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Protein Extraction by New Reversed Micelles with Di(Tridecyl) Phosphoric Acid

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ABSTRACT

Di(tridecyl) phosphoric acid (DTDPA) was synthesized as a new surfactant for protein extraction by reversed micelles. DTDPA can form stable reversed micelles in the concentration range above 1 mM. The size of the DTDPA reversed micelles was smaller than that of conventional AOT (sodium-di-2-ethylhexyl sulfosuccinate) reversed micelles under the same conditions. The formation of reversed micelles using DTDPA can be controlled by adjusting the aqueous pH. The extraction behavior of some proteins from an aqueous phase into the reversed micellar phase of DTDPA has been investigated by varying the experimental conditions (pH, ionic strength, surfactant concentration). It was found that reversed micelles with DTDPA can extract many proteins. In particular, hemoglobin, which is difficult to extract with an AOT reversed micelle, can be extracted in the DTDPA reversed micelles. In the new reversed micellar system, phase separation was very fast even at a high concentration of DTDPA.

INTRODUCTION

An oil and water usually do not mix homogeneously. However, if a small amount of surfactant is added to the solution, the oil and water can be mixed and a stable emulsion is formed. An amphiphilic molecule, which is composed of a hydrophilic group and hydrophobic long alkyl chains,

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can reduce the interfacial tension between an oil and water to almost zero. These molecules form a stable aggregate, including a small amount of water in an organic solvent. A molecular aggregate of less than about 10 nm is called a reversed micelle, and the organic solution is transparent. A reversed micellar solution has inner cores of water ("water pools"). The size of water pools almost corresponds to that of proteins, so that reversed micellar solutions have evoked interest as useful media to dissolve many enzymes in an organic solvent since 1979 (1).

In 1985, Göklen and Hatton indicated that a reversed micellar solution is an attractive tool for the extraction and separation of proteins (2). Since then, extraction and separation of many proteins have been investigated with reversed micellar solutions (3–9). However, in most works reported, the conventional surfactant AOT (sodium-di-2-ethylhexyl sulfosuccinate) has been used to form reversed micelles. Little attention has been given to finding new reversed micellar systems for protein extraction and separation. It is well known that the surfactant plays an important role in the extraction system because proteins are dissolved in water pools formed by surfactants in an organic media.

Peng and Luisi used lecithins of variable chain length to form reversed micelles (4), and Hilhorst et al. studied the reversed micellar system formed by a cationic surfactant (7). However, in these systems there was a crucial defect in that a cosurfactant is needed in order to obtain a water pool large enough to host large molecules like proteins. No studies have ever tried to protein extraction with reversed micelles formed by one component of a surfactant except for the AOT reversed micellar system. The purpose of this study is to develop a new reversed micellar system for the extraction and separation of proteins. In this technology it is necessary to design a new surfactant to form a stable reversed micelle and to determine the effect of the surfactant on the extraction behavior of proteins. In a previous study we synthesized dioleyl phosphoric acid (DOLPA) as a new surfactant (10), and it was found that an organophosphorous type of a surfactant is available for protein extraction. In the DOLPA system, however, there was a problem concerning phase separation in a concentration range higher than 0.1 M. In such a high DOLPA concentration range, a stable emulsion is formed at the oil–water interface and centrifugation is necessary to obtain clear solutions.

For this article we have synthesized di(tridecyl) phorphoric acid (DTDPA) as a new surfactant. This surfactant has the same hydrophilic part as DOLPA but it has different alkyl chains. Tridecyl alcohol, the hydrophobic part of DTDPA, is commercial available and is very cheap. The extraction behavior of some proteins from an aqueous phase into the

reversed micellar phase has been investigated with DTDPA, and the results have been compared with those of the conventional surfactant AOT.

EXPERIMENTAL

Materials

DTDPA was synthesized according to the reaction scheme shown in Fig. 1. Tridecyl alcohol, which includes several isomers, and phosphorus oxychloride in a 2:1 molar ratio were dissolved in dry benzene, and the solution was refluxed for 24 hours. Then dilute hydrochloric acid was added to the solution and it was stirred with a magnetic stirrer for 3 hours. After the upper organic solution was separated from the aqueous solution, the benzene solution was dried over anhydrous magnesium sulfate. The benzene was removed under reduced pressure. The final product was purified by a column chromatography, and it was identified by ^{1}H-NMR and FT-IR. Sodium-di-2-ethylhexyl sulfosuccinate (commercial name AOT) was supplied by Mitsui Cyanamid Co. and used without further purification. Sodium di(tridecyl) sulfosuccinate (commercial name ATR) was supplied as an ethanol solution by American Cyanamid Company. ATR was used after removing the ethanol. The detailed structure and abbreviation of the surfactants used in this study are shown in Fig. 2. Four kinds of proteins were used: cytochrome c (Bovine Heart, C-2037), hemoglobin (Bovine, H-2500), lysozyme (EC 3.2.1.17), and α-chymotrypsin (EC 3.4.21.1). All proteins were purchased from Sigma Chemical Co. and used as received. The other inorganic and organic reagents were reagent grade.

Protein Extraction by Reversed Micelles

Extraction experiment was carried out by a phase transfer method. Reversed micellar solution was prepared by adding an equal volume of organic phase to an aqueous phase containing a protein. In the extraction experiment the concentrations of lysozyme and α-chymotrypsin were 0.5 g/L and that of cytochrome c or hemoglobin was 0.2 g/L. The organic

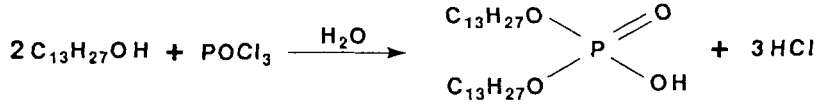

FIG. 1 Synthetic scheme of DTDPA.

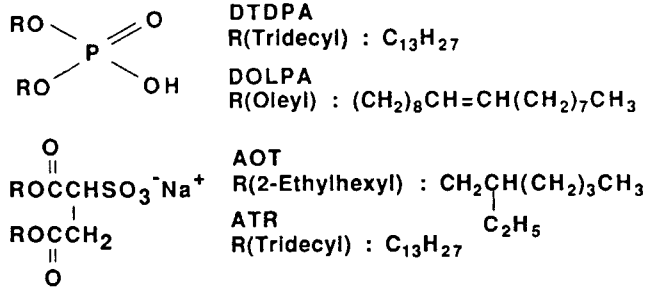

FIG. 2 Molecular structures and abbreviations of surfactants used.

phase was prepared by dissolving a surfactant in isooctane. The aqueous solution was prepared by adjusting the pH with a phosphate buffer of 50 mM and the ionic strength with potassium chloride. The reversed micellar solution was shaken in a flask immersed in a thermostated water bath at 303 K and allowed to reach equilibrium. After about 24 hours the two phases were separated. The water content in the organic solvent was determined by Karl Fischer titration (Mitsubishi Chemical Industries Limited CA-05). The concentration of proteins was measured using a UV/VIS spectrophotometer (JASCO UVIDEC-670) at 408 nm (cytochrome c), at 406 nm (hemoglobin), or at 282 nm (lysozyme and α-chymotrypsin). The aggregate number of reversed micelles made with different surfactants was measured with a Photal (Otsuka Elec.) dynamic light-scattering spectrometer (DLS-700) equipped with a 75-mW Ar laser.

RESULTS AND DISCUSSION

Characteristics of Reversed Micelles with DTDPA

Figure 3 indicates the relation between the concentration of surfactants and the amount of water taken into the reversed micelles. DTDPA can form reversed micelles in the concentration range above 1 mM. The water-to-surfactant mole ratio (W_0) in an organic solution is about 6 in the DTDPA system; this value is smaller than that of the conventional commercial surfactant AOT (about 10) under the same experimental conditions. DTDPA has the same hydrophobic group as ATR, but its W_0 value is different than that of ATR. On the other hand, AOT has the same hydrophilic group and a different hydrophobic group than ATR. The W_0 value of AOT is almost same as that of ATR. DTDPA also has the same hydrophilic group and a different hydrophobic group than DOLPA. How-

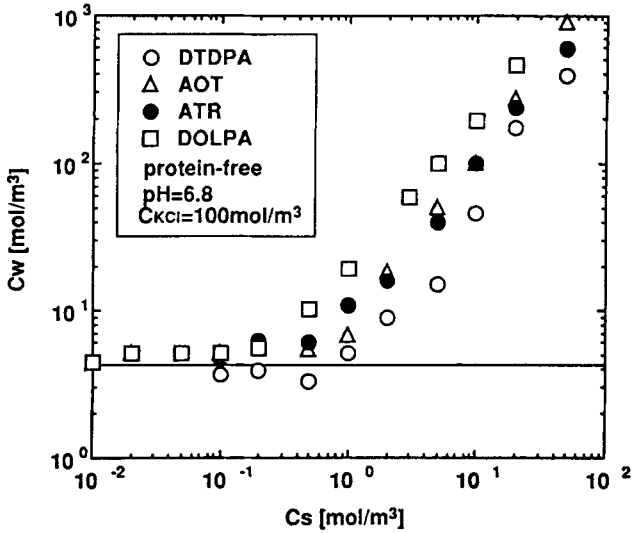

FIG. 3 Relation between concentration of surfactants and amount of water dissolved in reversed micelles. The solid line shows the saturated solubility of water to isooctane.

ever, the W_0 value of DTDPA is different than that of DOLPA. These results suggest that the formation of reversed micelles with a phosphoric-acid-type surfactant strongly depends on the structure of the hydrophobic part of the surfactant.

Table 1 shows the apparent molecular weight (weight-average) of an aggregate and the association number of a surfactant in reversed micelles made with different surfactants. The aggregates of DTDPA are smaller than those of DOLPA or AOT. One reason for this is that the tridecyl

TABLE 1
Molecular Weight and Association Number of Reversed Micelles[a]

Surfactant	MW_{RM}	MW_S	N
DTDPA	9,094	462.7	19
DOLPA	19,270	598.9	32
AOT	17,540	444.6	39

[a] MW_{RM} = molecular weight of reversed micelle. MW_S = molecular weight of surfactant. N = aggregate number. C_S = 20 mM, C_{KCl} = 0.1 M, pH 6.8.

group in the hydrophobic part is a bulky structure. We confirmed by ^{1}H-NMR that tridecyl alcohol includes several branched isomers, however, the detailed structure of the branched isomers cannot be identified. The DTDPA molecule cannot closely pack at the interface because of steric hindrance due to the hydrophobic part in the surfactant.

Figure 4 shows the relation between water content dissolved by reversed micelles and the pH of the aqueous solution. The water content when AOT was the surfactant was held constant throughout the experimental pH range. However, in the case of DTDPA the water content rapidly increases with a pH higher than 6. Reversed micelles can only be formed by anionic species of DTDPA. At low pH, reversed micelles are not formed because a proton in the phosphorus group of the surfactant does not dissociate. The formation of reversed micelles using DTDPA can be controlled by adjusting the aqueous pH.

Protein Extractions

Figure 5 shows the extraction of some proteins with DTDPA reversed micelles. Cytochrome c and lysozyme can be extracted completely under optimum conditions. Although 50% of α-chymotrypsin was extracted, a white membranous precipitate of α-chymotrypsin was observed at the interface in the high concentration range of DTDPA.

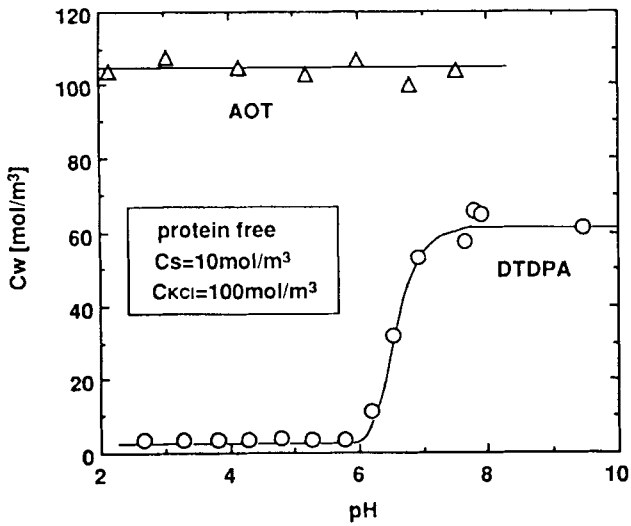

FIG. 4 Relation between water content dissolved by reversed micelles and pH in the aqueous solution.

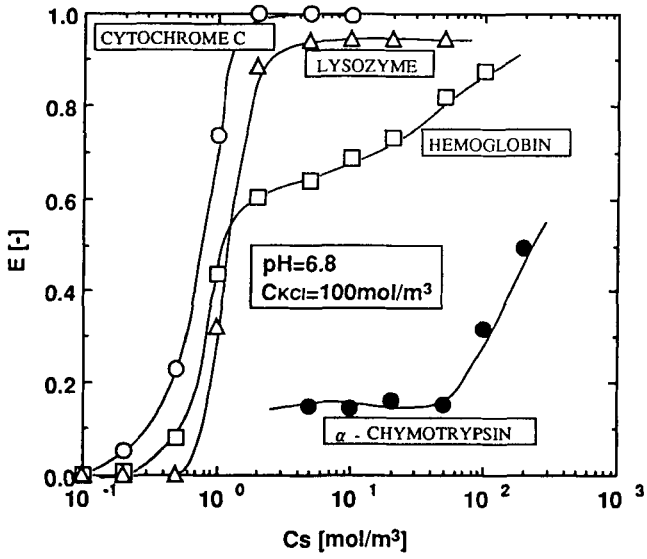

FIG. 5 Protein extraction with DTDPA reversed micelles.

Figure 6 indicates the pH dependence in the extraction of hemoglobin. Hemoglobin is not extracted with AOT reversed micelles due to its large molecular weight. However, hemoglobin can be extracted with DTDPA reversed micelles in a high concentration range of the surfactant. One reason is that the hydrophobic group of DTDPA is large compared to that of AOT. In order to dissolve a large protein in an organic solvent, high hydrophobicity of the surfactant is necessary. Larger proteins require a larger interaction with the charged residues on their surface in order to be transferred into reversed micelles. When ATR, which has the same hydrophobic group as DTDPA, was used, a red precipitate was produced at the oil–water interface. In the case of AOT, the same precipitate was produced. These results means that a phosphoric-acid type of surfactant is preferred for hemoglobin extraction. In the extraction of cytochrome c by AOT reversed micelles, the following adsorption process of surfactants has been reported for a low concentration range of AOT (11):

$$\text{cyt c} + n\text{AOT} \rightarrow (\text{cyt c})(\text{AOT})_n \rightarrow \text{precipitates}$$

In the AOT reversed micellar system, the red precipitates at the interface were filtered off and dried under reduced pressure. In order to determine whether the precipitate is a denatured hemoglobin or a complex of hemoglobin and AOT, we investigated the sample by elemental analysis.

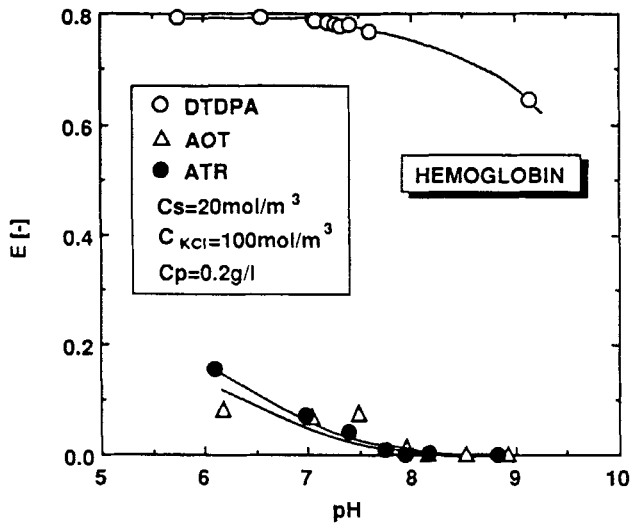

FIG. 6 Relation between degree of extraction of hemoglobin and pH in aqueous solution.

Table 2 shows the results of the elemental analysis of the red precipitates. From the results we found that the red precipitates are a complex of hemoglobin and about 120 molecules of AOT. The adsorption of an AOT molecule on the surface of hemoglobin changes the conformation of the protein, which causes denaturation of the protein at the oil–water interface.

Figure 7 shows the relation between protein extraction and pH in the aqueous solution with DTDPA reversed micelles. When the surface of a protein has a positive charge, reversed micelles of DTDPA can extract the proteins. At low pH, the proteins cannot be extracted because the reversed micelles are not formed, as shown in Fig. 4. The results obtained are consistent with the assumption that electrostatic interactions are dominant by reversed micelles in protein extraction (2, 8).

TABLE 2
Elemental Analysis Result of Red Precipitates

	C (%)	H (%)	N (%)
Hemoglobin	50.4	7.2	15.4
AOT	53.9	8.4	
Red precipitate	52.1	7.7	6.3
Hemoglobin$(AOT)_{120}$	52.4	7.9	6.6

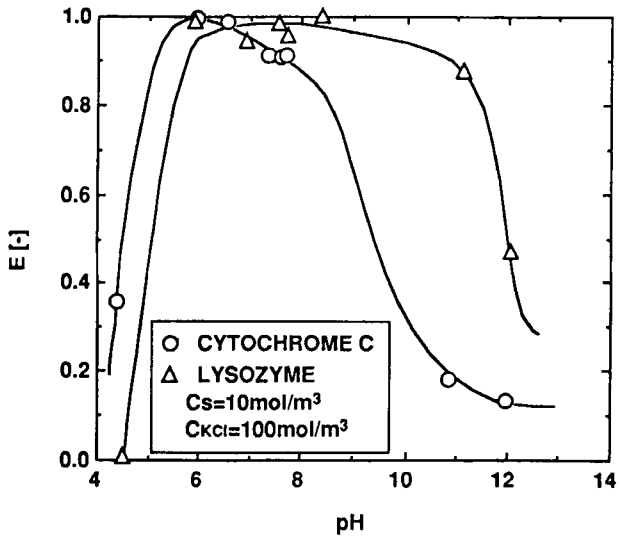

FIG. 7 Relation between the degree of extraction and aqueous pH with DTDPA reversed micelles.

Figure 8 shows the relation between protein extraction and the concentration of potassium chloride. The extracted ratio of the protein decreases with increasing concentration of potassium chloride. This decrease is due to electrostatic hindrance by the salt effect. The presence of a high concentration of salt diminishes the attractive interactions and can lead to expulsion of solubilized proteins. This characteristic can be used in the stripping operation of the extracted proteins.

Figure 9 shows the extracted ratio of protein by the other commercial surfactants. The protein used was cytochrome c. DTDPA shows a high extracted ratio compared with sulfuric-type surfactants. This result also indicates that a phosphoric group in the hydrophilic part of the surfactant is preferred for protein extraction. At a high concentration of AOT, cytochrome c is extracted completely; at a low concentration of AOT, a red precipitate is produced. This reason is that the concentration of AOT in an organic solution is not enough to transform cytochrome c into an amphilic complex. The driving force of cytochrome c extraction in reversed micelles is the transformation of native hydrophilic cytochrome c into an amphiphilic form due to adsorption of AOT (11). In the case of ATR, the extracted ratio increases with increasing concentration of ATR. However, reversed micelles of ATR cannot extract more than 60% of cytochrome c. The W_0 value of AOT is almost the same as that of ATR. This means

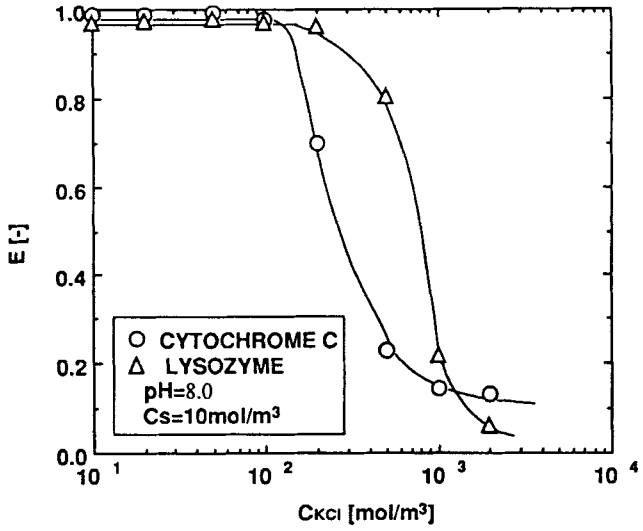

FIG. 8 Relation between degree of extraction and concentration of potassium chloride.

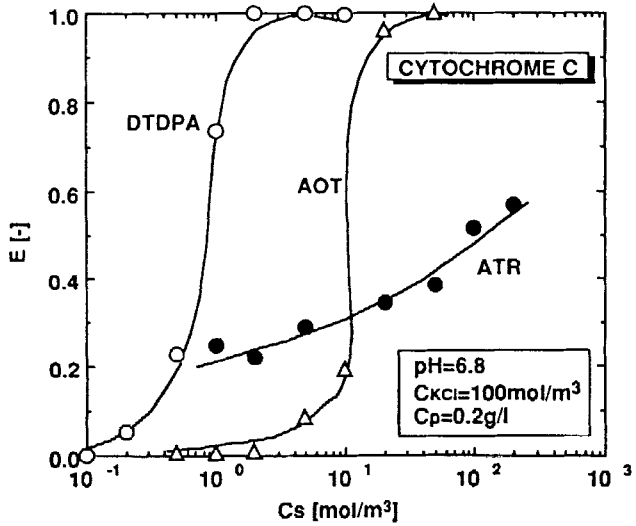

FIG. 9 Degree of extraction of cytochrome c by reversed micelles with several surfactants.

the reversed micelles of AOT are almost the same size as the reversed micelles formed by ATR. However, the extraction ability of AOT is much different than that of ATR. There is no relation between the size of reversed micelles and the transfer properties of proteins, even if the surfactants have the same hydrophilic group. This means that the structure of the hydrophobic group is an important factor in protein extraction.

Further, phase separation of reversed micelles with DTDPA is very fast in protein extraction even at concentrations above 0.1 M, which is not true for conventional systems.

CONCLUSIONS

A new reversed micellar system with DTDPA has been developed for the extraction and separation of proteins. The formation of reversed micelles with DTDPA can be controlled by adjusting the aqueous pH. The results of protein extraction are consistent with the assumption that electrostatic interactions are dominant. Hemoglobin, which is difficult to extract by conventional AOT reversed micelles, can be extracted in the DTDPA reversed micelles. Phase separation was very fast in the new reversed micelles, even with a high concentration of DTDPA.

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