Communication to the Editor

Protein Extraction Using the Sodium Bis(2-ethylhexyl) Phosphate (NaDEHP) Reverse Micellar System

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The reverse micellar system of sodium bis(2-ethylhexyl) phosphate (NaDEHP)/isooctane/brine was used for liquid–liquid extraction of proteins. We investigated the solubilization of cytochrome-c and α-chymotrypsin into the NaDEHP reverse micellar phase by varying the pH and NaCl concentration in the aqueous phase. At neutral pH and relatively low ionic strength, the proteins are extracted into the micellar phase with high yield. By contacting the micellar phase with a divalent cation (e.g., Ca^{2+}) aqueous solution, the reverse micelles are destabilized and release the protein molecules back into an aqueous solution for recovery. This method separates the proteins from the surfactant with very high overall efficiencies.

Key words: protein extraction • reverse micelle

INTRODUCTION

As a promising technique for protein isolation, transfer of proteins from an aqueous phase to a reverse micellar phase and vice versa has been extensively investigated.[1,3,7,11,14] It is generally believed that the primary driving force for solubilization of protein molecule into the reverse micelle water pool is the attractive electrostatic interaction between the protein molecule and the reverse micelle inner charge layer. Because the electrostatic interaction can be modified by changing the pH and ionic strength in the aqueous phase, the transfer efficiency of protein extraction is highly dependent on the pH and ionic strength of the aqueous phase.[9,12,13,16,17,19,23]

The most often-used surfactant in the protein extraction study, is bis(2-ethylhexyl) sodium sulfosuccinate (AOT). This is primarily due to the fact that AOT can form reverse micelles without a cosurfactant. Despite its popularity for researching model systems, AOT has a number of very serious limitations: Once proteins are extracted into the AOT reverse micellar phase, it is difficult to separate the proteins from the surfactant and the phase separation of the AOT system takes a long time. These problems[7,14] might be overcome by using other surfactant systems.

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In the present investigation, we used sodium bis(2-ethylhexyl) phosphate (NaDEHP) as the surfactant. NaDEHP is an anionic surfactant which has the same hydrocarbon tail as AOT, but a different polar head. The properties of the NaDEHP reverse micellar system are quite different from that of AOT. It has been known that NaDEPH can form a water-in-oil (w/o) microemulsion or reverse micellar solution under certain conditions.[5,6,18,21,22] Using our previous experience in extraction of heavy metal ions with NaDEHP reverse micelles, we concluded that this surfactant could be superior to AOT for extraction of proteins. The most attractive characteristic of NaDEHP is that its reverse micelles can be easily broken by converting the sodium salt, NaDEHP, to a non-surface-active divalent metal salt, $M(DEHP)_2$; also, the phase separation of NaDEHP is much faster than that of AOT. In addition, the surfactant can be readily recycled. The major goal of this study was to demonstrate that NaDEHP reverse micellar solutions can be used very effectively for protein extraction.

EXPERIMENTAL DETAILS

Materials

Cytochrome-c from the horse heart was obtained from Sigma, and α-chymotrypsin from the bovine pancreas was obtained from Fluka. Both proteins were used as received. Aqueous solutions (40 μM) of the proteins were prepared by dissolving the proteins in a solution of 0.025 *M* Tris-HCl and 0.2 *M* NaCl. Bis(2-ethylhexyl) phosphoric acid (HDEHP) was obtained from Aldrich with a purity of 97%. Tributyl phosphate of 99% purity was obtained from Fluka. *N*-glutaryl-L-phenylalanine-*p*-nitroanilide (GPNA) used for α-chymotrypsin enzymatic activity assay was obtained from Sigma with a purity of 99%. Spectroscopic grade isooctane was used as organic solvent, and deionized water was used for preparing all aqueous solutions.

Preparation of NaDEHP Reverse Micellar Phase

NaDEHP/isooctane/brine reverse micellar solutions were prepared by mixing equal volumes of 0.1 *M*

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HDEHP isooctane solution with 0.1 *M* NaOH and 0.2 *M* NaCl aqueous solution. The pH value of the aqueous phase was adjusted to 7 to 7.5, by adding NaOH or HCl solution. The NaDEHP reverse micelles were formed in the upper phase after the phase separation. Tributyl phosphate was added to the organic solution as a modifier to prevent the formation of the middle phase and to facilitate the phase separation. The mole ratios of TBP/NaDEHP were 1 and 2 for α-chymotrypsin and cytochrome-c extraction, respectively.

Forward Extraction

Forward extraction experiments were performed by contacting the reverse micellar phase with an equal volume of an aqueous protein solution in a Teflon-capped glass test tube at room temperature (24 ± 1°C). The tube was shaken vigorously for 5 min, then centrifuged (2000 rpm, 2 min) for phase separation. After centrifugation, both phases were transparent with a distinct phase boundary. The protein concentration in the aqueous phase was measured using UV spectroscopy and the protein concentration in the organic phase was determined by mass balance.

Backward Transfer

Backward transfer was carried out by using a 0.1 *M* $CaCl_2$ aqueous solution of neutral pH as the stripping solution. The protein loaded reverse micellar solution was contacted with an equal volume of the stripping solution in a glass tube by shaking the tube vigorously for 5 min, and then centrifuged mildly for phase separation.

Analytical Methods

Ultraviolet-visible spectroscopic measurements were carried out with a HP 8451A diode array spectrophotometer. Cytochrome-c concentration was determined using 410-nm (Soret band) absorbance, and α-chymotrypsin was determined using 280-nm absorbance. The water content in the organic phase, and the concentrations of the surfactant and TBP, before and after forward/backward transfers, were determined by infrared spectroscopy. Dynamic light scattering was used to measure the size of the reverse micelles before and after the forward/backward extraction.

Enzymatic Activity of α-Chymotrypsin after Recovery from the Micellar Phase

To make sure that the extraction process did not lead to protein denaturation we measured the α-chymotrypsin activity by using GPNA as substrate.[4] When α-chymotrypsin containing aqueous solution was added to the substrate, *p*-nitroaniline (yellow in color) was released. GPNA substrate solution was prepared by dissolving 20 mg of GPNA in 1 mL of methanol, and then added to 50 mL of 0.05 *M* Tris-HCl buffer of pH 7.5. The activity measurements were carried out by adding 0.5 mL of aqueous solution containing the enzyme to 2.5 mL substrate solution for 10 min, then 0.5 mL of 30% acetic acid was added to stop the reaction. The enzyme assay was carried out on both fresh and recovered α-chymotrypsin aqueous solutions. The overall recovery rate of the enzyme activity was determined by using the 410-nm UV absorbance band.

RESULTS AND DISCUSSION

Forward Transfer

Forward extraction was carried out by mixing 2 mL 100 m*M* NaDEHP reverse micelle solution with 2 mL of a protein aqueous solution with chosen pH and salt concentration. Under appropriate conditions, over 90% of the α-chymotrypsin and nearly 100% of the cytochrome-c can be transferred into the NaDEHP reverse micellar phase. After stopping shaking of the biphasic system of cytochrome-c aqueous solution/NaDEHP reverse micellar phase, a distinct phase boundary was obtained in less than 5 min by gravity only.

We observed that the addition of TBP was necessary to inhibit the formation of a middle phase or Windsor III microemulsion system. Without TBP, the NaDEHP/isooctane/aqueous salt solution system will form a Windsor I o/w microemulsion at low salt concentrations, and will form a third phase between the organic and aqueous phase over a wide range of salt (NaCl, KCl, etc.) concentration.

Effect of pH on the Protein Transfer Efficiency

As shown in Figure 1, the transfer percentage into the reverse micellar phase is a strong function of the aqueous phase pH. The isoelectric point pI values of cytochrome-c and α-chymotrypsin are 9.9 and 8.5, respectively. We see that the percent transfer sharply drops with pH near the protein pI value. This is due to the weakening of the attractive interaction between the protein molecule and the negatively charged reverse micelle inner charge layer.

Effect of Salt Concentration in the Aqueous Phase on the Transfer Efficiency

As shown in Figure 2, the transfer percentage is also a strong function of the NaCl concentration in the aqueous phase. In these measurements, the pH values in the aqueous solutions were maintained at pH 6 to 7. The primary effect of salt is through shielding of the electrostatic interaction between micellar wall and protein. As

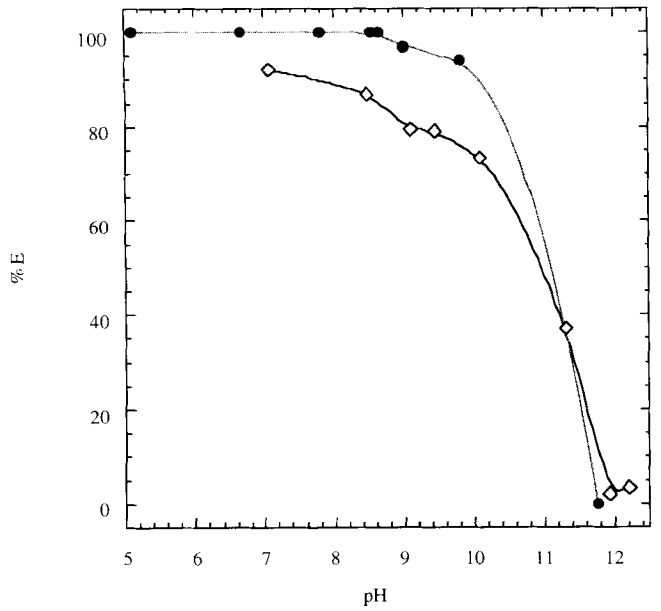

Figure 1. Effect of pH on protein transfer percentage. Solid circles: cytochrome-c; open diamonds: α-chymotrypsin.

salt concentration increases, the attractive interaction diminishes due to the Debye screening, and the transfer percentage declines.

Backward Transfer

In the past, the basic idea of backward transfer was to choose the pH and salt conditions that had minimal forward transfer efficiency. By using this approach, only a small portion of the protein is recovered.[8,12,14,20] In Rahaman et al.'s[20] work on the recovery of an extracellular alkaline protease, the backward yield was on the order of 10% to 20%. The difficulty of the backward transfer was attributed to kinetic limitations.

Recently, some alternative methods for recovering proteins from the reverse micellar phase have been investigated. Leser and Luisi used silica particles to absorb proteins as well as surfactant and water directly from the protein-loaded micellar phase.[14,15] Another method is to add a large amount of a second organic solvent, such as ethyl acetate, to destabilize the reverse micelle and release the protein.[2,23] Gupta et al.[10] dehydrated the micelle with molecular sieves to recover the protein.

We used a different approach to achieve overall recoveries of 98% for cytochrome-c and 67% for α-chymotrypsin. The method is based on the intrinsic properties of the surfactant NaDEHP. The alkali salts of HDEHP are surface active, and can readily form a reverse micelle system. However, the divalent metal salts, $M(DEHP)_2$, are not surface active. In addition, the divalent salts are very soluble in organic solvents and virtually insoluble in water. Therefore, the reverse micelle of NaDEHP can readily be broken down by converting the surfactant, NaDEHP, to a non-surface-active divalent salt, $M(DEHP)_2$. Hence, the protein in the micellar phase is released back to a fresh aqueous solution. We used 0.1 *M* $CaCl_2$ as the stripping solution to break the reverse micelles. When this aqueous solution is mixed with a protein-loaded NaDEHP reverse micellar phase, each calcium cation Ca^{2+} will react with two NaDEHP molecules and form a stable, relatively hydrophobic complex of $Ca(DEHP)_2$:

$$Ca^{2+} + 2\ NaDEHP \rightleftharpoons Ca(DEHP)_2 + 2\ Na^+$$

At a neutral pH condition, the equilibrium is shifted strongly to the right. Consequently, the NaDEHP reverse micelles are destroyed, and the proteins are released into the aqueous phase.

The divalent metal salt of HDEHP can be easily recycled and transferred back to NaDEHP. HDEHP has been extensively used as an extractant in hydrometallurgical industrial and nuclear fuel waste processing.

The diameter of the micelles were measured to be 5 nm before forward transfer by light scattering. The micelle size remains the same after forward extraction. After backward transfer, however, no micelles were detectable in the upper phase. We also made FTIR measurements to study the changes in the upper phase after the cytochrome-c forward/backward transfer. The infrared spectra showed the presence of significant amounts of water in the upper phase before and after forward transfer. However, the amount of water decreased by at least an order of magnitude after the backward transfer of the protein, confirming the light scattering results that there are no reverse micelles in the upper phase after contacting with $Ca(Cl)_2$. The IR specta also

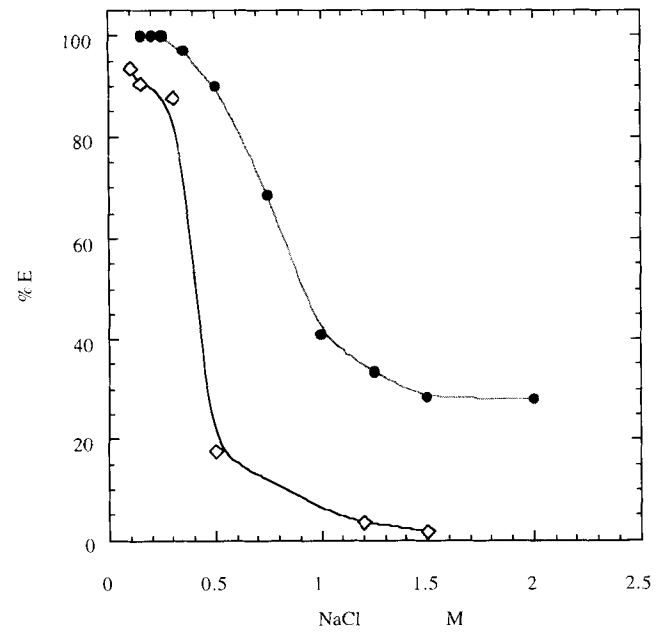

Figure 2. Effect of NaCl concentration on protein transfer. Solid circles: cytochrome-c; open diamonds: α-chymotrypsin.

showed that all of the surfactant and TBP remained in the upper phase.

α-Chymotrypsin

The extraction of α-chymotrypsin is similar to that of cytochrome-c, except the TBP concentration is 100 m*M* in the upper phase. We determined the overall protein recovery rate to be 67.6% by using absorbance at 280 nm. After phase separation of backward transfer, a very thin layer of white precipitate was observed at the interface of the organic and aqueous phases which is probably a mixture of the surfactant and the protein. This might be the reason for the relatively low recovery rate of α-chymotrypsin. We then measured the enzymatic activity of α-chymotrypsin using the enzymatric assay method described previously. The results of the activity assay showed that the extraction process did not denature α-chymotrypsin at all. On a per-unit weight basis there was no detectable activity loss.

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