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SHORT COMMUNICATION

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How can temperature affect reverse micellar extraction using sucrose fatty acid ester?

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Abstract The reverse micellar extraction of lysozyme using sucrose fatty acid ester was found to be greatly affected by the temperature in the extraction process. For example, lysozyme was perfectly extracted from the feed aqueous phase to the reverse micellar organic phase at 25°C, while it was not extracted at 5°C at all. After entrapping lysozyme into the reverse micelles, lysozyme was recovered from the reverse micellar organic phase to the recovery aqueous phase only by decreasing the temperature in the backward extraction. Moreover, lysozyme solubilized in the reverse micellar organic solution could be recovered without the recovery aqueous solution at 3°C, and its activity was retained at 95%.

Keywords Reverse micelle · Extraction · Protein · Nonionic surfactant · Temperature

Introduction

Recently the extraction of proteins using the reverse micelles has been considered as the novel methodology for the bioindustrial large-scale separation process [1–5]. The reverse micellar extraction consists of the forward transfer of proteins from the feed aqueous phase such as the fermentation broth to the water-immiscible reverse micellar organic phase and the backward transfer of proteins from the reverse micellar organic phase to the recovery aqueous phase. The driving force for entrapment of the protein into the reverse micelles is attributable to the affinity between the proteins and the surfactants of reverse micelle. The affinity consists of the noncovelent bonds such as the electrostatic interaction, the dipole–dipole interaction, the hydrogen bonding, the hydrophobic interaction, and so on. Because the ionic surfactants such as bis(2-ethylhexyl) sodium sulfosuccinate (AOT), didodecyldimethylammonium bromide (DDAB), and trioctylmethylammonium chloride (TOMAC) are generally used to form the reverse micelles, the electrostatic interaction between the charged hydrophilic group of surfactant, and the counter charged protein is considered to mainly work as the driving force of entrapping the protein into the reverse micelles. The electrostatic interaction of the protein with the surfactant is so strong that it enhances the transfer of proteins from the feed aqueous phase to the reverse micellar organic phase. However, the so-called protein-surfactant complex is firmly formed between the protein entrapped into the reverse micelle and the surfactants of reverse micelle, so that the backward extraction rate of the protein is relatively slow, and some protein cannot be recovered or are denatured. To improve the recovery of the protein in the reverse micellar extraction consisting of ionic surfactant, it has been reported that the polar solvent such as propanol was added to the reverse micellar organic phase containing the protein [6–9]. We have reported that the active proteins could be recovered from reverse micellar organic phase by forming the stable gas hydrate in the AOT reverse micelle at moderate pressures (3–5 atm) and around 0°C [8, 10]. Moreover, to reduce the interaction of the protein with the surfactant, nonionic surfactant or natural surfactant has been used instead of ionic surfactants [7, 11–14]. We have shown that the protein was efficiently extracted and its activity was kept by the reverse micellar extraction using nonionic surfactant sucrose fatty acid ester [15, 16].

In the present work, we investigated the effect of temperature on the reverse micellar extraction process using sucrose fatty acid ester, because the hydrophilicity of noionic surfactants is influenced by the temperature [17].

The transfer of protein from the feed aqueous phase to the reverse micellar organic phase was influenced by the operation temperature. Using the temperature dependency of the transfer of protein between the reverse micellar organic phase and the aqueous phase, the novel recovery process was proposed.

Experimental

DK-F-110, which was an equivalent weight mixture of sucrose monoester and polyester of stearic acids, was supplied from Dai-Ichi Kogyo Seiyaku (Kyoto, Japan). The surfactant was used without further purification. The hydrophile–lipophile balance (HLB) value of DK-F-110 was 11. Lysozyme from chicken egg white (EC 3.2.1.17, 46,400 U/mg solid, MW 14,300, pI 11.1) and *Micrococcus lysodeikticus* (ATCC No. 4698) were obtained from Sigma-Aldrich. Isooctane and *n*-butanol were obtained from Kanto Chemicals (Tokyo, Japan) and were of analytical grade.

The 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 the 50-ml screw vial for 1 h, at 120 rpm, and at temperatures ranging from 5 to 25°C. 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 0.01 M phosphate buffer solution at pH 7.2 containing 10 μM lysozyme.

On 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 was contacted with 10 ml of 0.01 M phosphate buffer solution at pH 7.2 as the recovery aqueous solution in the 50-ml screw vial for 1 h, at 120 rpm, and at temperatures ranging from 3 to 25°C. Moreover, 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 was contacted with a certain amount of 0.2 M phosphate buffer solution at pH 7.2 in the 50-ml screw vial at 3°C and 120 rpm for 1 h. The volumetric ratio of the recovery aqueous phase (V_{aq}) to the reverse micellar organic phase (V_{org}) was from 0 to 1. After the forward or backward extraction, the mixture was stood at the temperature, which was similar to that of the extraction operation, 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 spectrophotometically at 280 nm by UV/VIS spectrophotometer (Ubest-55, Japan Spectroscopic).

The forward extraction ratio (R_F) is defined as

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

where M_F and M_R are number of moles of lysozyme in feed aqueous phase before forward extraction and 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 number of moles of lysozyme in reverse micellar phase before backward extraction and 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 UV/VIS spectrophotometer.

The lysis of bacterium was the first order reaction. The lysis rate constant (k) is calculated by

$$\ln\left(A^o_{540}/A_{540}\right) = kt \tag{3}$$

where t, A^o_{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

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

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

The water content in the organic phase was determined by the optimized Karl Fisher potentiometric titration using a Hiranuma AQ-6 aquacounter.

To estimate the solubility of DK-F-110 in the organic solution, the turbid point of the organic phase at the different concentration of DK-F-110 was measured by the digital thermometer DMT-610 (Tokyo Denpa-Kiki).

Results and discussion

To elucidate the effect of the operation temperature upon the transfer from the feed aqueous phase to the DK-F-110 reverse micellar organic phase, lysozyme was used as a model protein because the lysozyme can sufficiently be solubilized in the feed aqueous phase in the range of the operation temperature measured. As shown in Fig. 1, the forward extraction ratio dropped when the temperature decreased below 20°C, and the extraction was not observed at 5°C. The reverse micellar organic phase became slightly turbid below 10°C. As demonstrated in the previous study, the solubility of sucrose fatty acid ester in apolar solvents is very low [15]. Consequently, the stability of the organic phase containing DK-F-110 might decrease in the range of

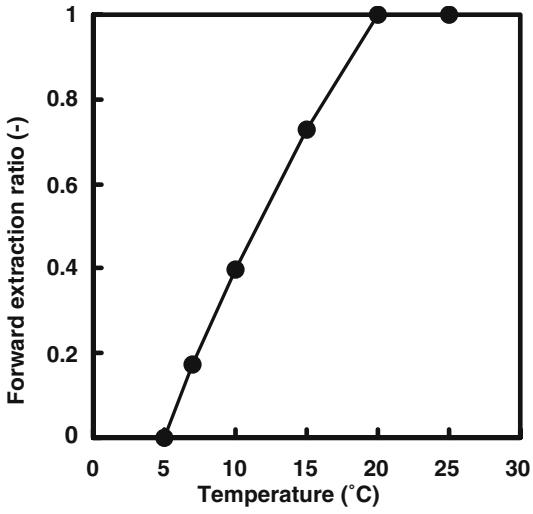

Fig. 1 Effect of temperature on the forward extraction ratio of lysozyme at pH 7.2. 0.01 M phosphate buffer solution at pH 7.2 containing 10 μM lysozyme was used as a feed aqueous phase, while an isooctane/*n*-butanol [7:3 (*v*/*v*)] containing 50 g/l sucrose fatty acid ester was used as an organic phase

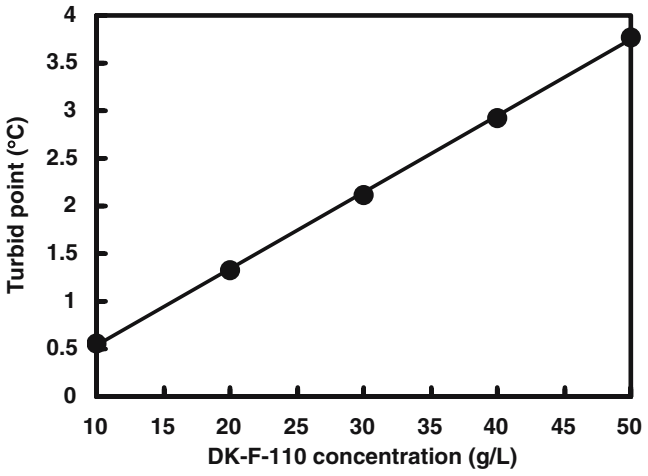

Fig. 2 Relationship between the turbid point and the initial DK-F-110 concentration in isooctane/*n*-butanol solution. The isooctane/*n*-butanol [7:3 (*v*/*v*)] containing the different initial concentration of DK-F-110 was used as an organic solution

low temperature due to the decrease of the solubility of DK-F-110.

To estimate the stability of organic phase containing DK-F-110, the solubility of surfactant at low temperature has been examined. It was difficult to determine the solubility of DK-F-110 quantitatively because DK-F-110 was a mixture of compounds esterified at the different degree. Consequently, we have observed the turbid point of the organic solution containing DK-F-110 at the different initial concentration of DK-F-110, when lowering gradually the temperature of the organic solution of DK-F-110 from 25°C. As seen in Fig. 2, the turbid point was strongly dependent upon the initial concentration of DK-F-110. The solubility of DK-F-110 decreased with a decrease in temperature, similarly to that of á-monoglycerol [18]. Moreover, the hydrophilicity of oxyethylated nonionic surfactants in an aqueous solution is well known to increase with a decrease in temperature because the hydrogen bonding between the hydrophilic group of surfactant and water is strengthened [17]. The hydroxyl groups of sucrose, which are the hydrophilic group of DK-F-110, are hydrated through the hydrogen bonding [19, 20]. From these points of view, it is considered that the surfactants in the organic phase tend to be distributed to the feed aqueous phase when the organic phase containing DK-F-110 is contacted with the feed aqueous phase, and the extraction efficiency is reduced because the extraction efficiency is dramatically influenced by the surfactant concentration in the organic phase [16].

Figure 3 shows the water content in the bulk organic phase or the reverse micelles against the temperature. The water content in the reverse micelles strongly depends upon the temperature. The water content in the reverse micelles at 5°C is 1.5 times lower than that at 25°C, while the water content in the bulk organic phase at 5°C is 1.2 times lower than that at 25°C. The decrease of the solubility of water in the reverse micelles results in the decrease of the solubilization of protein into the reverse micelle because the protein is entrapped into reverse micelle by accompanying with the water. The solubility of water is strongly dependent upon the concentration of DK-F-110 in the organic solution [15]. The decrease of the solubility of water in the reverse micelle would result from the decrease of the solubility of DK-F-110 due to the temperature drop as mentioned above. Moreover, DK-F-110 has high HLB value, and is more hydrophilic compared to AOT, DDAB, and TOMAC, which are used to form the reverse micelles. The HLB value of nonionic surfactants decreases with decreasing temperature [21]. The water-in-oil microemulsion would be destabilized at low temperature.

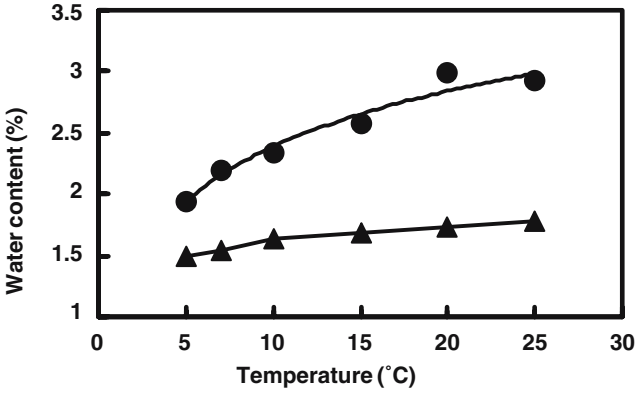

Fig. 3 Effect of temperature on the water contents in the reverse micelles (•) and in the bulk organic phase (▲) in the forward extraction. The isooctane/*n*-butanol [7:3 (*v*/*v*)] containing 50 g/l DK-F-110 was used as the reverse micellar organic solution

After the reverse micellar organic phase containing lysozyme and 0.01 M phosphate buffer solution at pH 7.2 as the recovery aqueous phase was mixed at 25°C, the mixture was incubated at the different temperature. Figure 4 shows the backward extraction ratio and the water content in the reverse micelles against the operation temperature. In the DK-F-110/*n*-butanol/isooctane system, the backward extraction ratio is strongly influenced by pH value of the buffer solution and the concentration of salt included in the buffer solution [15, 16]. In the present condition of the phosphate buffer solution, which is used as the recovery aqueous phase, lysozyme cannot be recovered at 25°C [16]. However, the backward extraction ratio dramatically increased with a decrease in temperature, and exhibited 0.94 at 3°C. The water content in the reverse micelles gradually decreased with a decrease in temperature, similarly to the case of the forward extraction. On the other hand, in TOMAC/Rewopal HV5 or AOT reverse micellar extraction system, it has been reported that an increase in temperature induced the recovery of protein from the reverse micellar organic phase [22, 23].

Figure 5 shows the backward extraction ratio and the water content in the reverse micelles against the volumetric ratio of the recovery aqueous phase (V_{aq}) to the reverse micellar organic phase (V_{org}) at 3°C. Although the volumetric ratio varied from 0 to 1.0, the backward extraction ratio did not almost change, and exhibited about 0.93. The water content was similarly constant, and showed around 2.3%. From these results, it is suggested that the protein recovery is not concerned with the recovery aqueous phase, and is attributable to the destabilization of reverse micelles.

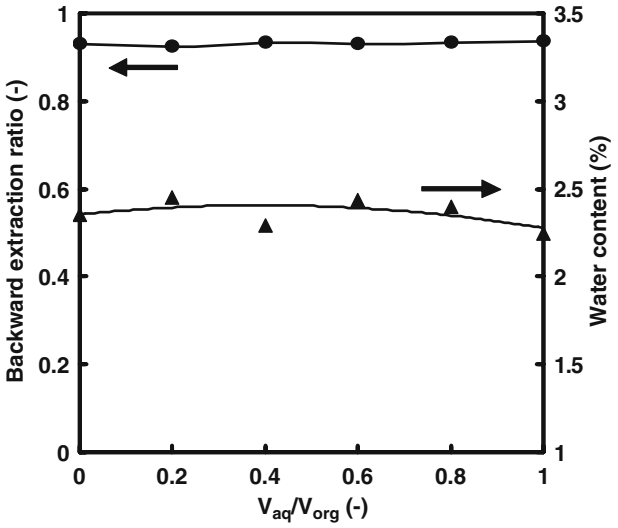

Fig. 5 Effect of the reverse micelles at the volumetric ratio of the recovery aqueous phase to the reverse micellar organic phase (V_{aq}/V_{org}) on the backward extraction ratio of lysozyme and the water content in the reverse micelles at 3°C. The reverse micellar organic solution containing 10 μM lysozyme and 23.7 g/l of 0.01 M phosphate buffer at pH 7.2 was used as a the reverse micellar organic phase, while 0.01 M phosphate buffer solution at pH 7.2 was used as the recovery aqueous phase

Figure 6 shows the backward extraction ratio and the water content in the reverse micelles against the operation

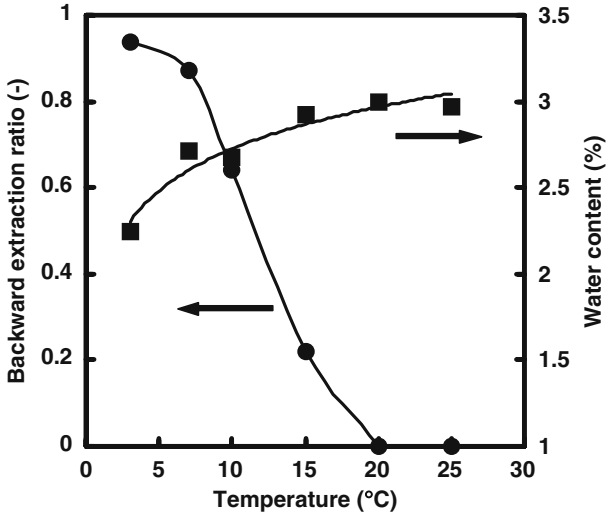

Fig. 4 Effect of temperature on the backward extraction ratio of lysozyme and water content in the reverse micelles at the volumetric ratio of the recovery aqueous phase to the reverse micellar organic phase (V_{aq}/V_{org})=1. The reverse micellar organic solution containing 10 μM lysozyme and 23.7 g/l of 0.01 M phosphate buffer at pH 7.2 was used as the reverse micellar organic phase, while 0.01 M phosphate buffer solution at pH 7.2 was used as the recovery aqueous phase

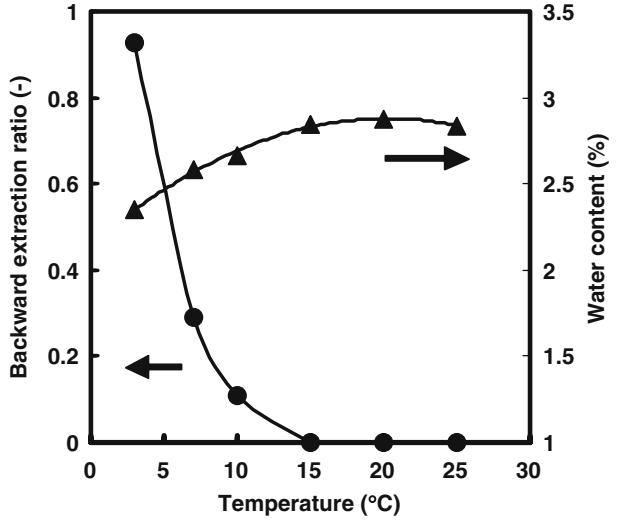

Fig. 6 Effect of temperature on the backward extraction ratio of lysozyme and water content in the reverse micelles at the volumetric ratio of the recovery aqueous phase to the reverse micellar organic phase (V_{aq}/V_{org})=0. The reverse micellar organic solution containing 10 μM lysozyme and 23.7 g/l of 0.01 M phosphate buffer at pH 7.2 was used as the reverse micellar organic phase

temperature at V_{aq}/V_{org}=0. Lysozyme was not recovered above 15°C, while the backward extraction ratio increased with decreasing operation temperature below 15°C, and was 0.93 at 3°C. In the range form 3 to 15°C, the change of the backward extaction ratio at V_{aq}/V_{org}=0 was sharper, compared to that at V_{aq}/V_{org}=1 in Fig 4. On the other hand, the water content gradually decreased when lowering the operation temperature. Lysozyme was precipitated out with the surfactant and could easily be separated from the organic phase. The recycle of the reverse micellar organic phase is considered to be important from the standpoint of the industrial application. To reuse the reverse micellar organic solution after recovering the protein, the addition of surfactant is necessary to keep the sufficient amount of surfactant needed for the extraction of protein. Lysozyme recovered could easily be resolubilized with the buffer solution. The remaining activity of lysozyme recovered at 3°C and V_{aq}/V_{org}=0 was 95%.

Conclusion

Temperature strongly affects the solubilization of lysozyme into DK-F-110 reverse micellar organic phase, and any protein is not transferred from the feed aqueous phase to the reverse micellar organic phase at 5°C, while the protein is perfectly transferred at 25°C. The water solubility in the reverse micelles is also reduced by decreasing the temperature. On the basis of these results, we have examined that the operation temperature is lowered to recover lysozyme from the reverse micellar organic phase. Lysozyme is efficiently recovered regardless of the volumetric ratio of the recovery aqueous phase (V_{aq}) to the reverse micellar organic phase (V_{org}) at 3°C. When the recovery is carried out without the recovery aqueous phase (V_{aq}/V_{org}=0), lysozyme is highly concentrated. Moreover, lysozyme exhibits the high remaining activity when the recovery is operated at low temperature.

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