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ORIGINAL CONTRIBUTION

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Forward and backward extractions of cytochrome c using reverse micellar system of sucrose fatty acid ester

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Abstract The effects of solvent, pH, and ionic strength on the reverse micellar extraction of cytochrome c have been examined, when sucrose fatty acid esters were employed as surfactants of reverse micelles. The transparent and stable reverse micellar organic phase was formed, when the mixture of isooctane and *n*-butanol (7:3 v/v) was used as the bulk organic phase. The high forward extraction ratio was obtained under mild alkaline and low ionic strength conditions, while the high backward extraction ratio was obtained for acidic pH values or at high ionic strength. The activity of cytochrome c recovered from the reverse micellar phase was sufficiently retained.

Keywords Reverse micelle · Extraction · Protein · Nonionic surfactant · Sucrose fatty acid ester

Introduction

Nowadays, various proteins have widely been produced in the research on biochemical study and in chemical, food, and pharmaceutical industries by means of the conventional fermentation and new biotechnological techniques such as recombinant DNA [1]. The reverse micelles, which are the nanometer-sized aggregates of the surfactant molecules and water molecules dispersed in the hydrophobic organic phase like octane, have recently been applied to the liquid–liquid extraction of protein, since various hydrophilic biological molecules can be solubilized into the micro water pool of the reverse micelle [2–7]. The process of so-called reverse micellar extraction, in general, consists of the forward extraction of the protein from the feed aqueous phase to the reverse micellar organic phase and the backward extraction of the protein from the reverse micellar organic phase to the recovery aqueous phase. As seen in most previous studies using the ionic surfactants such as bis(2-ethylhexyl) sodium sulfosuccinate (AOT), trioctylmethylammonium chloride (TOMAC), and cetyltrimethylammonium bromide (CTAB), the forward extraction is relatively carried out at the rapid rate and at the high efficiency, while the backward extraction proceeds at the low rate; and it is often difficult to recover the proteins from the reverse micellar organic phase, and the proteins are often denatured and deactivated due to the diffusion-determining on the interface between the organic and aqueous phases, the strong electrostatic interaction of the ionic surfactants with the proteins entrapped in the reverse micelles, and so on. In order to improve the protein recovery, hydrophilic organic solvents such as isopropanol (IPA) and ethyl acetate are added, which cause the reverse micellar organic phase containing the protein to be unstable, and thereby the proteins entrapped in the reverse micelle are transferred to the recovery aqueous phase [8]. As the other methodology improving the protein recovery, we have reported that the gas such as 1,1,1,2-tetrafluoroethane was added to the AOT reverse micellar organic solution containing the protein at moderate pressure (3–5 atm) and around 273 K [9, 10]. The gas molecules form the gas hydrates with the water molecules in the reverse micelles, and thereby the solid protein is precipitated out with the gas hydrate. On the other hand, in

order to avoid the undesirable strong interaction between the hydrophilic group of surfactant and the protein entrapped in the reverse micelles, the use of the nonionic surfactants such as Tween and Span and the natural surfactant such as lecithin have been proposed [11–15]. However, for instance, in the reverse micellar extraction using nonionic surfactant sucrose fatty acid ester, the protein cannot be backward-extracted by contacting the reverse micellar organic phase with the recovery aqueous solution, although the high forward extraction efficiency is achieved [11].

In order to achieve the high activity yield of protein through the reverse micellar extraction, many studies using the ionic surfactant on the forward extraction process and the improvement of backward extraction process have been reported, whereas the studies using nonionic surfactant have been rare. In our present work, we have addressed a question of whether the protein was conventionally backward-extracted at the high efficiency and at the fine repeatability by optimizing the kind of bulk organic solvent in the reverse micellar organic phase containing the crude nonionic surfactant for the industrial use. The reverse micellar extraction using nonionic surfactants such as series of alkyl sorbitan esters (Spans, Tweens), linear alcohol ethoxylate (Neodol 91–2.5), alkyl phenol ethoxylates (lgepal CO-520, Trycol 6985), and sucrose fatty acid ester (DK-F-110) has been studied [11–14]. We have employed the sucrose fatty acid ester as a nonionic surfactant, since sucrose fatty acid ester is widely utilized as an emulsifier in the food industry and prevents the denaturation of milk protein [16, 17]. Cytochrome c was used as a model protein, since cytochrome c has been thoroughly investigated in such extraction [3, 4, 10–13].

Experimental

The majority of investigations in this field have employed either the anionic surfactant Aerosol OT (AOT) or cationic quaternary ammonium salts. The investigations described here employed the nonionic surfactant sucrose fatty acid ester DK-F-110, which was an equivalent weight mixture of sucrose monoester and polyester (diester to pentaester) of stearic acids, supplied from Dai-Ichi Kogyo Seiyaku (Kyoto, Japan). The surfactant was used without further purification. The hydrophile–lipophile balance (HLB) value of the DK-F-110 was 11. Cytochrome c from horse heart (MW = 12,400, pI = 10.1) was obtained from Sigma-Aldrich Co. Hexane, isooctane, hexadecane, IPA, *n*-butanol, and 1-octanol were obtained from Kanto Chemicals (Tokyo, Japan), and were of analytical grade.

In order to measure the solubility of DK-F-110 in organic solvents at 25 °C, the saturated surfactant solution was prepared, evaporated, dried in vacuo, and then the dried surfactant was weighed.

All equilibrium microemulsions were prepared as follows. Ten milliliters of the organic solution containing a certain amount of DK-F-110 and 10 mL of 0.1 M KCl aqueous solution were mixed in the 50-mL screw vial, incubated at 25 °C and 80 rpm for 30 min, and then stood at 25 °C overnight. The phase behavior of the mixture was visually estimated. The strong or middle turbidity was visually observed, and the low turbidity was spectrophotometically checked by UV–Vis spectrophotometer (Ubest-55, Japan Spectroscopic Co. Ltd.).

The solubilization of water to the organic phase containing DK-F-110 was carried out as follows. Ten milliliters of isooctane/*n*-butanol (7:3 (v/v)) containing a certain amount of DK-F-110 and 10 ml of distilled water were mixed in the 50-mL screw vial at 25 °C and 120 rpm for 1 h. After the incubation, the organic phase was centrifuged at 4000 rpm for 30 min. The water concentration of the organic phase after centrifugation was determined by the optimized Karl Fisher potentiometric titration using a Hiranuma AQ-6 aquacounter.

The forward extraction was carried out by mixing equal volumes (10 mL) of the organic solution and the aqueous solution in the 50-mL screw vial at 25 °C and 120 rpm for 1 h. The organic phase was an isooctane/*n*-butanol (7:3 (v/v)) containing 50 g/L DK-F-110, while the aqueous phase was a 10 μM cytochrome c buffered solution at an appropriate pH value and with a certain amount of KCl. The feed and recovery aqueous solutions used in this study were an acetate buffer solution at pH 4, phosphate buffer solutions at pH 6, 7, and 8, borate buffer solutions at pH 9 and 10, and sodium hydrogen phosphate buffer solutions at pH 11 and 12. The concentration of buffer solution was prepared at 0.01 M.

In the process of the backward extraction, 10 mL of the reverse micellar organic solution containing 13.5 μM cytochrome c and 132 μL of pH 8 phosphate buffer were mixed with 10 mL of the recovery aqueous solution in the 50-mL screw vial at 25 °C and 120 rpm for 1 h. The pH values and KCl concentrations in the recovery aqueous solution were prepared similarly to the case of the feed aqueous solution. After the forward and backward extractions, the mixture was stood until the organic phase and the aqueous phase were separated distinctly. The volume of organic phase was checked by a graduated cylinder. The organic phase was centrifuged at 4000 rpm for 30 min. After centrifugation, the concentration of cytochrome c in the organic phase was measured spectrophotometically at 415 nm by UV–Vis spectrophotometer (Ubest-55, Japan Spectroscopic Co. Ltd.). The forward and backward extraction ratios were defined as follows: forward extraction ratio = (concentration of cytochrome c in reverse micellar organic phase after forward extraction)/(concentration of cytochrome c in feed aqueous phase before forward extraction);

backward extraction ratio = [(concentration of cytochrome c in reverse micellar organic phase before backward extraction) − (concentration of cytochrome c in reverse micellar organic phase after backward extraction)]/(concentration of cytochrome c in reverse micellar organic phase before backward extraction).

The UV–Vis spectra of cytochrome c in the reverse micellar organic phase and in the recovery aqueous phase after the forward and backward extractions showed the reduced type having peaks at 415, 520, and 550 nm, while the spectrum in the feed aqueous phase showed the oxidized type having a peak at 407 nm. In order to oxidize cytochrome c recovered from the reverse micellar organic phase, the aqueous solutions of potassium hexacyanoferrate(III) and iron(III) chloride were added to the recovery aqueous solution after the backward extraction. The activity of oxidized cytochrome c in the recovery aqueous solution was determined by measuring the reduction rate of cytochrome c with ascorbic acid [18].

Results and discussion

The kind and concentration of constituents such as nonionic surfactants, oils, water, and electrolytes strongly affect the phase behavior and stability of microemulsions [12, 13, 19]. In order to establish the extraction system having the characteristics of the easy operation and the high repeatability, we have considered that the following conditions were necessary to the system: (1) Surfactants should be sufficiently soluble in the organic phase to obtain the high solubility capability. (2) Surfactants should readily be soluble in the organic solvent, since difficult soluble surfactants easily tend to precipitate out. (3) After mixing the organic phase with the aqueous phase, those two phases should be separated clearly and rapidly. (4) After mixing the organic phase with the aqueous phase, those two phases should be transparent, since the extraction efficiency is estimated by the spectroscopic analysis. According to those conditions, we have examined the screening for the extraction system. Some examples in the screening are depicted as follows.

As sucrose fatty acid ester DK-F-110 consists of sucrose as a hydrophilic group and stearic acid as a hydrophobic group, the HLB value is higher and more hydrophilic, compared to bis(2-ethylhexyl) sodium sulfosuccinate (AOT) and cationic quaternary ammonium salts such as didodecyldimethylammonium bromide (DDAB) and trioctylmethylammonium chloride (TOMAC). Consequently, in order to prepare the organic solution containing the sufficient amount of DK-F-110 for the formation of reverse micelles, after dissolving DK-F-110 in alcohol such as ethanol and propylene glycol, the apolar organic solvent is mixed with the alcohol solution of DK-F-110. For instance, the solubility of DK-F-110 in isooctane and in isooctane/IPA (isooctane:IPA = 7:3(v/v)) were 3.00 and 92.5 g/L, respectively. The solubility in hexadecane/IPA (hexadecane:IPA = 7:3(v/v)) was 10.4 g/L, which was one-ninth compared with that in isooctane/IPA. Therefore, when using the same kind of alcohol, the smaller the carbon number of the hydrophobic solvent was, the more soluble DK-F-110 was in the solvent. However, it was considered that the solvent with a small carbon number was not suitable for an organic phase of the reverse micellar extraction process, since the volatility of solvent increased with a decrease in the carbon number, and DK-F-110 is easily precipitated out by the fugacity of solvent.

In order to carry out the stable reverse micellar extraction, the condition of the clear phase separation between the aqueous phase and the organic phase is needed [13]. The organic phase containing DK-F-110 in 8:2 volumetric ratio (hydrophobic solvent : IPA) was mixed with 0.1 M KCl aqueous phase. As seen in Table 1, the solubility of DK-F-110 to hexadecane/IPA was low. When using hexane/IPA or isooctane/IPA, the clear

Table 1 Phase behavior of organic and aqueous phases using the different hydrocarbon

Solvent	Phase	DK-F-110 (g/L)			
		10	30	50	70
Hexane + IPA	Organic phase	+ +	+ +	–	–
	Aqueous phase	–	+	+ +	+ +
Isooctane + IPA	Organic phase	+	+ + +	+ + +	[a]
	Aqueous phase	+	+ +	+ +	[a]
Hexadecane + IPA	Organic phase	–	[b]	[b]	[b]
	Aqueous phase	+ +			

After mixing 10 mL of the organic solution containing a certain amount of DK-F-110 with 10 mL of 0.1 M KCl aqueous solution, the mixture was incubated at 25 °C and 80 rpm for 30 min, and then stood at 25 °C overnight. The strong or middle turbidity was visually observed, and the low turbidity was spectrophotometrically checked: (–) no turbidity, (+) low turbidity, (+ +) middle turbidity, (+ + +) strong turbidity

[a] Aqueous and organic phases were not separated

[b] DK-F-110 was not dissolved perfectly

phase separation between the organic and the aqueous phases were observed, but the phases were turbid to some extent. The time for the phase separation using hexane/IPA was longer than that using isooctane/IPA.

Isooctane was mixed with different alcohol. Since alcohols enhance the solubility of surfactants, and play a role as a cosurfactant [12], it is important to select an appropriate alcohol. As shown in Table 2, the turbidity in both organic and aqueous phases strongly depended upon the kind of alcohol and the volumetric ratio. When the volumetric ratio in isooctane/*n*-butanol or isooctane/octanol was 7:3, the organic and aqueous phases were transparent, and the interface between the phases was distinct. Isooctane/*n*-butanol was employed for the forward and backward extractions, since it took a long time to dissolve DK-F-110 in isooctane/octanol.

First, we have investigated the critical micelle concentration (CMC) so as to prepare the reverse micellar organic phase. In general, there are differences in CMC values by various methods of determination [20]. As our aim is to investigate the surfactant concentration exhibiting the sufficient solubilization capability for the protein, we have assessed the solubility of water into the organic phase, since the amount of water solubilized into the reverse micelles is parallel to that of the protein solubilized into the reverse micelles [12]. The measurement of the water concentration was carried out by contacting isooctane/*n*-butanol containing a certain amount of DK-F-110 with distilled water. Figure 1 shows the water concentration against DK-F-110 concentration in organic phase. It should be noted that the surfactant and *n*-butanol contained in the organic phase would partially be distributed to the water phase, since DK-F-110 has the HLB value at 11, and is generally utilized for the formation of the oil-in-water emulsion. As shown in Fig. 1, the increase in water concentration was observed above 10 g/L DK-F-110. Since the organic phase measured in the range from 0 to 50 g/L DK-F-110 is transparent, the apparent critical micelle concentration is considered to exist around 10 g/L DK-F-110. It has been reported that in DK-F-110/IPA/hexane, the critical micelle concentration was 0.5 g/L [11]. The CMC value in the present system is 20 times larger than that in the DK-F-110/IPA/hexane. Since the water solubility in the present system without DK-F-110 is 70 times larger than that in the IPA/hexane without DK-F-110 and the bulk organic phase is much more hydrophilic, the apparent CMC value in the present system is considered to be larger compared to that in the DK-F-110/IPA/hexane. As the stable phase behavior and the sufficient forward extraction were observed at 50 g/L DK-F-110, the extraction experiments shown below were carried out at 50 g/L DK-F-110.

Cytochrome c entrapped in the reverse micelles exhibited the UV–Vis spectrum having peaks at 415, 520, and 550 nm, which were characteristic of the reduced form [21]. Thus, cytochrome c was reduced by a reducing agent in the reverse micelles. In DK-F-110/IPA/hexane system the similar spectrum change of cytochrome c has been reported [11], although the different kind of bulk organic solvent in the reverse micellar solution was used. The surfactant employed at the present work is a crude product, which is supplied as an emulsifier by the manufacturer [16]. Consequently, impurities are probably included in DK-F-110. One candidate reducing cytochrome c is D-glucose. D-glucose functions as a reducing agent, and is easily formed with D-fructose by the hydrolysis of sucrose, which is the hydrophilic group of DK-F-110.

Figure 2 shows the effect of the pH value of feed aqueous solution on the forward extraction ratio of cytochrome c using DK-F-110 reverse micellar solution. The forward extraction ratio strongly depends upon the pH value of the feed aqueous phase, shows the bell-shaped curve, and exhibited 0.80 at pH 8. At higher pH value, the reverse micellar organic phase after contact with the feed aqueous phase became more turbid, and was too turbid to measure the extraction ratio above pH 10.5. At the range below pH 8, the extraction ratio drastically decreases with a decrease in the pH value.

Table 2 Phase behavior of organic and aqueous phases using the different alcohol

Solvent	Phase	Isooctane : alcohol (v/v)		
		9:1	8:2	7:3
Isooctane + IPA	Organic phase	+ + +	+ + +	+ + +
	Aqueous phase	+ + +	+ + +	+
Isooctane + *n*-butanol	Organic phase	+	+	–
	Aqueous phase	+ +	–	–
Isooctane + octanol	Organic phase	[a]	–	–
	Aqueous phase	[a]	–	–

After mixing 10 mL of the organic solution containing 50 g/L DK-F-110 with 10 mL of 0.1 M KCl aqueous solution, the mixture was incubated at 25 °C and 80 rpm for 30 min, and then stood at 25 °C overnight. The strong or middle turbidity was visually observed, and the low turbidity was spectrophotometrically checked: (–) no turbidity, (+) low turbidity, (+ +) middle turbidity, (+ + +) strong turbidity

[a]50 g/L DK-F-110 was not dissolved perfectly

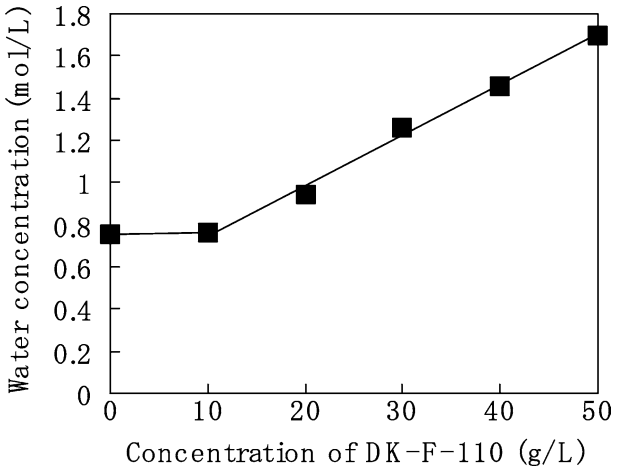

Fig. 1 Dependence of water concentration in the organic phase containing DK-F-110 on the concentration of DK-F-110

The relationship between pH and the forward extraction ratio is not attributable to the kind of bulk organic solvent but to the interaction of the protein with the surfactant, since the similar dependence of pH on the forward extraction ratio depicted at the present system has been reported in the DK-F-110/IPA/hexane system [11]. This pH dependence in the present system roughly resembles that in the reverse micellar extraction system using ionic surfactants, although the range in high extraction ratio using DK-F-110 reverse micellar solution is much narrower than that using AOT reverse micellar solution, which is given from pH 6.5 to 10 [3]. In the case of AOT reverse micellar extraction, the forward extraction is mainly enhanced by the electrostatic interaction between the strong negative-charged hydrophilic group of surfactant molecules and the positive-charged protein [3]. In the Tween 85/IPA/hexane system,

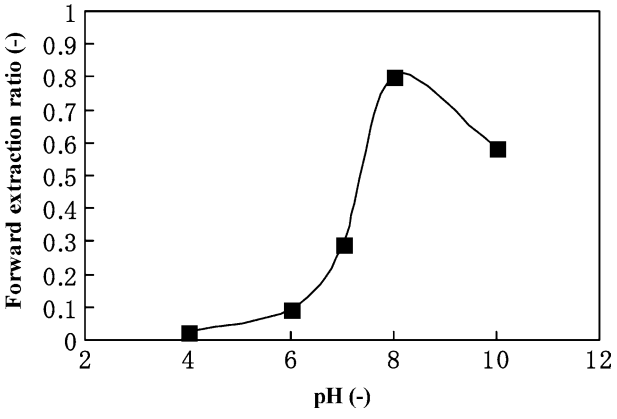

Fig. 2 Effect of pH of the feed aqueous solution on the forward extraction ratio of cytochrome c using DK-F-110 reverse micellar organic solution. A buffer solution of 0.01 M containing 10 μM cytochrome c was used as a feed aqueous solution

cytochrome c is sufficiently extracted with water from the feed aqueous phase to the reverse micellar organic phase at neutral pH by increasing surfactant and co-surfactant concentrations [12] Increases in surfactant and cosurfactant concentrations result in an increase in the solubilization capability of water, and thereby the extraction of protein is enhanced. The present system exhibits the sufficient solubilization capability of water, as shown in Fig. 1. In the alkyl sorbitan esters (Tween 85 and Span 20)/IPA/hexane system, cytochrome c can be extracted, whereas in the linear alcohol ethoxylate (Neodol 91–2.5)/hexane or decane system none of the cytochrome c is extracted [13]. Consequently, the sorbitan moiety is essential for the extraction of cytochrome c. DK-F-110 has sucrose moiety as the hydrophilic group. Proteins interact with saccharides in vitro as well as in vivo, and thereby are stabilized [22, 23] In addition, a week electrostatic interaction between surfactants and proteins is the underlying extraction mechanism, since the measurement of zeta potential illustrates that Tween 85 and several other nonionic surfactants possess a net negative charge at neutral pH [14, 24]. This charge has a pK_a of around 4.0, and is attributable to a carboxylic acid moiety, which exists as an impurity [14]. Sucrose fatty acid esters are industrially produced by the transesterification between the sucrose and fatty acid methyl ester through the formation of sucrate in the presence of alkaline catalysts [25]. This reaction reversibly proceeds. Consequently, impurities are probably included in the crude product for the industrial use to some extent. In the present system, the week electrostatic interaction might make contribution to the extraction of proteins, similar to the case using other nonionic surfactants such as Tweens. On the other hand, it is recommended by manufacturer (Dai-Ichi Kogyo Seiyaku) that sucrose fatty acid esters are used as an emulsifier around neutral pH, since sucrose fatty acid esters are chemically unstable owing to the hydrolysis of ester bond and hydrolysis of sucrose under the existence of water at acidic and alkali pH.

The protein is solubilized with water into the reverse micelles. The amount of water entrapped in the reverse micelles is influenced by the concentration and kind of salt [26]. In order to address the question of whether the salt concentration affects the transfer of cytochrome c from the feed aqueous phase to the reverse micellar organic phase, potassium chloride was added into the feed aqueous phase. As shown in Fig. 3, the forward extraction ratio is influenced by KCl concentration of the feed aqueous phase at pH 8, and dramatically decreases in the range from 0 to 0.02 M. In comparison with the forward extraction using AOT reverse micellar organic solution, the same tendency was observed in about one-twentieth KCl concentration [3]. In the alkyl sorbitan esters (Tween 85 and Span-20)/IPA/hexane system, the extraction of cytochrome c reduces by more

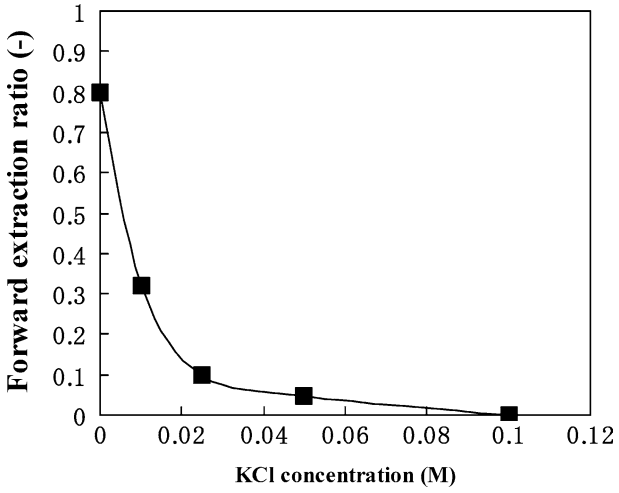

Fig. 3 Effect of KCl concentration of the feed aqueous solution at pH 8 on the forward extraction ratio of cytochrome c using DK-F-110 reverse micellar organic solution

than an order of magnitude at the ionic strength of 0.001 M phosphate [13]. In the nonionic surfactant system, an increase in the ionic strength results in a decrease in the water concentration in the organic phase due to a salting-out [19]. It is supposed that the addition of KCl into the feed aqueous solution mainly leads to the salting out, reduces the water concentration in the reverse micelles, and thereby the transfer of cytochrome c is inhibited. Moreover, the addition of KCl in the present system might shield the week electrostatic interaction of the positive-charged proteins with the negative-charged impurities included in DK-F-110.

Figure 4 shows the forward extraction ratio against the phosphate buffer concentration of the feed aqueous phase at pH 8. Similar to the case of KCl, the transfer of cytochrome c to the reverse micellar phase is strictly dependent upon the concentration of buffer. As shown in Fig. 4, the smaller the buffer concentration is, the more efficient the forward extraction is. However, in the practical separation process, the buffering effect is usually necessary for the stabilization of protein of interest.

Figure 5 shows the transfer of cytochrome c from the reverse micellar organic phase to the recovery aqueous phase against the pH value of the recovery aqueous phase. Cytochrome c can significantly be recovered by contacting with the recovery aqueous phase. The rate on the backward extraction is similar to that on the forward extraction, and the extraction reaches at equilibrium within 1 h. The curve has the low minimal backward extraction ratio around pH 8, and in the acidic pH values, high extraction ratios are exhibited compared to those in the alkaline pH values, and at pH 4 the extraction ratio is 0.9. Regarding the DK-F-110/IPA/hexane system exhibiting the behavior of the forward extraction similar to that in the present system, it has been reported that it was difficult to backward-extract the protein from the reverse micellar organic phase with the recovery aqueous phase, and the addition of an alcohol such as IPA or ethanol to the recovery aqueous phase was needed [11]. The phase behavior and stability of microemulsions consisting of water, oils, nonionic surfactants, and electrolytes are significantly influenced by the kind and concentration of those constituents [19]. The choice of organic solvents in the present work was advantageous to the backward extraction.

The effect of KCl concentration of the recovery aqueous phase at pH 8 on the backward extraction ratio is shown in Fig. 6. The backward extraction ratio drastically increases with an increase in the KCl

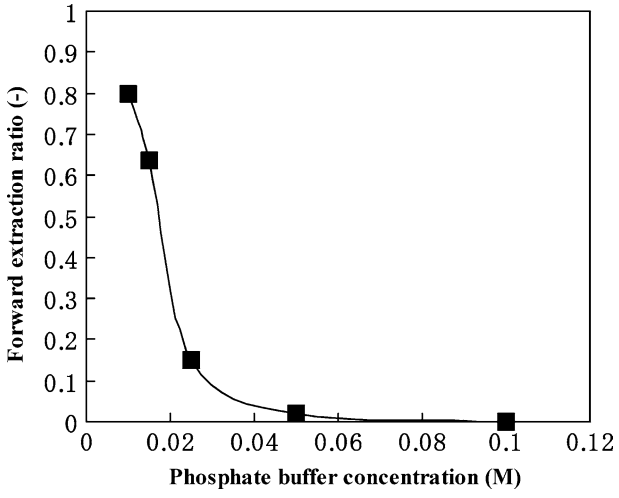

Fig. 4 Effect of phosphate buffer concentration of the feed aqueous solution at pH 8 on the forward extraction ratio of cytochrome c using DK-F-110 reverse micellar organic solution

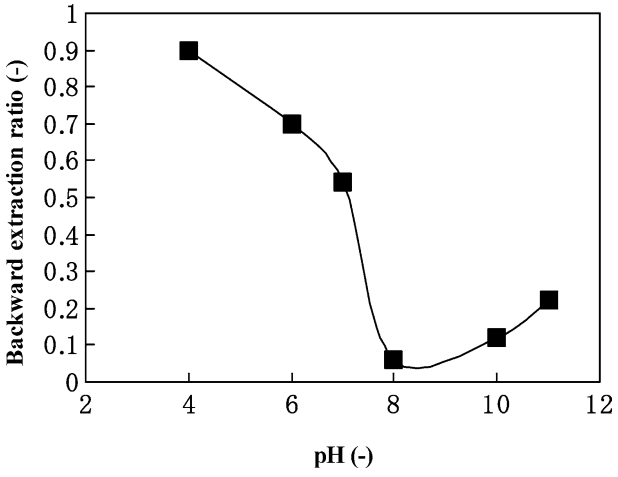

Fig. 5 Effect of pH of the recovery aqueous solution on the backward extraction ratio of cytochrome c from DK-F-110 reverse micellar organic solution containing 13.5 μM cytochrome c. A buffer solution of 0.01 M was used as a recovery aqueous solution

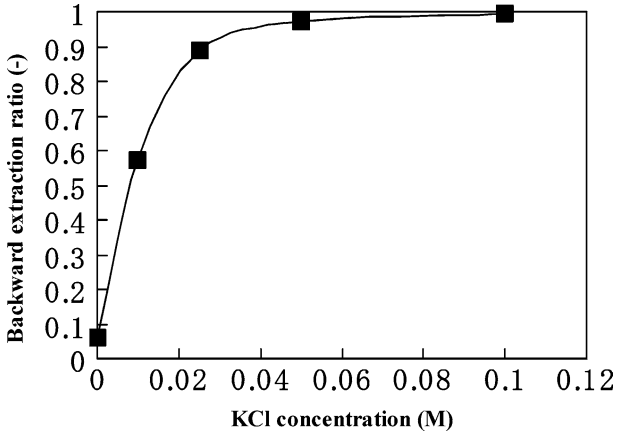

Fig. 6 Effect of KCl concentration of the recovery aqueous solution at pH 8 on the backward extraction ratio of cytochrome c using DK-F-110 reverse micellar organic solution

concentration, and reaches 1.0 at 0.1 M KCl. The range of ionic strength in the present system is much lower than that in the AOT/isooctane system [3]. In the AOT/isooctane system, the addition of salt causes the electrostatic interaction of the positive-charged proteins with negative-charged hydrophilic groups of AOT to be shielded.

The residual activities of cytochrome c recovered from the reverse micellar organic phase by acetate buffer solution at pH 4 and phosphate buffer solutions at pH 8 containing 0.1 M KCl, as a recovery aqueous solution exhibited 85 and 93 %, respectively. Saccharides are well known to work as a protein stabilizer in vitro as well as in vivo [22, 23]. Moreover, it has been reported that on the extraction of rebonuclease A through AOT/isooctane reverse micelles, the addition of sucrose into the feed aqueous solution improved both the extraction ratio and the residual activity, since the preferential hydration of the protein reduced the interaction of the protein with the surfactant[27]. Hydrophilic sucrose groups of DK-F-110 would enhance the residual activity of cytochrome c.

Conclusion

It has been demonstrated that the DK-F-110/*n*-butanol/isooctane system forms the stable reverse micelles, which can solubilize water, and achieve not only the high forward extraction efficiency but also the high backward extraction efficiency. The UV–Vis spectra of cytochrome c in the reverse micellar phase and in the recovery aqueous phase after the forward and backward extractions indicate that DK-F-110 reduces the protein. The forward extraction ratio is sensitively influenced by the pH value and the ionic strength of the feed aqueous phase, and exhibits 0.8 when 0.01 M phosphate buffer solution at pH 8 is used as the feed aqueous phase. The backward extraction ratio is similarly dependent upon the pH value and the ionic strength of the recovery aqueous phase, and cytochrome c encapsulated in the DK-F-110 reverse micelles is perfectly recovered when 0.01 M phosphate buffer solution at pH 8 containing 0.1 M KCl is used as the recovery aqueous phase. The activity of cytochrome c recovered from the DK-F-110 reverse micellar organic phase is highly retained.

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