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SHORT COMMUNICATION

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Application of sucrose fatty acid ester to reverse micellar extraction of lysozyme

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Abstract Reverse micellar extraction of lysozyme has been carried out using an organic solution containing a mixture of monoester and polyester of sucrose fatty acid ester. The forward extraction of lysozyme from the feed aqueous phase to the reverse micellar organic phase of the mixture of monoester and polyester of sucrose fatty acid ester at pH 7.2 was strongly dependent upon the weight fraction of monoester, while any amount of lysozyme was not extracted only by using monoester or polyester. The forward extraction ratio dramatically increased with an increase in the concentration of fatty acid ester, and was high around neutral pH and at low ionic strength. The backward extraction of lysozyme from the reverse micellar organic phase to the recovery aqueous phase exhibited high efficiency at acidic pH value or at high ionic strength. The addition of sucrose into the recovery aqueous phase promoted the backward extraction ratio, and caused the activity of lysozyme recovered from the reverse micellar phase to be retained perfectly.

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

Introduction

The reverse micelles are thermodynamically stable aggregates of surfactant in non-polar solvent, and have a diameter in the nanometer range [1]. The fundamental biomaterials such as amino acids and polypeptides can easily be solubilized into the water pool located in the center of the reverse micelle [2–7]. The high solubilization ability of reverse micelle has recently been applied to the extraction of protein [8–12]. The proteins are extracted from the feed aqueous phase to the reverse micellar organic phase by having the feed aqueous phase containing the proteins of interest in contact with the reverse micellar organic phase. The ionic surfactant such as bis(2-ethylhexyl)sodium sulfosuccinate (AOT) is mainly used to prepare the reverse micellar organic phase. The extraction of protein is promoted by the electrostatic interaction of the charged protein with the counter-charged hydrophilic group of surfactant. However, when the protein–surfactant complex is firmly formed due to the strong electrostatic force, the protein cannot be recovered from the reverse micellar phase to the recovery aqueous phase only by the backward extraction, or is denatured. In order to weaken the rigid protein–surfactant complex and promote the recovery of protein from the reverse micellar phase, the polar organic solvent or the gas was added to the reverse micellar organic phase containing the protein [13–17]. Moreover, nonionic surfactant or natural surfactant has been used to reduce the interaction of the protein with the surfactant [14, 18–21, 37].

As a part of our ongoing research efforts aimed at enhancing the activity yield in the reverse micellar extraction, we are exploring further systems of affecting the recovery efficiency in the reverse micellar extraction. In our previous study, we have reported that cytochrome c can be transferred between the aqueous phase and the reverse micellar organic phase at a high efficiency not only by the forward extraction but also by the backward extraction using the DK-F-110/*n*-butanol/isooctane system [37]. DK-F-110 is nonionic surfactant sucrose fatty acid ester, and is

widely used as an emulsifier in a great variety of food and cosmetics formulations [22, 23]. In order to test the generality of the observed phenomena in our previous study, in the present study we have investigated how the other protein affects the extraction efficiency in the DK-F-110/*n*-butanol/isooctane system. Lysozyme was used as a model protein. Lysozyme is an important enzyme used as a food preservative and a pharmaceutical [24], but can hardly be recovered only by contacting the AOT reverse micellar organic phase containing lysozyme with the recovery aqueous phase on the reverse micellar extraction system [17, 25].

Experimental

DK-F-SS (sucrose fatty acid monoester of stearic acid, purity 99%), DK-F-10 (sucrose fatty acid polyester, diester to pentaester of stearic acid, purity 99%), and DK-F-110, which was an equivalent weight mixture of sucrose monoester and polyester of stearic acids, were supplied from Dai-Ichi Kogyo Seiyaku (Kyoto, Japan). The surfactant was used without further purification. The hydrophile-lipophile balance (HLB) values of DK-F-10, DK-F-110, and DK-F-SS were 1, 11, and 19, respectively. Lysozyme from chicken egg white (EC 3.2.1.17, 46,400 units/mg solid, MW=14,300, p*I*=11.1), *Micrococcus lysodeikticus* (ATCC No. 4698), and sucrose were obtained from Sigma-Aldrich Co. Isooctane and *n*-butanol were obtained from Kanto Chemicals (Tokyo, Japan), and were of analytical grade.

Forward extraction was carried out by mixing equal volumes (10 ml) of the organic solution of sucrose fatty acid ester and the feed aqueous solution of lysozyme in a 50-ml screw vial at 25°C and 120 rpm for 1 h. The organic phase was mainly an isooctane/*n*-butanol (7:3 (v/v)) containing 50 g/l DK-F-110, while the aqueous phase was a 10-μM lysozyme solution at an appropriate pH value and with a certain amount of KCl. The feed and recovery aqueous solutions used in the present work were acetate buffer solutions at pH 4–5 and phosphate buffer solutions at pH 5–8. The concentration of buffer solution was at 0.01 M.

In the backward extraction, 10 ml of the reverse micellar organic solution containing 10 μM lysozyme and 23.7 g/l of 0.01 M phosphate buffer at pH 7.2, which was prepared by the injection method [8], was contacted 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 left to stand until the organic phase and the aqueous phase were separated distinctly, and the organic phase was centrifuged at 4,000 rpm for 30 min. After centrifugation, the concentration of lysozyme in the organic phase was measured spectrophotometrically at 280 nm by a UV/VIS spectrophotometer (Ubest-55, Japan Spectroscopic). The water concentration of the organic phase after centrifugation was determined by an optimized Karl Fisher potentiometric titration using a Hiranuma AQ-6 aquacounter.

The forward extraction ratio (R_F) is defined as

$$R_F = M_R/M_F \tag{1}$$

where M_F and M_R are the number of moles of lysozyme in feed aqueous phase before forward extraction and the number of moles of lysozyme in reverse micellar organic phase after forward extraction, respectively. The backward extraction ratio (R_B) is defined as

$$R_B = (M_{RO} - M_{RA})/M_{RO} \tag{2}$$

where M_{RO} and M_{RA} are the number of moles of lysozyme in reverse micellar phase before backward extraction and the number of moles of lysozyme in reverse micellar organic phase after backward extraction, respectively.

After adding 100 μl of the recovery aqueous solution containing lysozyme to 3 ml of 0.1 M phosphate buffer solution at pH 7 containing 250 mg/l *M.lysodeikticus* at 25°C, the absorbance was continuously measured at 540 nm by a UV/VIS spectrophotometer. The lysis of bacterium was a first-order reaction. The lysis rate constant (k) is calculated by

$$In\left(A^0_{540}/A_{540}\right) = kt \tag{3}$$

where t, A^0_{540}, and A_{540} are the reaction time, the absorbance of the substrate solution at 540 nm at T=0, and the absorbance of the substrate solution at 540 nm at T=t, respectively. The remaining activity (R. A.) is defined as

$$\text{R.A.} = 100 \times k/k_0 \tag{4}$$

where k_0 and k are the lysis rate constants of native enzyme without the extraction and enzyme backward-extracted, respectively.

Results and discussion

The geometry and HLB of surfactants affect the formation and stability of micelles [26]. In order to elucidate the effect of those factors on the extraction efficiency, we have examined the forward extraction of lysozyme at various compositions of monoester (DK-F-SS) and polyester (DK-F-10) of sucrose fatty acid ester. The transfer of lysozyme from the feed aqueous phase to the reverse micellar organic phase strongly depended upon the composition of sucrose

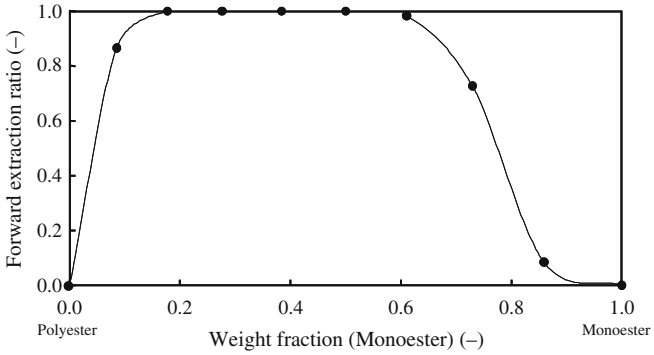

Fig. 1 Effect of composition of sucrose fatty acid ester on the forward extraction ratio of lysozyme at pH 7.2 and 25°C. A 0.01-M phosphate buffer solution containing 10 μM lysozyme was used as a feed aqueous solution, while an isooctane/*n*-butanol (7:3 (v/v)) containing 50 g/l sucrose fatty acid ester was used as an organic phase

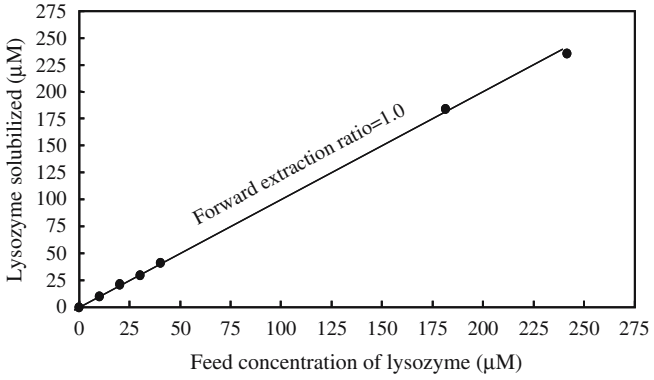

Fig. 3 Solubilization of lysozyme in DK-F-110 reverse micellar organic phase at pH 7.2 and 25°C. 0.01 M phosphate buffer solution containing a certain amount of lysozyme was used as a feed aqueous solution, while an isooctane/*n*-butanol (7:3 (v/v)) containing 50 g/l DK-F-110 was used as an organic phase

fatty acid ester, as shown in Fig. 1. Especially, when using only polyester or monoester of sucrose fatty acid ester, the transfer was not observed at all. Polyesters consist of the diester to pentaester of stearic acid, and the highly esterified ones would especially exhibit a steric hindrance to the assembly, so that it would be difficult to form the reverse micelles [26, 27]. On the other hand, monoesters have high HLB value, and are more hydrophilic, compared to bis(2-ethylhexyl)sodium sulfosuccinate (AOT) and cationic quaternary ammonium salts such as didodecyldimethylammonium bromide (DDAB) and trioctylmethylammonium chloride (TOMAC), which are used to form the reverse micelles. It is considered that the stable reverse micelles cannot be formed only by monoesters. In the weight fraction of monoester from 0.2 to 0.6, the high forward extraction ratio was exhibited.

Figure 2 shows the plot of the forward extraction ratio of lysozyme against the concentration of sucrose fatty acid ester. The forward extraction ratio was influenced by the concentration of DK-F-110 consisting of an equivalent weight mixture of sucrose monoester and polyester of stearic acids. The forward extraction ratio dramatically increases from 10 to 30 g/l DK-F-110, while the transfer of lysozyme was hardly observed using DK-F-SS or DK-F-10 at any surfactant concentration examined. The solubility of water to the organic phase gradually increased beyond 10 g/l DK-F-110 [37]. The critical micelle concentration would be more than 10 g/l. Therefore, the forward and backward extractions have been examined using 50 g/l DK-F-110 below.

The effect of the lysozyme concentration in the feed aqueous phase on the solubilization of lysozyme in the reverse micellar organic phase is shown in Fig. 3. The linear line in the figure represents the concentration of lysozyme solubilized when lysozyme is transferred from the feed aqueous phase to the reverse micellar organic phase perfectly, and the plots are the experimental results. Under the experimental condition, lysozyme was effi-

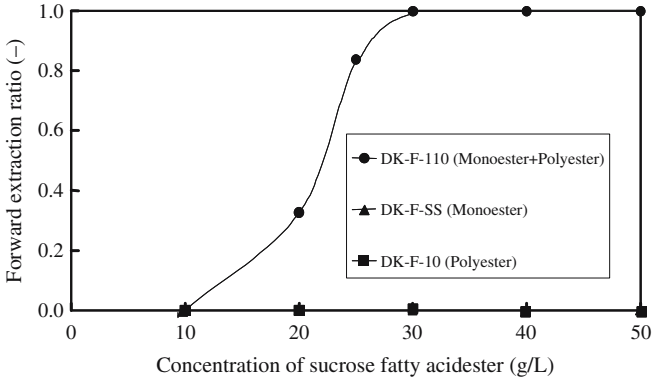

Fig. 2 Effect of concentration of sucrose fatty acid ester on the forward extraction ratio of lysozyme at pH 7.2 and 25°C. A 0.01-M phosphate buffer solution containing 10 μM lysozyme was used as a feed aqueous solution, while an isooctane/*n*-butanol (7:3 (v/v)) containing a certain amount of sucrose fatty acid ester was used as an organic phase

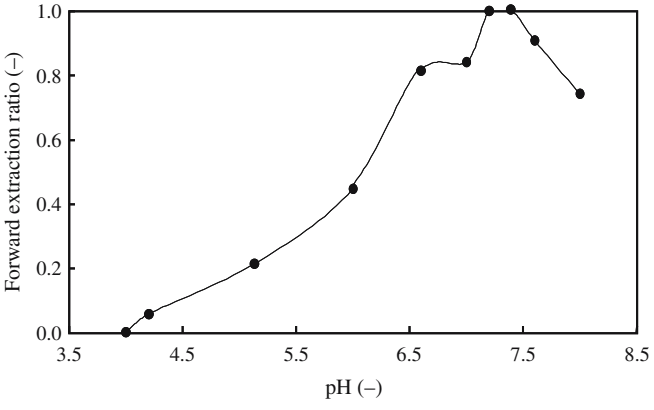

Fig. 4 Effect of pH of the feed aqueous solution on the forward extraction ratio of lysozyme using 50 g/l DK-F-110 reverse micellar organic solution. A 0.01 M buffer solution containing 10 μM lysozyme was used as a feed aqueous solution

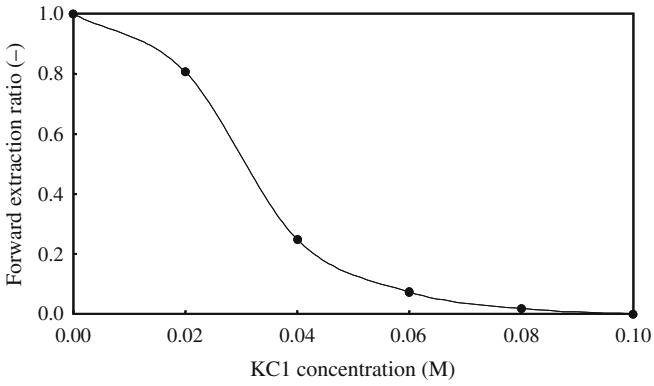

Fig. 5 Effect of KCl concentration of the feed aqueous solution on the forward extraction ratio of lysozyme using 50 g/l DK-F-110 reverse micellar organic solution. A 0.01 M phosphate buffer solution containing 10 μM lysozyme was used as a feed aqueous solution

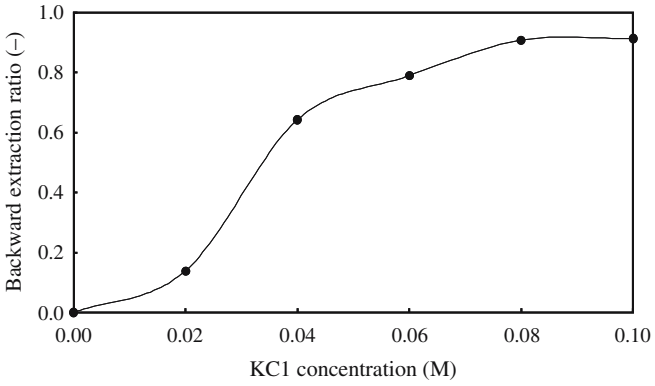

Fig. 7 Effect of KCl concentration of the recovery aqueous solution at pH 7.2 on the backward extraction ratio of lysozyme using 50 g/l DK-F-110 reverse micellar organic solution

ciently extracted from the feed aqueous phase to the reverse micellar organic phase. Especially, till the lysozyme concentration of feed aqueous phase was 50 μM, the forward extraction ratio was 1.0. The extraction processes shown below were carried out at 10 μM lysozyme.

Figure 4 shows the effect of the pH of the feed aqueous solution on the forward extraction ratio of lysozyme. The forward extraction ratio was strongly dependent upon the pH of the feed aqueous phase and the maximal ratio was 1.0 in the range between pH 7.2 and 7.4. However, turbidity appeared in the feed aqueous and the reverse micellar organic phases after mixing the feed aqueous phase with the reverse micellar organic phase as the pH of the feed aqueous phase increased beyond pH 7.4. The low extraction ratio is shown under acidic conditions. The pH dependency upon the forward extraction ratio in the present work is similar to that in our previous work using cytochrome c, although the maximal peak is shifted to the acidic side in the present work [37]. Lysozyme

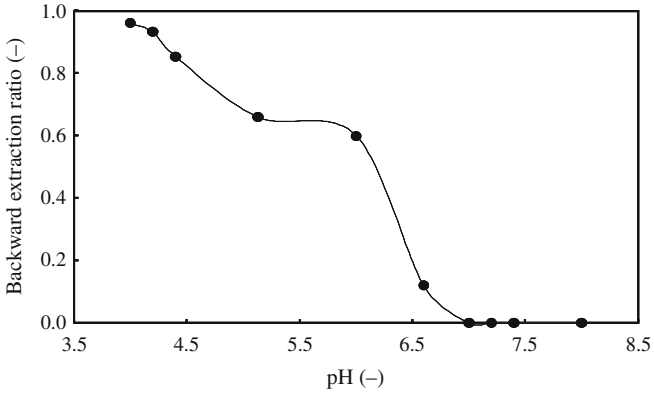

Fig. 6 Effect of pH of the recovery aqueous solution on the backward extraction ratio of lysozyme using 50 g/l DK-F-110 reverse micellar organic solution containing 10 μM lysozyme. A 0.01 M buffer solution was used as a recovery aqueous solution

entrapped in DK-F-110 reverse micelle would interact with the sucrose moieties of surfactants, as the proteins interact with saccharides such as sucrose in vitro as well as in vivo, and thereby are stabilized [33, 34]. Furthermore, positive-charged lysozyme would interact with the free fatty acid through the electrostatic force, as the sucrose fatty acid esters used in the present work exhibit an acid value of less than 5. It has been reported that a net negative charge at neutral pH is observed by the measurement of zeta potential of Tween 85 and several other nonionic surfactants, and is attributable to a carboxylic acid moiety, which exists as an impurity [20, 28]. On the other hand, it is recommended by the manufacturer (Dai-Ichi Kogyo Seiyaku) that sucrose fatty acid esters are used for emulsification around neutral pH, as 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 alkaline pH.

Table 1 Backward extraction ratios and remaining activities of lysozyme at various pH values[a]

pH	Adduct	Backward extraction ratio	Remaining activity (%)
4.00	None	0.96	53
	0.75 M sucrose	0.93	68
5.13	None	0.66	67
	0.1 M KCl	0.69	73
	0.75 M sucrose	0.86	68
7.20	None	0.00	–
	0.1 M KCl	0.91	67
	0.1 M KCl + 0.75 M sucrose	0.94	97

[a]After contacting 10 ml of the reverse micellar organic solution containing 10 μM lysozyme and 23.7 g/l of 0.01 M phosphate buffer at pH 7.2 with 10 ml of the recovery aqueous solution in the 50-ml screw vial, the mixture was incubated at 25°C and 120 rpm for 1 h

The ionic strength affects the interaction of the protein with the surfactant such as AOT and the stability of reverse micelles [9, 29, 30]. As KCl concentration of the feed aqueous phase increases at pH 7.2, the forward extraction ratio is reduced as shown in Fig. 5. In the range from 0 to 0.06 M KCl, the forward extraction ratio descends from 1.0 to less than 0.1, although the decrease tendency in this case is milder than that in the case using cytochrome c [37]. The protein is solubilized with water to reverse micelles. The content of water solubilized in reverse micelles tends to decrease with an increase in salt concentration [29, 30]. However, the water content in the DK-F-110 reverse micellar organic phase was about 2.9 wt.% in the range of KCl concentration measured in the present work, and was unchangeable. On the other hand, the electrostatic interaction of lysozyme with free fatty acids included as the impurity in surfactants would decrease with an increase in KCl concentration, as the positive-charged proteins and the negative-charged fatty acids are respectively surrounded by counter ions. Thereby, the electrostatic interaction between the protein and the fatty acid is weakened, similar to the case of AOT reverse micellar extraction [9].

In general, the transfer of protein from the reverse micellar organic phase to the recovery aqueous phase is much slower than that from the feed aqueous phase to the reverse micellar organic phase, or is difficult [17, 31, 32]. The backward extraction reached equilibrium within 60 min under the recovery conditions examined in the present study. The plot of the backward extraction ratio against the pH of the recovery aqueous phase is shown in Fig. 6. The backward extraction ratio is high at acidic pH, while no transfer of lysozyme from the reverse micellar organic phase to the recovery aqueous phase is shown at neutral and alkaline pH. Figure 7 shows the effect of KCl concentration of the recovery aqueous phase at pH 7.2 on backward extraction ratio. The transfer of lysozyme from reverse micellar organic phase to the recovery aqueous phase is enhanced by increasing KCl concentration. However, the effect of KCl addition in this case is inferior to that in the case using cytochrome c [37]. On the other hand, ionic surfactants such as AOT generally bind to the proteins and initiate the unfolding of the tertiary structure, while nonionic surfactants do not alter the tertiary structure of the protein [36]. Similarly, AOT induces the strong interaction of AOT with lysozyme, resulting in the difficulty of backward extraction of lysozyme from the reverse micellar organic phase to the recovery aqueous phase and the denaturation of lysozyme [28].

In order to elucidate the effective backward extraction condition, KCl and sucrose were added to the recovery aqueous phase in the range from acidic pH to neutral pH. The middle column in Table 1 shows the observed backward extraction ratio. The effect of adduct is enhanced as the pH of recovery aqueous phase increases. The addition of sucrose causes the backward extraction ratio at pH 5.13 to gradually increase and reach 0.86, which is a 1.3-fold efficiency compared to that without sucrose, although sucrose does not affect the backward extraction ratio at pH 4 or 7.2. The last column in Table 1 shows the remaining activity of lysozyme recovered from the reverse micellar organic phase. The addition of sucrose improves the remaining activity at pH 4, 5.13, and 7.2. The activity of lysozyme is kept at pH 7.2 perfectly. Proteins are stabilized by the addition of saccharides into the aqueous solution of proteins [33, 34]. Moreover, it has been reported that on the AOT reverse micellar extraction of ribonuclease A, the addition of sucrose into the feed aqueous solution improved not only the extraction ratio but also the remaining activity due to the preferential hydration of the protein [35]. As shown in Table 1, the activity yield, which is the backward extraction ratio multiplied by the remaining activity, is high when using pH 7.2 phosphate buffer containing 0.1 M KCl and 0.75 M sucrose as the recovery aqueous phase.

Conclusion

We have demonstrated that in the sucrose fatty acid ester/*n*-butane/isooctane system, the forward extraction of lysozyme strongly depends upon the geometry of sucrose fatty acid ester. The concentration of DK-F-110 affects the solubility of lysozyme. DK-F-110 reverse micelles exhibit high solubility of lysozyme. Lysozyme entrapped in the reverse micelles can easily be recovered by the buffer with KCl and sucrose, and its activity is highly maintained. Thus, DK-F-110 suitably interacts with lysozyme through the extraction process, and provides efficient extraction and high activity.

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