we choose Aliquat 336/isooctane reversed micelles as the extractant to extract the crude enzyme preparation from *B. subtilis*. The purpose of this study is not only to separate and purify α-amylase from the crude enzyme preparation, but also to recover selectively neutral protease at the end of extraction.

MATERIALS AND METHODS

Chemicals

The crude enzyme preparation from *B. subtilis* was obtained from Wuxi Enzyme Reagent Factory, China. It was the mixture of two enzymes with a total protein of 0.17 mg/mg (crude enzyme), which contained α-amylase (640 μ/mg, pI = 5.4) and neutral protease (20 μ/mg, pI = 7.5). Aliquat 336 is a type of trialkyl-methyl ammonium chloride with the number of carbon atoms from 8 to 11 in alkyl groups. It was purchased from Fluka. All other chemicals were purchased from a local market and were of analytic grade. Experiments were performed at room temperature.

Forward and Backward Extraction Conditions

Aliquat 336 can form reversed micelles in apolar solvent with the addition of cosolvent (or cosurfactant) (*6*). In this article, *n*-butanol was used as the cosolvent, and the reversed micellar solution contained 50 m*M* Aliquat 336 and 1.0% (v/v) *n*-butanol in isooctane. The aqueous phase was a 30-m*M* borate-buffered solution (from pH 8 to 11) with the two enzymes from *B. subtilis*. The total protein in the aqueous was controlled within the range of about 1.0 mg protein/mL aqueous solution.

Experiments were carried out in tightly stoppered 50 m*M* glass flasks. In the forward extraction, equal volumes (usually 5 mL) of micellar solution and aqueous phase were mixed at 250 rpm for 1 min. In the backward extraction, the enzyme-loaded micellar solution from the forward extraction was mixed with equal volume of aqueous stripping solution (30 m*M* phosphate buffer, pH 6.0 + 0.4*M* KCl) at 250 rpm for 1 min. In each case, 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

Protein concentration was determined by measuring the absorbance of the aqueous and reversed micellar solution at 280 nm on a model 751G UV/Vis spectrophotometer. The method of Lowery (*7*) 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 is defined as the amount of enzyme that liberates 1 mg of maltose from starch at 37°C in 1 min at pH 6.0. Neutral protease activity was determined by using casein as the substrate. One unit is defined as the amount of enzyme that liberates 1 μg of tyrosine at 37°C in 1 min at pH 7.2. The separation factor in the stripping solution is defined as the ratio of the purification factors of α-amylase to that of neutral protease. The separation factor in the raffinate is defined as the ratio of the purification factors of neutral protease to that of α-amylase. 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

The separation and purification of the crude enzyme preparation were achieved by performing a full forward and backward extraction cycle. The feasibility of this process was tested by following the activity recovery of two enzyme activities in the crude enzyme preparation at the end of extraction cycle. The extraction conditions were selected according to the only criterion, which was to retain the maximum enzyme activity.

After the preliminary experiment, the data indicated that α-amylase from *B. subtilis* was mostly transferred into the stripping solution via reversed micellar solution, whereas neutral protease remained in the raffinate at the end of extraction cycle. Many factors can affect the transfer processes of these two enzymes, but only the parameter of the pH values in the initial aqueous solution would be discussed in the following sections. Since different pH values in the initial aqueous phase can favor or not favor the transfer of protein toward the reversed micelles (*8*), it might be very significant to investigate the effect of pH on the recovery of enzyme activity during the extraction procedure.

Recovery of α-Amylase

The first consideration is the case in the stripping solution. After a forward and backward extraction cycle, the effect of initial aqueous pH on the activity recovery of α-amylase and neutral protease in the stripping solution was measured, as presented in Fig. 1A. The figure shows that the activity recoveries of two enzymes are all pH dependent. It is found

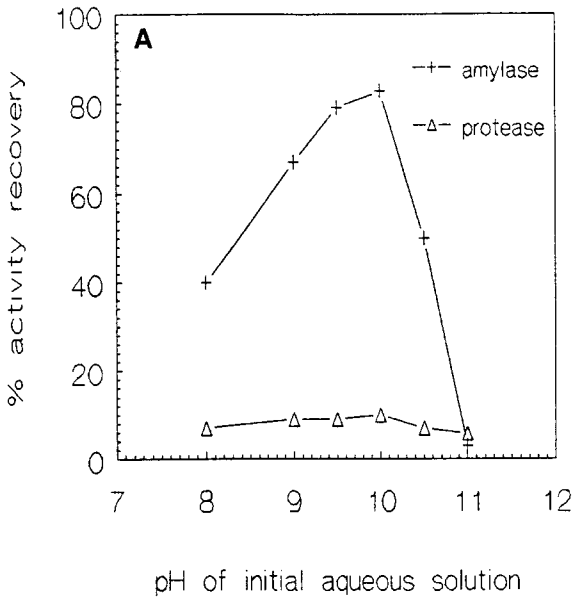

Fig. 1A. The influence of the initial aqueous pH on the activity recovery of α-amylase and neutral protease in the stripping solution. Forward extraction conditions—organic phase: 50 m*M* Aliquat 336/isooctane/1% (v/v) *n*-butanol. Aqueous phase: 30 m*M* buffer containing 6.0 mg crude enzyme/mL aqueous phase. Backward extraction conditions: 30 m*M* buffer at pH 6 + 0.4*M* KCl. +, Amylase; △, protease.

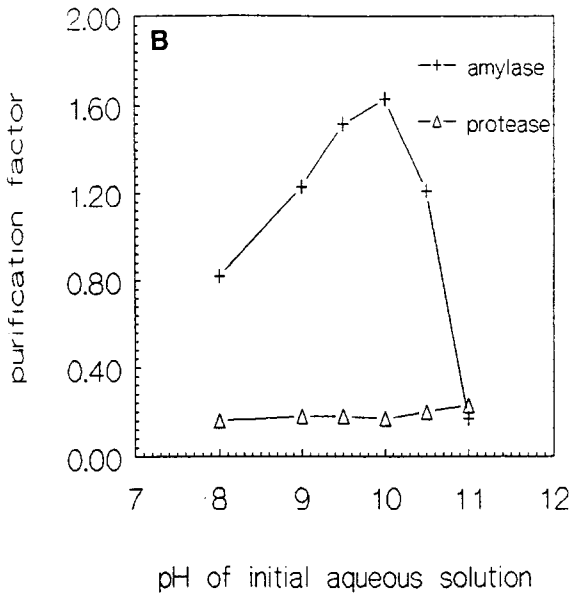

Fig. 1B. The influence of the initial aqueous pH on the purification factors of α-amylase and neutral protease in the stripping solution. Forward extraction conditions—organic phase: 50 m*M* Aliquat 336/isooctane/1% (v/v) *n*-butanol. Aqueous phase: 30 m*M* buffer containing 6.0 mg crude enzyme/mL aqueous phase. Backward extraction conditions: 30 m*M* buffer at pH 6 + 0.4*M* KCl. +, Amylase; △, protease.

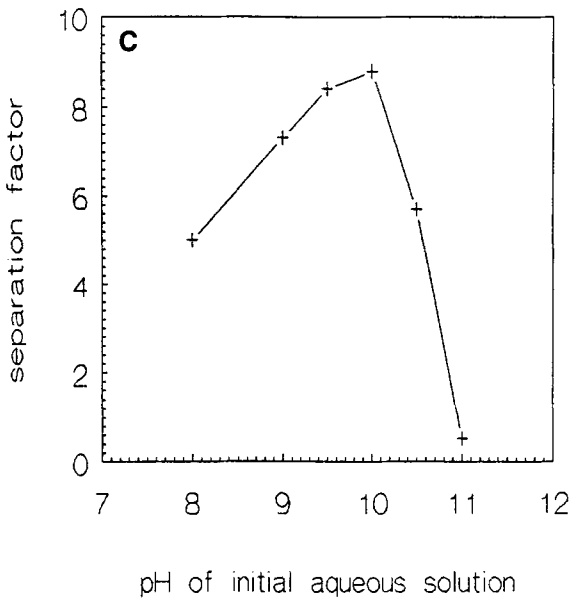

Fig. 1C. The influence of the initial aqueous pH on the separation factors of α-amylase and neutral protease in the stripping solution. Forward extraction conditions—organic phase: 50 m*M* Aliquat 336/isooctane/1% (v/v) *n*-butanol. Aqueous phase: 30 m*M* buffer containing 6.0 mg crude enzyme/mL aqueous phase. Backward extraction conditions: 30 m*M* buffer at pH 6 + 0.4*M* KCl.

that the activity recovery of α-amylase is much higher than that of neutral protease and has a maximum around pH 10. As much as 85% of total α-amylase activity can be recovered at pH 10. Concerning neutral protease in the stripping solution, however, its overall activity recovery is found to be lower than 10% in the whole experimental pH range.

The purification factors of the two enzymes in the stripping solution as a function of the initial aqueous pH is presented in Fig. 1B, which shows that the purification factors of α-amylase are also much higher than that of neutral protease and have a maximum around pH 10. α-Amylase can be concentrated about 1.6-fold at pH 10. The purification factors of neutral protease, however, are quite lower than 1.0 (about 0.2), which means that the specific activity of neutral protease is drastically decreased in the stripping solution. This observation suggests that α-amylase can be separated and purified from the crude enzyme preparation in the stripping solution.

To determine this separation extent quantatively, the separation factors of α-amylase to neutral protease in the stripping solution are calculated and presented in Fig. 1C, which shows that maximal separation factor can reach about 9 around pH 10.

Recovery of Neutral Protease

The second consideration is the case in the raffinate. Figure 2A shows the activity recovery of α-amylase and neutral protease in the raffinate

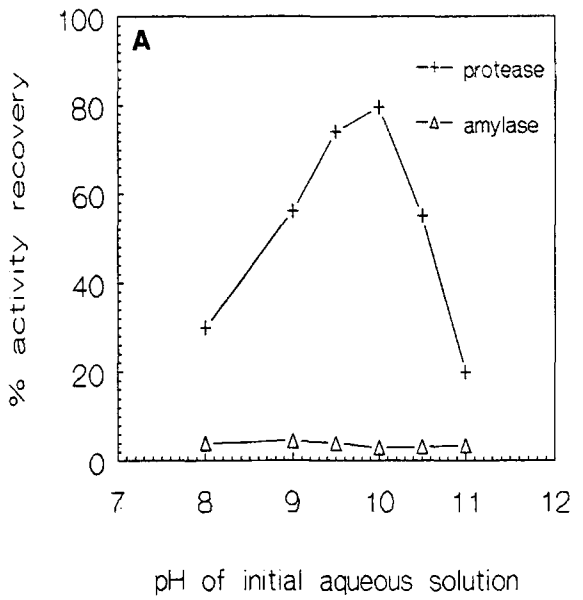

Fig. 2A. The influence of the initial aqueous pH on the activity recovery of α-amylase and neutral protease in the raffinate. Forward extraction conditions—organic phase: 50 m*M* Aliquat 336/isooctane/1% (v/v) *n*-butanol. Aqueous phase: 30 m*M* buffer containing 6.0 mg crude enzyme/mL aqueous phase. Backward extraction conditions: 30 m*M* buffer at pH 6 + 0.4*M* KCl. +, Protease; △, amylase.

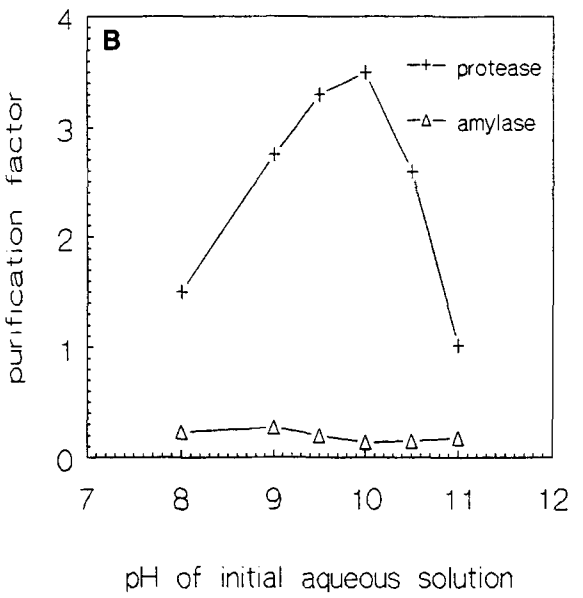

Fig. 2B. The influence of the initial aqueous pH on the purification factors of α-amylase and neutral protease in the raffinate. Forward extraction conditions—organic phase: 50 m*M* Aliquat 336/isooctane/1% (v/v) *n*-butanol. Aqueous phase: 30 m*M* buffer containing 6.0 mg crude enzyme/mL aqueous phase. Backward extraction conditions: 30 m*M* buffer at pH 6 + 0.4*M* KCl. +, Protease; △, amylase.

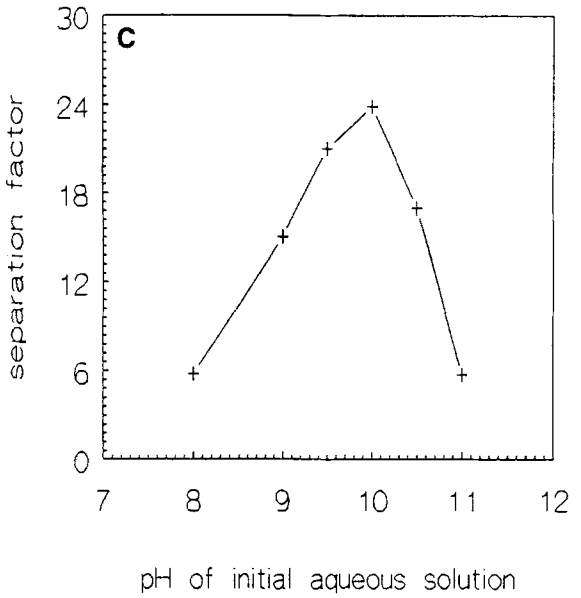

Fig. 2C. The influence of the initial aqueous pH on the separation factors of neutral protease to α-amylase in the raffinate. Forward extraction conditions—organic phase: 50 m*M* Aliquat 336/isooctane/1% (v/v) *n*-butanol. Aqueous phase: 30 m*M* buffer containing 6.0 mg crude enzyme/mL aqueous phase. Backward extraction conditions: 30 m*M* buffer at pH 6 + 0.4*M* KCl.

varying as a function of the initial aqueous pH. Notice that the activity recovery of neutral protease in the raffinate is much higher than that of α-amylase and can reach about 80% around pH 10. The activity recovery of α-amylase in the raffinate, however, is very low, which is never higher than 5%.

The purification factors of the two enzymes in the raffinate as a function of the initial aqueous pH are presented in Fig. 2B. It is found that the purification factors of neutral protease are also higher than that of α-amylase and can reach a maximum around pH 10. The neutral protease can be concentrated about 3.5-fold at pH 10. However, the purification factor of α-amylase is found to be much lower than 1.0, meaning that the specific activity of α-amylase is exceedingly decreased in the raffinate.

Figure 2C presents the separation factor of neutral protease to α-amylase in the raffinate, which indicates that the separation factor can reach as high as about 24 at pH 10. This observation implies that neutral protease from *B. subtilis* can also be effectively recovered by liquid–liquid reversed micellar extraction.

In summary, it might be concluded that if the pH values in the initial aqueous solution are suitable (such at pH 10), the two enzymes from *B. subtilis* could be effectively separated and purified by liquid–liquid reversed micellar extraction. The experimental data indicated that α-amylase was separated and purified in the stripping solution with about 85% of its

activity recovered and concentrated about 1.6-fold; neutral protease was recovered and purified in the raffinate with about 80% of its activity recovered and concentrated about 3.5-fold.

ACKNOWLEDGMENT

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