



BACK-EXTRACTION MECHANISM OF LYSOZYME FROM REVERSE MICELLES IN SODIUM BIS 2-ETHYLHEXYL SULFOSUCCINATE AND LONG CHAIN ALKYL AMINE MIXED SYSTEMS

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ABSTRACT

Extraction and back-extraction of lysozyme are investigated using a mixed micellar system of sodium bis(2-ethylhexyl) sulfosuccinate and long chain alkyl amines. Either di-*n*-octylamine or di-2-ethylhexylamine is used as the amine added. In these systems, reverse micelles is not formed at acidic pH range. Lysozyme extracted at pH which is slightly lower than the isoelectric point of lysozyme is successfully back-extracted by destruction of the micelles at acidic pH range. By increasing the amine concentration, the pH values, at which the back-extraction of lysozyme began, are raised, and the activity of the back-extracted lysozyme decreases. Linear relationship between the concentration of the amine added in the system and that of lysozyme back-extracted exists .

Keywords: Reverse Micelles, Lysozyme, Back-extraction, Long Chain Alkyl Amine

INTRODUCTION

Reverse micelles, which are self-organization molecular aggregates of surfactants in apolar organic solvent, have been extensively investigated as media for the extraction of proteins [1, 2]. The extraction of proteins is

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controlled by the interaction between reverse micelles and proteins, such as electrostatic interaction between the ionic surfactant and the charged surface of proteins, hydrophobic interaction between the interface of reverse micelles and the hydrophobic part of proteins, and the steric exclusion effect from the micelles [3,4].

One intensively studied surfactant is sodium bis 2-ethylhexyl sulfosuccinate (AOT). Extraction of protein with the usual AOT reverse micellar system is controlled using the changes of either the surface characteristics of proteins by pH or the size of reverse micelles by the salt concentration, for example, forward extraction can be carried out at pH lower than the protein's isoelectric point (pI), where proteins have positive charge, and the back-extraction at pH higher than pI, where proteins have negative charge. Among these interactions, electrostatic interaction can be most effective for the extraction of proteins. However, at the same time, the electrostatic interaction often causes a structural change and inactivation of proteins. It is necessary to control the interactions for the effective extraction of proteins and also to eliminate the interactions in the back-extraction process.

One of most important problems in reverse micellar extraction processes of protein is a difficulty of back-extraction process. If it is possible to greatly change the characteristics and structure of reverse micelles by means of external stimulation, it will be possible to construct a more effective separation process of proteins. Some studies have been reported on the control of the formation of reverse micelles by pH [5-8], pressure [9] and temperature [10] for the purpose of developing novel and effective extraction and back-extraction processes for proteins. Previously, the addition of various long chain alkyl amines to the AOT reverse micellar system has been found effective to control the formation of reverse micelles and the extraction of proteins by pH change [11]. The formation of reverse micelles in this mixed system, AOT and long chain alkyl amine, is controlled by the intermolecular interaction between the anionic surfactant AOT and the cationic salt formed from the added amine by decreasing pH. In this paper, either di-*n*-octylamine (DOA) or di-2-ethylhexylamine (DEHA) was used for the additives to the AOT reverse micelles. Extraction and back-extraction of lysozyme were carried out using the DOA-AOT and DEHA-AOT mixed micellar systems. The effects of the concentrations of AOT, the amine and lysozyme on the back-extraction were examined in detail. Based on those results, the back-extraction mechanism of lysozyme in the mixed micellar system was discussed.

MATERIALS

The surfactant constructing reverse micelles was sodium bis(2-ethylhexyl) sulfosuccinate (AOT) from Nacalai Tesque Co. Bis(2-ethylhexyl) amine (DEHA) and di-*n*-octylamine (DOA), which were used as additives for modification of the reverse micellar system, were obtained

from Wako Pure Chemical Co. AOT was dissolved in isooctane, and then the long chain alkyl amine was dissolved in the solution. A buffer solution containing NaCl or CaCl₂ (0.1-1.0 kmol/m³) was used as an aqueous phase. Acetic acid-sodium acetate (pH 3-6), dimethyl glutamate-NaOH (pH 5-8), glycine-NaOH (pH 9-11), and Na₂HPO₄-NaOH (pH 11-12) were used as buffers at 50 mol/m³ concentrations in all experiments. Egg white lysozyme was purchased from Wako and dissolved in the buffer solution containing salts.

METHODS

Extraction and back-extraction of lysozyme were carried out by the phase transfer method. Same volumes of the organic and aqueous phases were placed in a screw capped sample tube. The two phases were dispersed completely by a magnetic stirrer for 30 min. at 298 K. After mixing, the solution was separated into two phases by centrifugation at 3500 rpm for 15 min and the organic and aqueous phases were collected, respectively. Back-extraction of lysozyme from the micellar phase was carried out by contacting the organic phase extracted lysozyme with a new aqueous phase in the same manner as for the forward extraction.

The water concentration in the micellar organic phase was determined by Karl-Fisher titration using a Kyoto Electronics MKS-1S. The concentration of lysozyme in the aqueous and organic phases, was measured by adsorption at 280 nm with a Hitachi UV 3200. Lysozyme activity was measured by the lysis rate of *Micrococcus lysodeketicus* [12].

RESULTS AND DISCUSSION

Forward extraction of lysozyme

Extraction of lysozyme was carried out at 0.1 kmol/m³ NaCl using the AOT, DEHA-AOT and DOA-AOT systems. The effects of pH on the extraction of lysozyme are shown in Fig. 1. In the AOT system, lysozyme extraction effectively occurred at a wide pH range around neutral pH. In the pH range lower than 5, the extracted fraction of lysozyme into the organic phase, E_f , was decreased, whereas the removed fraction of lysozyme from the aqueous phase, R_f , is very high and a large number of aggregates was observed at the interface and in the aqueous phase. Lysozyme is considered to be denatured by strong electrostatic interaction with AOT.

In the DEHA-AOT and DOA-AOT mixed systems, E_f was also decreased with pH values lower than a certain pH, a pH higher than that in the AOT system. Under this condition, R_f decreased with pH and the aggregates were not formed. The decrease in both E_f and R_f almost corresponded to the decrease in the water extraction. Because the reverse micelles do not form at acidic pH by the interaction with the alkyl ammonium ion, it is considered that lysozyme is not extracted and that the interaction between lysozyme and AOT was suppressed.

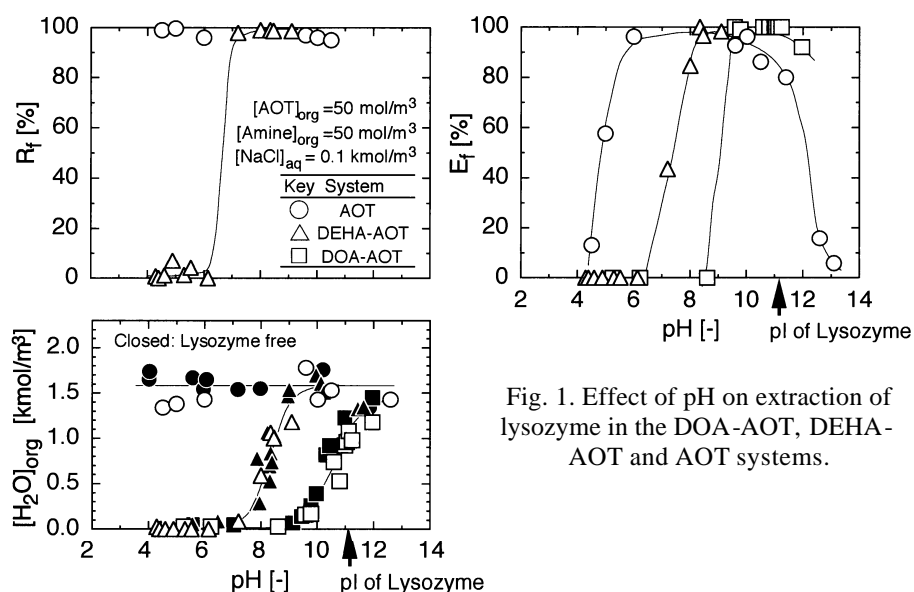


Fig. 1. Effect of pH on extraction of lysozyme in the DOA-AOT, DEHA-AOT and AOT systems.

BACK-EXTRACTION OF LYSOZYME FROM THE MICELLAR PHASE

Effect of pH

Back-extraction of lysozyme extracted into the micellar phase at pH 9.0 and 0.1 M NaCl was carried out by contact with a new aqueous phase. The effects of pH on the back-extracted fraction of lysozyme into the aqueous phase, E_b , the removed fraction of lysozyme from the organic phase, R_b , the concentration of water in the organic phase, and the residual activity of the back-extracted lysozyme, RSA , are shown in Fig. 2. In the AOT system, lysozyme could not be back-extracted using the aqueous phase containing 0.1 M NaCl, regardless of the pH values. By increasing NaCl concentration up to 1.5 M, lysozyme could be back-extracted in the pH range higher than the pI. The size exclusion effect from the reverse micelles by the increase in the salt concentration is required for the back-extraction of lysozyme in the AOT system. Because lysozyme has a very high pI (pI=11.1) and relatively small size, back-extraction of lysozyme in the AOT system requires that the aqueous phase have at very high pH and high salt concentration.

In the mixed micellar systems, lysozyme was effectively back-extracted at a more acidic pH range than that of the forward extraction, in which the water extraction is very low and the reverse micelles are not formed. There is a difference between the increase behavior of E_b and that of R_b by lowering pH, that is, the pH region where lysozyme is not back-

extracted to the aqueous phase, though it decreases from the organic phase, is observed. *RSA* was high and decreased moderately with pH. Back-extraction of lysozyme in the mixed system is considered to be carried out by the destruction of the reverse micelles that are caused by the complex formation between AOT and the cationic ammonium ion formed in the acidic pH range [11].

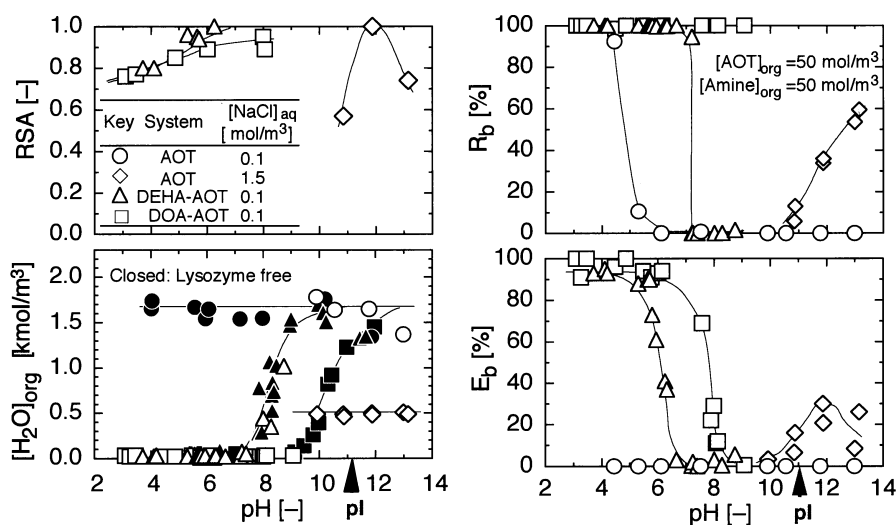


Fig. 2. Effect of pH on back-extraction of lysozyme in the DOA-AOT, DEHA-AOT and AOT systems.

Effects of the concentrations of the long chain alkyl amine

Back-extraction of lysozyme extracted using the mixed micellar systems increasing the amine concentrations at a fixed AOT concentration at 50 mol/m^3 was carried out at various pH values. Effect of pH on the back-extraction for the DEHA-AOT system is shown in Fig. 3 and for the DOA-AOT system in Fig. 4. In the DEHA-AOT system at 45 mol/m^3 of DEHA, lysozyme is not back-extracted regardless of the pH values even though R_b increases with an decrease in pH. In this condition, a large amount of aggregates of lysozyme was observed at the interface and in the aqueous phase. At 47 mol/m^3 or above of the DEHA concentration, lysozyme is back-extracted at acidic pH range. The pH values, at which the back-extraction of lysozyme began, are raised by increasing the concentration of DEHA.

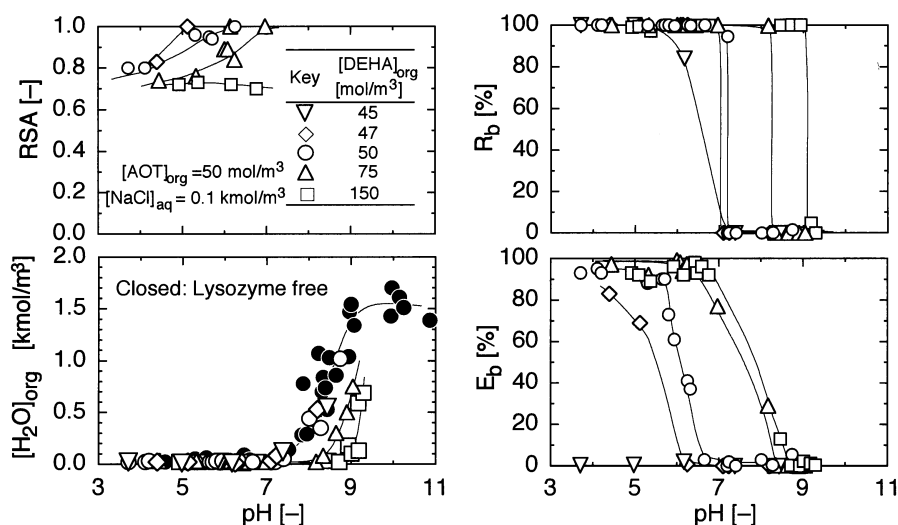


Fig. 3. Effect of pH on back-extraction of lysozyme at various concentrations of DEHA.

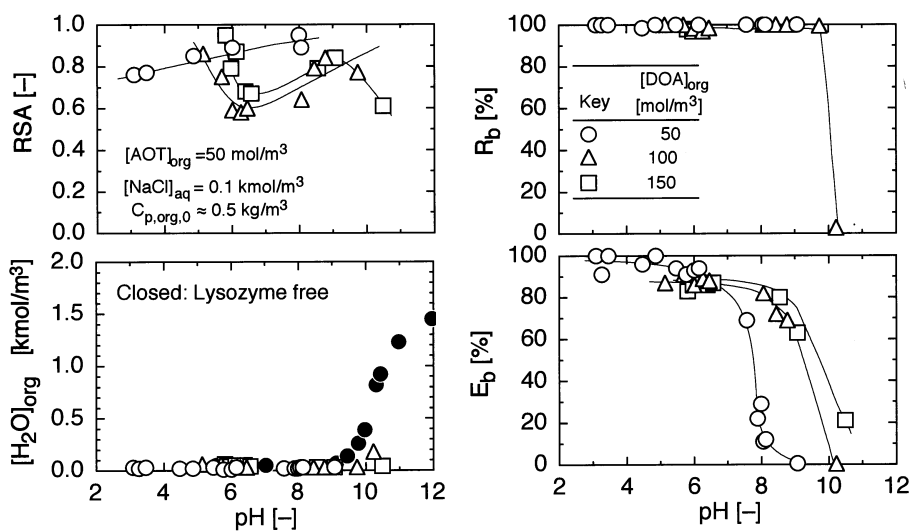


Fig. 4. Effect of pH on back-extraction of lysozyme at various concentrations of DOA.

In the DOA-AOT system, the same tendency is observed. The concentration of the ammonium salt increases by the increase in the amine concentration, hence the destruction of reverse micelles is effectively occurred from high pH. However, the activity of the back-extracted lysozyme decreased at high amine concentration. It is obvious that

lysozyme is denatured by addition of the amine to excess. When the concentrations of both AOT and DOA was changed with keeping at equimolar ratio, no significant difference in the results was observed.

Relationship between the concentrations of back-extracted lysozyme and DOA

The effects of the concentrations of AOT, DOA and lysozyme on the back-extraction were studied in detail. Back-extraction of lysozyme extracted using the DOA-AOT mixed micellar system at various concentrations of AOT, DOA and lysozyme was carried out at pH 4.0 where the all molecules of DOA is estimated to be converted to its ammonium ion. Relationship between the concentration of lysozyme back-extracted into the aqueous phase and that of DOA at various AOT concentrations is shown in Fig. 5. In the concentration range of DOA lower than a specific one, which depends on the AOT concentration, no lysozyme was back-extracted in the aqueous phase. In these cases, no lysozyme remained in the organic phase and large amounts of aggregates were observed at the interface. The DOA concentration, which began the back-extraction, increased with an increase in the AOT concentration. In the concentration range of DOA higher than the specific one, the concentration of lysozyme back-extracted to the aqueous phase increased linearly with that of DOA. The slope of the straight line at each AOT concentration was almost same.

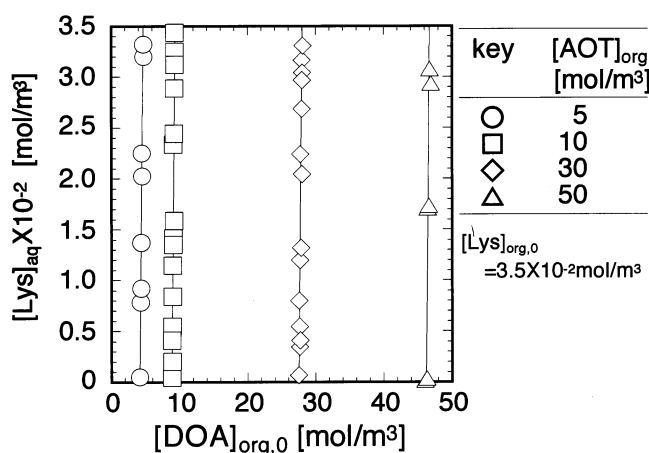


Fig. 5. Relationship between concentrations of lysozyme back-extracted and DOA used at various initial AOT concentrations.

Relationship between the concentration of lysozyme back-extracted into the aqueous phase and that of DOA at various initial lysozyme concentrations is shown in Fig. 6.

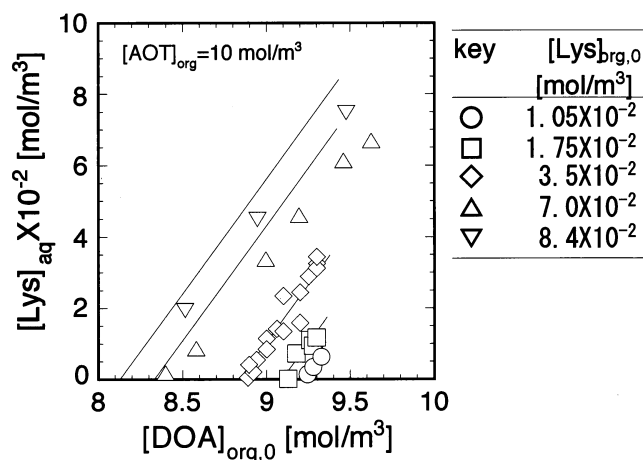


Fig. 6. Relationship between concentrations of lysozyme back-extracted and DOA used at various initial lysozyme concentrations.

The DOA concentration, which began the back-extraction, decreased with an increase in the lysozyme concentration. A linear relationship between the concentration of lysozyme and that of DOA was also observed with same slope at any lysozyme concentration. It is considered that the slope of the straight lines in Fig. 5 and 6 shows the reciprocal of the average number of DOA molecules needed to back-extract lysozyme from the organic phase. These results suggest that a quantitative relationship among the concentrations of the amine, AOT and lysozyme is present. The quantitative analysis on the interaction between AOT and the amines have been reported in previous work [11]. However, the quantitative analysis on the interaction among with AOT, alkylamine and proteins can not be sufficiently clarified. Further investigation is necessary in order to clarify the quantitative analysis on the mechanism of extraction and back-extraction.

Back-extraction mechanism of lysozyme in the mixed micellar system

The experimental results of the effects of pH and the amine concentration on the back-extraction in the mixed systems of long chain alkyl amine and AOT show that two stages exist in the back-extraction of lysozyme by the destruction of reverse micelles which is caused by the electrostatic interaction between AOT and the ammonium ion formed. One is the stage in which lysozyme is not back-extracted to the aqueous phase though the reverse micelles is destructed and lysozyme decreases from the organic phase, and is observed at pH region where E_b is low though R_b is high and at lower concentration of the amine than the concentration which the back-extraction is began by increasing the amine concentration. Another

is the stage in which the concentration of the lysozyme back-extracted increases in proportion to the amine concentration.

On the bases of these facts, qualitative back-extraction mechanism of lysozyme in the mixed micellar system is suggested as shown in Fig. 7.

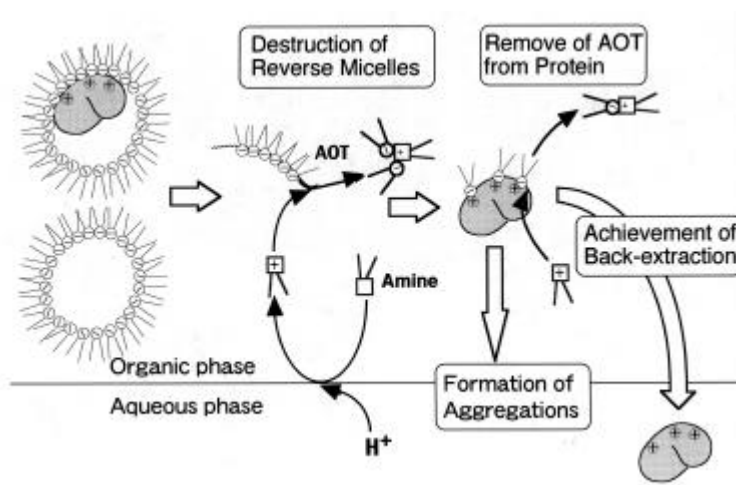


Fig. 7. Schematic diagram of the back-extraction of lysozyme from a long chain alkyl amine-AOT reverse micellar phase by formation of ion-pare complex.

Lysozyme extracted in the reverse micelle has positive charge on its surface, and a part of AOT is adsorbed to the surface of lysozyme by the electrostatic interaction. The amine in the organic phase will be converted to the ammonium ion by contacting with the aqueous phase. First step of the back-extraction is destruction of reverse micelles by the formation of ion-pare complex between AOT and the ammonium ion. Second step is remove of AOT from the surface of lysozyme by the ammonium ion. It is considered that the ammonium ion react preferentially with AOT molecule forming reverse micelles compared with that adsorbed on the surface of lysozyme. In case of pH region in which the ammonium ion is not formed at the sufficient concentration or insufficient addition of the amine, the AOT molecules adsorbed on the surface of lysozyme could not be removed and lysozyme bound with AOT would not dissolved both in the organic and the aqueous phases, and then would be form aggregates by denaturation. By sufficient addition of the amines or sufficient formation of the ammonium ion at further lower pH region, the AOT adsorbed on the lysozyme surface would be removed by the formation of ion-pare complex between AOT and the ammonium ion and then lysozyme would be back-extracted to the aqueous phase.

CONCLUSIONS

The addition of long chain alkyl amines to the AOT reverse micellar system can effectively control the formation of reverse micelles by pH change in the aqueous phase. Extraction of lysozyme is also controlled by the formation of the reverse micelles, and does not completely occur in the pH range in which no water is extracted into the organic phase. Lysozyme extracted into the mixed micellar systems can be successfully back-extracted with high activity yield by destruction of the micelles in the acidic pH range in which no water is extracted into the organic phase. By increasing the amine concentration, the pH values, at which the back-extraction of lysozyme began, are raised, and the activity of the back-extracted lysozyme decreases. Linear relationship exists between the concentration of the amine added in the system and that of lysozyme back-extracted.

Nomenclature

E_f	= extracted fraction of protein into organic phase [%]
E_b	= back extracted fraction of protein into aqueous phase [%]
R_f	= removed fraction of protein from aqueous phase [%]
R_b	= removed fraction of protein from organic phase [%]
RSA	= residual specific activity of lysozyme back-extracted [%]
[]	= concentration of spices in bracket [kmol/m ³]

Subscript

0	= initial state
aq	= aqueous phase
org	= organic phase

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