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Effect of chaotropes in reverse micellar extraction of kallikrein

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ABSTRACT

Reverse micellar extraction is a promising technique in large-scale bioseparation. However, low recovery and high salt concentration in back extraction limit its application. In CTAB/*n*-octane/*n*-hexanol reverse micellar system, the enzyme, pancreatic kallikrein could be effectively enwrapped into reverse micelles in forward extraction, but was difficult to be released during back extraction. In this study, dilute chaotropes (urea and GuHCl) were introduced to enhance the release of enzyme instead of high salts in back extraction. Kallikrein enwrapped in reverse micelles was released effectively in the presence of dilute urea and GuHCl during back extraction. Nearly 100% activity recovery of kallikrein from commercial product was obtained by adding 0.60 M urea, and for kallikrein from crude material, the recovery increased greatly by adding 0.80 M urea and 0.08 M GuHCl in the stripping solution. The mechanism of chaotrope for enhancing the release of enzyme from micelles was explored and dynamic light scatter analysis showed that the chaotrope would influence the sizes of micelles during reverse micellar extraction.

1. Introduction

In the past three decades, reverse micellar extraction has attracted considerable attention due to its potential application in bioseparation engineering area [1]. The process of reverse micellar extraction consists of two steps including forward and back extractions. In the forward extraction, most of biomolecules could be transferred effectively from the initial aqueous phase to reverse micelles phase. And then, the biomolecules are released from the reverse micelles to a fresh aqueous stripping solution in the back extraction step. However, the recovery in the later step is very low, indicating that biomolecule enwrapped in reverse micelle was not easily released. Low recovery in the back extraction limits the application of reverse micellar extraction.

To promote the release of biomolecules from reverse micelles, optimization of ionic strength and pH in the stripping solution are usually adopted in back extraction, but interesting molecules usually could not be released effectively. In recent years, many researchers have made great efforts to improve protein recovery by using other methods. Protein enwrapped in AOT reverse micelle was released by adding a counterionic surfactant (TOMAC or DTAB) [2]. Cytochrome C in AOT reverse micelle system reached more than 80% recovery when tetrafluoroethane was used to form gas hydrate in back extraction [3]. Acetone as stripping solution instead of aqueous solution was used to extract lysozyme from reverse micelle, and above 70% lysozyme was recovered [4]. Alkyl halides including R_4Cl, R_8Br and R_4I can obviously enhance BSA back transfer at low ionic strength [5]. Low concentration ethanol in stripping solution could avoid inactivation of proteins in back extraction in AOT reverse micelle system [6].

Chaotropes (chaotropic agent) can influence the three dimensional structure of macromolecules such as proteins by noncovalent forces like hydrogen bonds and hydrophobic effects, thus denaturing them at high concentrations. Urea and guanidine hydrochloride (GuHCl) are the extensively used chaotropes in protein folding field. Effect of urea on micelle formation was explored to obtain understanding of unfolding and refolding of proteins in some studies [7]. GuHCl is seldom reported to be used in the studies about micelles, and only two reports could be seen in forward extraction of reverse micelles to improve recovery of enzyme [8,9]. Until now, no study focuses on promoting target molecules release with the addition of chaotropes in back extraction of reverse micelles.

Kallikrein is an enzyme present in blood plasma, pancreas, and other body fluids that catalyzes the proteolysis of kininogen to kinin, and used for the therapy of microcirculation disturbance. Kallikrein is conventionally separated by fractional acetone precipitation from pancreas, and then usually purified by chromatography, such as ion exchange [10], affinity adsorption etc. [11]. Those chromatography techniques are not easily scaled up in industry.

In this study, the purification of pancreatic kallikrein was performed using an easily scale-up system of reverse micelle with cationic surfactant CTAB in *n*-octane and *n*-hexanol. Kallikrein could be extracted well into the reversed micelles, but was difficult to be released from reverse micelles in back extraction. This study focuses on the improvement of back extraction, and the chaotropes

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(urea and GuHCl) was introduced to promote the release of target molecules from reverse micelles in back extraction. Dynamic light scatter analysis and water content in micelles was applied to explore effect of chaotropes on reverse micelles.

2. Materials and methods

2.1. Materials

Commercial product of porcine pancreatic kallikrein (PI = 3.9, MW = 26.8 kDa) was purchased from Dongkaiyuan Biochemical Institute (Beijing, China), and the crude material of porcine pancreatic kallikrein was friendly provided by Lantian Biochemical Factory (Changshu, China). Cetyl trimethylammonium bromide (CTAB), ethanol, *n*-octane, *n*-hexanol, and acetic acid were all supplied by Lingfeng Com. (Shanghai, China), and N-benzoyl-L-arginine ethyl ester (BAEE) was from Sigma. All chemicals were of analytical grade.

2.2. Methods

2.2.1. Forward extraction

0.1 g commercial product of kallikrein with specific activity (305.6 U mg^{-1}) was dissolved in 100 ml sodium carbonate buffer system (pH 9.0, 0.05 M) and used as aqueous phase of forward extraction. 1.0 g crude material of kallikrein with specific activity (31.3 U mg^{-1}) was added in 100 ml sodium carbonate buffer system (pH 9.0, 0.05 M) and dissolved by magnetic stirrer for 30 min, and then the supernatant collected by centrifuge was used as aqueous phase of forward extraction. In the aqueous phase, pH was adjusted to 9.0 by 0.1 M Tris–HCl, and 0.1 M KBr was added to adjust ionic strength. The organic phase was *n*-octane containing 20.0 mM CTAB as surfactant to form reverse micelles and *n*-hexanol (1:5, v/v) as the co-surfactant of CTAB.

During forward extraction, 3.0 ml solution of organic phase was added into equal volume of aqueous phase containing kallikrein from commercial product or crude material. The two-phase systems were mixed for 5 min on a vortex, and then were settled for 5 min for phase separation. The activity of kallikrein in aqueous phase was measured and the extraction percentage of forward extraction was calculated according to Eq. (1). The organic phase containing kallikrein was transferred into another tube for back extraction.

2.2.2. Back extraction

The stripping solution was 0.2 M acetic acid-sodium acetate buffer (pH 4.0) with different kinds of salts with different concentration (NaCl, KCl, KBr, $CaCl_2$ and $MgCl_2$). Ethanol was also added into the stripping solution to reduce the precipitate formation of complex. Here, chaotropes (urea and GuHCl) were firstly introduced in reverse micellar extraction to promote the release of the enzyme from micelles.

After forward extraction, the stripping solution was added to above organic phase containing reverse micelles (1:1, v/v) and mixed for 5 min on a vortex and then settled for above 10 min for phase separation. The activity of kallikrein in the stripping solution was measured and the extraction recovery of back extraction was calculated according to Eq. (2).

2.2.3. Analysis

Kallikrein activity was measured at 25 °C by using the method suggested by Schwert and Takenaka [12], in which BAEE was hydrolyzed by Kallikrein to cause an increase of absorbance at 253 nm. Considering BAEE is also hydrolyzed by trypsin, trypsin inhibitor was required when measuring the activity of kallikrein because the pancreatic crude material may contain trypsin. Protein concentration in the aqueous phase was detected by using Bradford method. Water content in the organic phase was measured by Karl Fischer method (SKF1, Super Scientech, Shanghai). Three duplicates were performed each experimental point for measurement of activity, protein concentration and water content. Sodium dodecyl sulfate (SDS)-polyacryl amide gel electrophoresis (PAGE) was performed using the electrophoresis apparatus (VE-180, Tanon, Shanghai, China) with 5.0% (w/v) stacking gel and 12.0% (w/v) separation gel.

After forward and back extraction, the size of reverse micelle was measured by dynamic light scattering analysis. It was performed with a multiple tau digital correlator (ALV-5000, ALV-Langen, Germany) and a JDS Uniphase He–Ne laser (vertically polarized beam, wavelength 632.8 nm. The range of scattering wave vectors (q) was 2.8×10^{-3} to 2.6×10^{-2} nm^{-1}, and the temperature was controlled to within 0.2 °C using a thermostat water bath.

2.2.4. Calculation

The activity of enzyme enwrapped in reverse micelle cannot be measured directly, and it was estimated by total activity of material minus that of residual aqueous phases after forward extraction. So the recoveries of forward and back extraction were evaluated based on the activity of enzyme in aqueous phases after forward extraction and back extraction. Their formulae were shown as follows.

The recovery of forward extraction (E) was obtained by Eq. (1):

$$E = \frac{U_{rmf} \times V_{rmf}}{U_{aqo} \times V_{aqo}} \times 100\% \quad (1)$$

The recovery of back extraction (E') was obtained by Eq. (2):

$$E' = \frac{U_{aqb} \times V_{aqb}}{U_{rmf} \times V_{rmf}} \times 100\% \quad (2)$$

The activity recovery of kallikrein (R) was obtained by Eq. (3)

$$R = \frac{Uaqb \times Vaqb}{Uaq0 \times Vaq0} \times 100\% \quad (3)$$

The purification factor (F) was obtained by Eq. (4),

$$F = \frac{U_{aqb}}{U_{aq0}} \times 100\% \quad (4)$$

where V was the volume of solution (ml), U was the activity of kallikrein (U ml^{-1} for Eqs. (1)–(3); U mg^{-1} protein for Eq. (4)), *aqo* was the initial aqueous phase with commercial or crude materials before forward extraction, *aqb* was the aqueous phase with purified kallikrein after back extraction, *rmf* was the organic phase with kallikrein enwrapped in reverse micelle.

3. Results and discussions

3.1. Back extraction without chaotrope

In forward extraction, nearly 100.0% kallikrein from commercial product was extracted into reverse micelles, as well as about 95.0% for crude material by optimized conditions such as pH, ion strength and concentration of surfactant (detailed data not shown). These conditions affect the size of reverse micelles and electrostatic interactions between proteins and reverse micelles, thus affect the extraction recovery. Hence they are usually used as effective methods to enhance the recovery in reverse micelle extraction. However, although those factors were also extensively optimized in back extraction, Kallikrein enwrapped in reverse micelle could not be released completely by those traditional methods. For example, several salts with different concentration (NaCl, KCl, KBr, $CaCl_2$ and $MgCl_2$) were added to adjust the ionic strength (data were not given). At 1.5 M KBr (pH 4.0) stripping phase, the back extraction recovery of kallikreins from commercial purity and crude materials were less than 20.0% under optimized conditions. Meanwhile, it was observed that the separation of two phases took long time, and there was white precipitate on the interface. This precipitate should be the complex of protein and surfactant molecule caused by the electrostatic interaction between surfactant polar head and oppositely charged groups of enzyme, and the interaction also induces the micelle aggregation, resulting in the loss of interesting protein and low recovery of back extraction [13].

When ethanol was added into the back striping solution containing 1.5 M KBr, the phases separation time was shorten greatly (only 10 min), and the recovery of back extraction was improved (Fig. 1). With the increase of ethanol concentration from 0 to 15.0%, the recovery of back extraction increased from 17.5% to 81.2% for kallikrein from commercial product. The similar trend also happens for kallikrein from crude material, and its maximum recovery was 39.5% in the presence of 20.0% ethanol. Ethanol molecule shows amphiphilic property as a co-surfactant. Its hydrophobic hydrocarbon chains can weaken the hydrophobic interactions between protein and surfactant/solvent, thus suppressing the formation of micellar cluster. However, ethanol with hydrophilic hydroxyl group and high water-miscible makes the interface more flexible, thus reverse micelles may easily coalesce at the interface and proteins can better penetrate through the interfacial layer of reverse micelles into the stripping phase [13]. But high concentration of ethanol has denaturing effect for proteins and causes some loss of kallikrein activity.

3.2. Effect of chaotrope urea on back extraction

Although the ethanol in stripping phase could improve the recovery of back extraction, about 20.0% activity for commercial