

EFFECTIVE PURIFICATION METHOD OF LARGE MOLECULAR WEIGHT PROTEINS USING CONVENTIONAL AOT REVERSE MICELLES

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Hydrophilic bulky proteins with molecular weights larger than 60 kDa, such as catalase, β -galactosidase, BSA and hemoglobin, can be easily solubilized into a micro water pool of AOT reverse micelles by the injection method. Those proteins and enzymes solubilized into reverse micelles maintain their activities and native structures, and can be back-extracted effectively to a new aqueous phase when the system pH is kept higher than their isoelectric points, *pI*, with low salt concentration, i.e. high water content. The back-extraction of hemoglobin is also partially achieved under these conditions. The efficiency of back-extraction is strongly influenced by the pH values in the feed protein solution and in the aqueous solution used for back-extraction, as well as KCl concentration in the solution. The pH values of both solutions should be higher than the protein *pI* together with high *W_o* or low salt concentration to preserve native structures and activities, since monomers and oligomers with relatively large molecular weight are likely to be denatured by association with ionic surfactant mediated by salt. In this case, both steric and electrostatic interactions between bulky proteins and micelles play dominant roles in the separation. By reducing interactions with micelles or surfactants, proteins solubilized in micelles can be effectively stabilized, and easily back-extracted to the aqueous phase without inactivation. The present method suggests another way for the effective bioseparation of bulky proteins with high yields of activity.

Introduction

Solubilization and extraction of proteins into reverse micelles are carried out by phase transfer, injection, or powder methods (Luisi, 1985). A number of studies have been carried out, mainly using the phase transfer method, for the extraction and separation of proteins and enzymes (Fletcher and Parrott, 1989; Leodidis and Hatton, 1989; Nishiki *et al.*, 1993). Extraction of proteins into micelles is controlled by steric, electrostatic, and hydrophobic interactions between proteins and micelles, and relatively large monomeric and oligomeric proteins have been believed to be rather difficult to be extracted into reverse micelles (Goto *et al.*, 1990; Kinugasa *et al.*, 1994; Kuboi *et al.*, 1990a). Such proteins are likely to be excluded by steric interactions with micelles or irreversibly denatured in the reverse micellar systems (Kuboi *et al.*, 1990b), and thus cannot be readily back-extracted from the reverse micelles (Marcozzi *et al.*, 1994). The solubilization of proteins into reverse micelles for the study of enzymology in organic solvents is usually performed by the injection method (Khmelnitsky *et al.*, 1989). However, a few very limited studies on extraction have been made using the

injection method for the solubilization and separation of proteins by reverse micelles (Giovenco *et al.*, 1987; Kuboi *et al.*, 1990b).

In the previous work (Shiomori *et al.*, 1994), a bulky protein, β -galactosidase (464 kDa), was found to be solubilized in an active state into AOT reverse micelles by the injection method, and was also back-extracted effectively from the micellar phase to a new aqueous phase when water content, *W_o*, of micellar phase, i. e. micellar size, was properly selected. Although the effects of pH and salt on the protein extraction in reverse micellar systems are important factors (Golken and Hatton, 1987; Kuboi *et al.*, 1990a; Leodidis and Hatton, 1989; Nishiki *et al.*, 1993), it has not been studied yet in previous works. Since the interactions between proteins and ionic surfactants are governed by pH values and salt concentrations, these influences both in the injection and back-extraction steps are rather important. The purpose of this paper is to investigate these effects on protein extraction and purification processes in more detail and also to extend it to other bulky or oligomeric proteins, such as BSA, catalase and hemoglobin. These results were also compared with a conventional process using the phase transfer method.

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Table 1 Properties of used protein and the concentrations of protein solubilized in organic phase and effective micelles for protein solubilization

Proteins	<i>M. W.</i> [kDa]	<i>pI</i> [-]	<i>HFS</i> [kJ/mol]	<i>dp</i> [nm]	[Protein] _{org,0} [mol/m ³]	<i>C_{m,eff}</i> ^(d) [mol/m ³]
β-Galactosidase	464	5.0	-447	10.5±2.0 ^(a) 6.85 ^(b)	1.6×10 ⁻⁴ 3.7×10 ⁻⁴	3.7×10 ⁻⁴ 1.6×10 ⁻⁷
Catalase	240	5.5	-75.7	5.2 ^(b)	1.5×10 ⁻³	1.7×10 ⁻¹
BSA	66	4.9	-231	3.5 ^(b)	5.5×10 ⁻³	1.6×10 ⁰
Hemoglobin	65	6.8-7.0	-208	6.7 ^(c)	1.1×10 ⁻³	1.1×10 ⁻²

a) present study (measured by DLS), b) from Horiike *et al.*, 1989 (measured by gel chromatography), c) from Porter and Michaels (measured by gel chromatography), d) calculated from protein diameter and micellar size distribution at *W_o* = 20 (Yamada *et al.*, 1994)

1. Experimental

1.1 Materials

Bis (2-ethylhexyl) sodium sulfosuccinate (AOT) was purchased from Nacalai Tesque Co, and used without further purification. β-Galactosidase from *E. coli*, bovine serum albumin (BSA) and hemoglobin from bovine were purchased from Sigma Chemical Co. Catalase from bovine liver was obtained from Wako Pure Chemical Industries Co. The proteins were used without further purification. The molecular weight (*M.W.*), isoelectric point (*pI*), surface hydrophobicity (*HFS*, Kuboi *et al.*, 1990c) and size of the proteins used are shown in Table 1. Acetic acid-sodium acetate (*pH* 3.6-5.6), Tris-HCl (*pH* 7.2-9) and glycine-NaOH (*pH* 8.5-11) buffer were used. The concentration of these buffers were 10 mM in all cases. BCA protein assay reagent was purchased from Sigma Chemical Co. All the other chemicals used were of reagent grade.

1.2 Methods

Proteins were solubilized into 200 mM AOT/isooctane solution by the injection method; the feed buffer solution (0.72 ml) dissolving protein (1-5 mg/ml) was injected into 10 ml of AOT/isooctane solution, and shaken vigorously until a clear solution was obtained. The concentrations of proteins solubilized in the micellar solution are shown in Table 1. Initial *W_o* of the prepared reverse micellar solution, *W_{o,inj}*, was kept at 20 in all experiments as in the previous work (Shiomori *et al.*, 1994) in order to maintain constant *W_o* values in both the solubilization and back-extraction steps. No salts were added in the injected feed solution to prevent non-specific hydrophobic interaction between proteins and AOT and to keep a large value of *W_{o,inj}*. In the freshly prepared reverse micellar solution for all proteins used, aggregation or turbidity of the micellar solution was not observed for at least one day.

Back-extraction of the protein from the reverse micelles was carried out by contacting 5 ml of the reverse micellar solution containing proteins with 5 ml of the buffer solution with KCl for 3 *hs*, and then the solution was centrifuged at 5,000 *rpm* for 15 minutes. The *pH* value of the feed solution injected into the reverse micelles, *pH_{inj}*, and the *pH* value, *pH_{aq}*, and the KCl concentration in the aqueous solution used for the back-extraction were varied in the experiments.

Protein extraction by the phase transfer method was

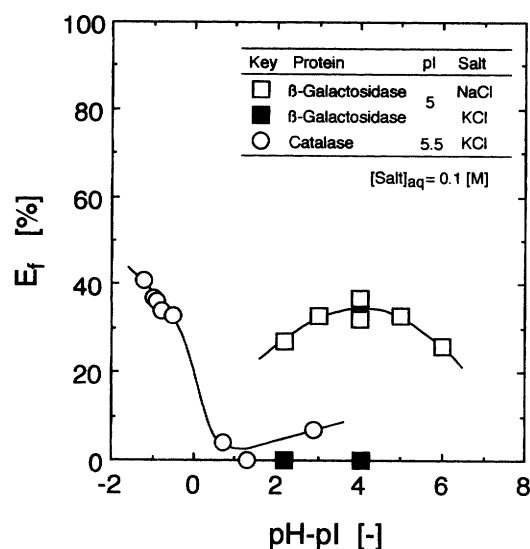


Fig. 1 Effect of *pH* on extraction of catalase and β-galactosidase by phase transfer method

carried out by contacting 5 ml of 200 mM AOT/isooctane solution with 5 ml of buffer solution solubilized protein and salt for 3 *hrs*. Back-extraction of the protein extracted by phase transfer was carried out by the same procedure as in the injection method.

The separation of β-galactosidase from cell extracts was also carried out. Preparation of cell extracts was carried out in the same manner as in the previous work (Shiomori *et al.*, 1994). The cell extracts were solubilized into reverse micellar solutions by the injection method, and back-extraction was effected by the same procedure as described above. The activity of β-galactosidase and the concentration of total soluble protein in the aqueous phase were measured as follows.

The protein concentrations in the micellar organic phase and in the aqueous phase were determined using a Hitachi UV-3200 spectrophotometer. BSA and β-galactosidase were monitored at 280 nm and hemoglobin and catalase at 406 nm. The concentration of catalase in the aqueous phase was determined by the BCA method (Smith *et al.*, 1985). The concentration of total soluble protein was measured by the pyrogallol red method (Fujita *et al.*, 1983). The activity of catalase was measured by the decomposition rate of H₂O₂ at 298 K according to the literature (Sumner and Dounce, 1937). β-Galactosidase activity was measured by the hydrolysis rate of o-nitrophenylgalactopyranoside (Steers *et al.*, 1971).

The fraction of protein back-extracted to the aqueous phase, *E_b*, was calculated as the ratio of the amount of protein recovered in the aqueous phase to that in the organic phase prepared by the injection method. In the case of the back-extraction of hemoglobin, the amount of protein recovered in the aqueous phase differed from that back-extracted from the organic phase. This difference was defined as the amount of precipitate of the protein. The percentage of precipitate of protein, *P*, was also calculated as the ratio of the amount of precipitate of the protein to the amount of protein in the initial organic phase. The resid-

Table 2 Extraction and back-extraction of enzyme by phase transfer method

Enzyme	Extraction				Back-extraction			
	<i>pH</i>	Salt	E_f [%]	W_o	<i>pH</i>	Salt	E_b [%]	<i>RSA</i> [%]
Catalase	4.6	KCl 0.1M	36	20.9	8.3	KCl 0.1M	34	0
β -Galactosidase	8.0	NaCl 0.1M	39	45.4	7.2	KCl 0.1M	52	20

a) Based on extracted concentration in forward extraction

ual activity, *RSA*, of the enzyme in the back-extracted solution was defined as the ratio of the specific activity of the enzyme back-extracted to that of the feed. The size of β -galactosidase was measured by using the dynamic light scattering method (DLS, Otsuka Electronics DLS-700Ar). Water content in the organic phase, W_o ($=[H_2O]/[AOT]$), was determined by Karl-Fisher titration.

2. Results and Discussion

2.1 Extraction and back-extraction of bulky proteins by the phase transfer method

Extraction of catalase and β -galactosidase by the phase transfer method was carried out at various *pH* values and a salt concentration of 0.1 M in the aqueous phase. The effect of *pH* on the extraction is shown in Fig. 1, in which the extracted fraction of protein, E_f , was plotted against the *pH* deviation from *pI* of the protein. In spite of their large molecular weight, catalase and β -galactosidase were both partly extracted to the reverse micellar phase. Catalase was extracted at *pH* values less than the *pI* value of 5.5. The extracted fraction of catalase, E_f , sharply decreased with increase in *pH* value, and E_f was negligible above the *pI* as expected (Leodidis and Hatton, 1989; Kuboi *et al.*, 1990a; Nishiki *et al.*, 1993). β -Galactosidase was extracted at *pH* values above the *pI* of 5 with 0.1 M NaCl, a maximum of extraction being obtained at *pH* 9.0 with the fraction of 40%. The reason for this unusual *pH* dependence of β -galactosidase extraction is not clear at present. However, since β -galactosidase extracted in the micelles is strongly inactivated as described below, this unexpected extraction is likely to be caused by the denatured protein, whose surface hydrophobicity may be changed by the binding of AOT molecules. On the other hand, when 0.1 M KCl was used for extraction, β -galactosidase was not extracted at *pH* 7.2 and 9.0. A *pH* range lower than 6 was not used for the extraction of β -galactosidase because of its significant denaturation and precipitation.

Back-extraction of enzymes, which were initially extracted to the micellar phase by the phase transfer method, was performed by contacting the micellar phase with another new aqueous phase. The conditions and results for the extraction and the back-extraction of catalase and β -galactosidase are shown in Table 2. Thirty four percent of catalase extracted to the micellar phase was back-extracted to a new aqueous phase, but completely lost enzyme activity. Catalase is considered to be denatured by

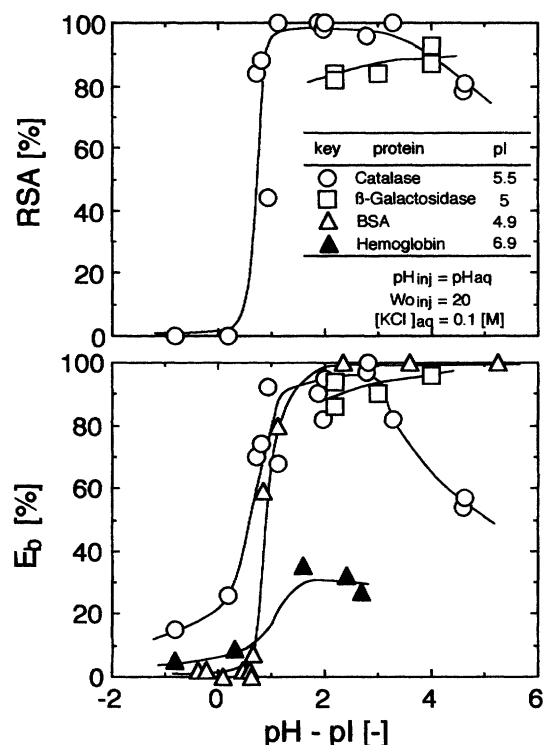


Fig. 2 Effect of *pH* on back-extraction of protein solubilized into reverse micelles by injection method keeping $pH_{inj} = pH_{aq}$

electrostatic interaction with AOT in the extraction step. The back-extracted fraction of β -galactosidase was 52%, and the relative specific activity, *RSA*, of the fractor was 20%. β -Galactosidase is also considered to be inactivated in the extraction step due to interaction with the micellar inner wall or AOT molecules. It was difficult to extract catalase and β -galactosidase by the phase transfer method, and back-extracted enzymes from the micellar phase were strongly inactivated.

2.2 Solubilization of bulky proteins by the injection method

Solubilization of large molecular weight proteins into AOT reverse micelles was attempted by use of the injection method. The maximum amount of proteins solubilized in reverse micelles can be determined by the effective micelle concentration for the protein, $C_{m,eff}$, as shown in the previous works (Shiomori *et al.*, 1994; Yamada *et al.*, 1994). $C_{m,eff}$ is the concentration of micelles larger than the protein size. The activity and the stability of β -galactosidase was strongly influenced by the W_o value, and successfully interpreted by the solubilization mechanism based on reverse micellar size distribution and $C_{m,eff}$. According to the work, lower $C_{m,eff}$ compared with the concentration of a protein to be solubilized in the micelles, is likely to cause protein denaturation due to the steric exclusion of the excess protein from micelles into organic solution. And thus, sufficiently higher $C_{m,eff}$ is required for the solubilization of a protein without its inactivation. $C_{m,eff}$ for each protein at $W_o = 20$ was calculated from the diameter of protein and the micellar size distribution as described before (Yamada *et al.*, 1994). $C_{m,eff}$ is calculated

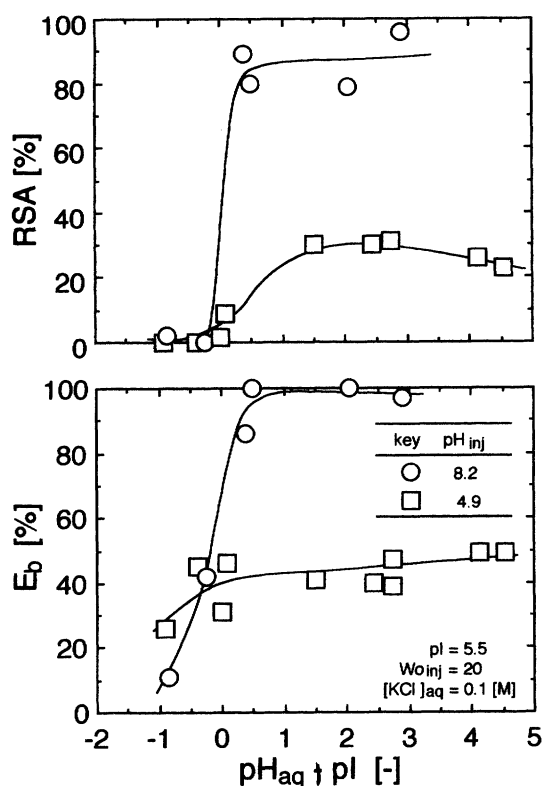


Fig. 3 Effect of pH_{aq} on back-extraction of catalase solubilized at pH_{inj} 4.9 or 8.2

shown in Table 1. $C_{m,eff}$ for catalase, BSA and hemoglobin are large enough compared with the protein concentration. $C_{m,eff}$ for very large molecular weight proteins such as β -galactosidase are very sensitive to the size of proteins, to be solubilized. The size of β -galactosidase, obtained by DLS is shown in Table 1 together with that of the literature (Horiike *et al.*, 1983). $C_{m,eff}$ were estimated in the range of 1.6×10^{-7} - 8.1×10^{-3} mol/m³ for the size of β -galactosidase of 12.5 - 6.85 nm. Therefore, the present $C_{m,eff}$ may be suitable for the solubilization of β -galactosidase. Actually all these proteins were easily and completely solubilized into organic reverse micellar solution without any precipitate at $Wo = 20$ and various pH_{inj} from 4 through 10. The prepared transparent organic solutions were used for the following back-extraction.

2.3 Effect of pH on the back-extraction

Back-extraction of proteins such as catalase, β -galactosidase, BSA and hemoglobin, solubilized into reverse micelles at various pH_{inj} was carried out at the same pH_{aq} as pH_{inj} ($pH_{aq} = pH_{inj}$) and 0.1M KCl. The results are shown in Fig. 2. The fraction of the protein back-extracted to the aqueous phase, E_b , and the relative activity, RSA , of the back-extracted enzyme are plotted against the pH deviation from pI of the protein. All proteins solubilized at higher pH than pI in the reverse micelles are shown to be back-extracted to the new aqueous phase with the same pH . In the pH range at or below the pI , it was difficult to back-extract proteins to the aqueous phase. Back-extraction of catalase was very efficient and complete at pH 6.5-8.0, and the deactivation of catalase was not observed. Although

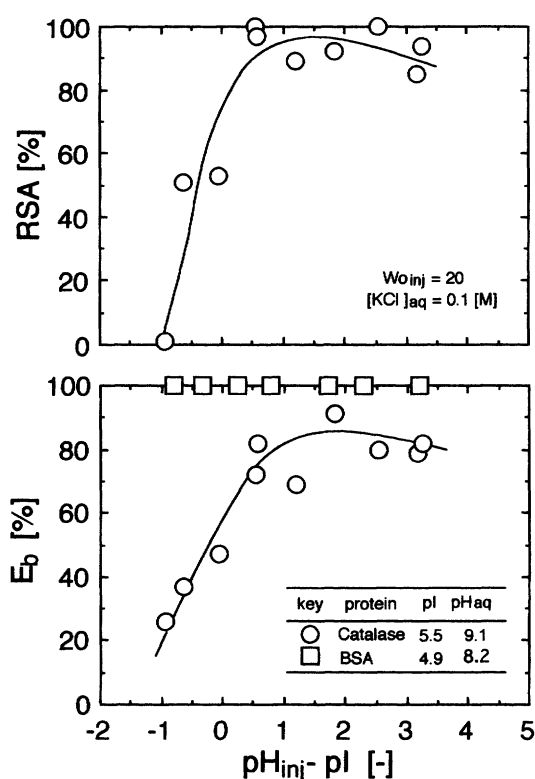


Fig. 4 Effect of pH_{inj} on back-extraction of catalase and BSA

both E_b and RSA of the catalase at pH higher than 8 decreased with pH , this is probably due to the instability of catalase in alkaline pH . In fact, catalase in bulk aqueous solution under similar conditions rapidly lost activity. The back-extraction of β -galactosidase was also possible at pH 7.2-9.0, and the activity loss during the process was very small. BSA was completely back-extracted at pH - pI values greater than 2. In contrast, the fraction of hemoglobin back-extracted was about 30%, and lower than other proteins.

Usually, large molecular weight proteins are very difficult to be solubilized into reverse micelles and tend to be precipitated in the phase transfer method (Goto *et al.*, 1990). By using the present method, all proteins including hemoglobin, could be (at least partly) solubilized into the reverse micelles to form a transparent clear solution without any precipitate, and then back-extracted to the aqueous phase. The above results of effective back-extraction of protein can be rationally explained by the steric exclusion and electrostatic repulsion between the solubilized protein and the anionic surfactant, AOT. If proteins carry their negative charge evenly on their surface at pH above pI , proteins may be forced to be in the center of the micro water pool in micelles and thus are easily back-extracted by the steric and electrostatic repulsion between protein and AOT.

2.4 Effects of pH_{inj} and pH_{aq}

The effects of pH both on the back-extraction step and on the solubilization step were further studied separately. Catalase solubilized at $pH_{inj} = 4.9$ or 8.5 was back-extracted at various pH_{aq} as shown in Fig. 3. When

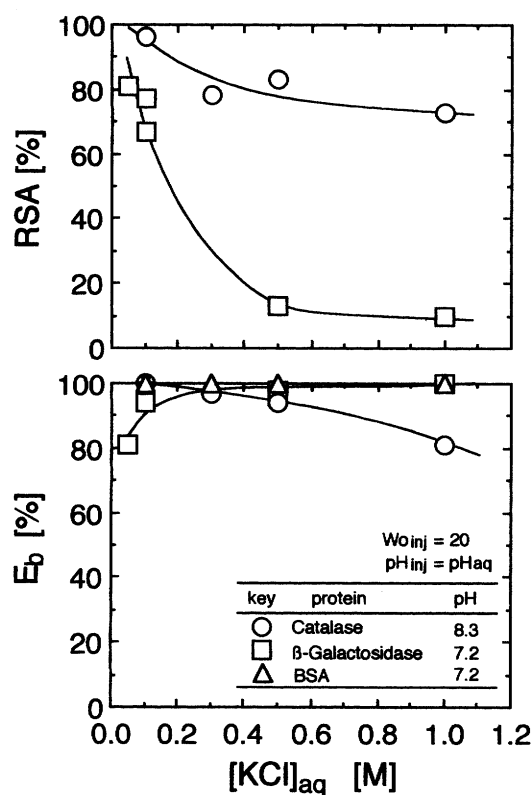


Fig. 5 Effect of KCl concentration in aqueous phase on back-extraction of protein keeping $pH_{inj}=pH_{aq}$

catalase was solubilized at $pH_{inj}=8.2$, catalase was back-extracted completely at pH_{aq} higher than pI and catalase activity after back-extraction was almost as high as the injected one ($RSA \approx 90\%$). At pH_{aq} lower than pI , E_b was lower than that obtained with pH_{aq} higher than pI , and catalase activity was completely lost. It is clear that catalase is back-extracted by an increase in the electrostatic repulsion between catalase and AOT with rising pH_{aq} value.

When catalase was solubilized at $pH_{inj}=4.9$, it was difficult to back-extract catalase even at pH_{aq} higher than pI , and E_b and RSA were lower than those at $pH_{inj}=8.2$. This indicates that catalase solubilized at $pH_{inj}=4.9$ ($pH_{inj} < pI$) is irreversibly denatured in the solubilization step or in the reverse micelles in the face of electrostatic interaction with AOT. It also indicates why the extracted proteins have often been denatured and are difficult to back-extract when using the phase transfer method. In the case of the phase transfer method, extraction of catalase is achieved at pH less than pI , and thus catalase is considered to be inactivated by electrostatic interaction in the extraction step.

In order to clarify the effect of pH_{inj} , the protein solubilized at various pH_{inj} was back-extracted under fixed condition of pH_{aq} . The results for catalase and BSA are shown in **Fig. 4**. At pH_{inj} higher than pI , back-extraction of catalase was efficient and the activity loss was small. The electrostatic interaction between catalase and the micelles is reduced by increasing pH_{inj} , and catalase retains its native structure in the solubilization step and in the water pool of the micelles.

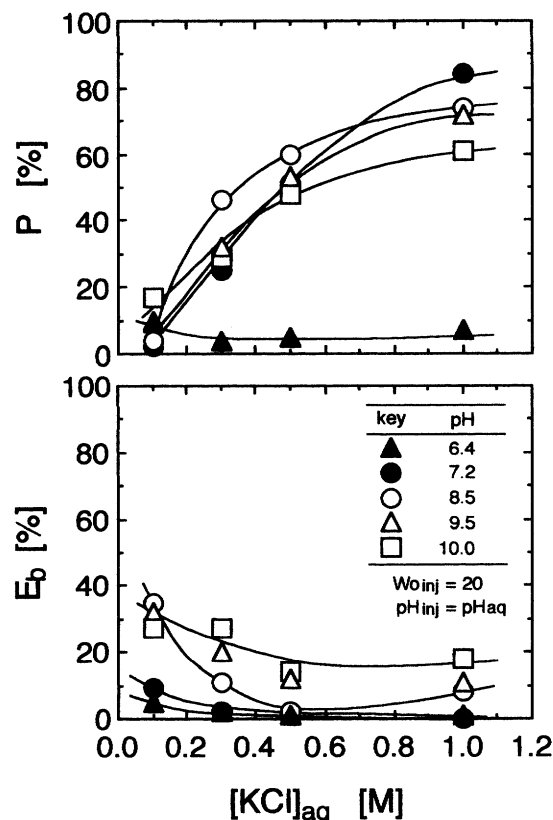


Fig. 6 Effect of KCl concentration in aqueous phase on back-extraction of hemoglobin keeping $pH_{inj}=pH_{aq}$

In contrast to catalase, BSA was completely back-extracted to the aqueous phase independent of the pH_{inj} . This suggests that BSA solubilized, even at pH_{inj} lower than pI , is not denatured both in the solubilization step and standing condition in the micelles, probably because it is a monomeric stable protein. The above effect of pH_{inj} may also depend on the nature of proteins.

2.5 Effect of KCl concentration in the aqueous phase

The back-extraction of protein from micelles, while maintaining $pH_{inj} = pH_{aq}$, was carried out at various concentrations of KCl in the aqueous phase. The effect of KCl concentration on the back-extraction of proteins at pH higher than pI is shown in **Fig. 5**. For catalase, E_b and RSA decreased only slightly with the increase in KCl concentration. For β -galactosidase, E_b was independent of KCl concentration, while RSA decreased drastically with KCl concentration. β -Galactosidase was strongly denatured in the high KCl concentration. This is probably due to the large increase in the surface hydrophobicity of β -galactosidase with the addition of KCl as previously found (Kuboi *et al.*, 1993). In contrast to β -galactosidase, hydrophilic BSA was completely back-extracted for all KCl concentrations.

The effect of KCl concentration on the back-extraction of hemoglobin at various pH is shown in **Fig. 6**. At $pH=6.4$ ($pH < pI$), hemoglobin could not be back-extracted to the aqueous phase, and was mostly retained in the reverse micelles. In the pH range greater than the pI of hemoglobin ($pI=6.8-7.0$), back-extraction of hemoglobin was partly

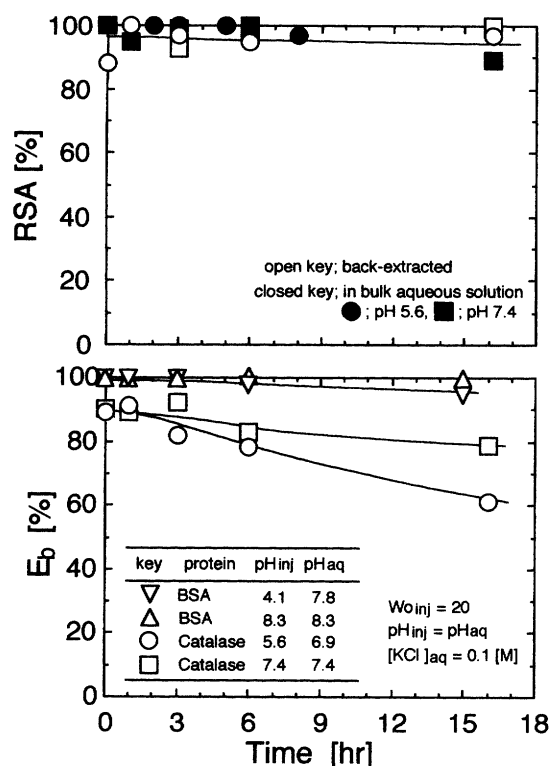


Fig. 7 Effect of incubation time after solubilization on back-extraction of catalase and BSA

achieved and most effective at 0.1M KCl. However, the back-extracted fraction of hemoglobin, E_b , decreased with the increase in KCl concentration, and the amount of precipitate increased significantly. It is known that the regions of interface between subunits are hydrophobic parts. These hydrophobic parts are likely to interact with the surfactant. It is considered, therefore, that hemoglobin, which has four subunits, is denatured to form the precipitate caused by the hydrophobic interaction with surfactant molecules at high concentrations of KCl. However, the degree of denaturation of hemoglobin may be decreased with increasing pH value due to the electrostatic repulsion with surfactant molecules. These results show that the condition of high salt concentration tends to cause the deactivation and precipitation of protein due to hydrophobic interaction with AOT. The degree of deactivation and precipitation appeared most markedly for hemoglobin.

2.6 Effect of incubation time of protein in reverse micelles

The protein solubilized into reverse micelles was incubated at 298K, and after standing for certain fixed periods a back-extraction was performed. The effect of incubation time on the E_b and RSA of the back-extracted proteins is expected to reflect the stability of the protein in reverse micelles. The results of catalase and BSA are shown in Fig. 7. The values of E_b and RSA were plotted against incubation time, together with the activity of catalase in the bulk aqueous solution. In the latter, catalase was very stable and a drop in activity was not observed for 16 hrs. In the cases of catalase and BSA solubilized at pH_{inj} 7.4 and 8.3, respectively, these pH values were higher than pI , and E_b

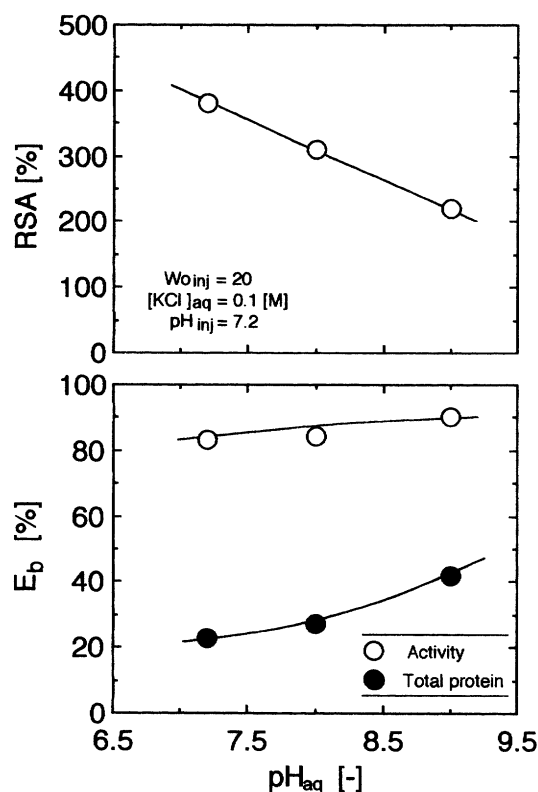


Fig. 8 Effect of pH_{aq} on back-extraction of β -galactosidase from crude cell extracts solubilized in reverse micelles

and RSA values were approximately constant for 16 hrs. This shows that the protein solubilized under conditions of electrostatic repulsion within reverse micelles is quite stable and maintains the native structure for a long time.

When catalase and BSA were solubilized at pH_{inj} 5.6 and 4.1, respectively, these pH values were approximately equal or lower than pI . E_b decreased slightly with incubation time. The RSA of catalase, however, is constant and the back-extracted catalase in the aqueous phase is not inactivated. Since only native catalase can be back-extracted, catalase denatured in the micellar water pool is considered not to be back-extracted from the micellar phase. The increase in electrostatic interaction between the proteins and the micellar inner wall causes denaturation of the proteins.

2.7 Effect of pH of back-extraction solution on separation of β -galactosidase from cell extracts.

The separation of β -galactosidase from crude extracts of *E. coli* cells was carried out as an application of the present work. The crude extracts from *E. coli* cell were solubilized into reverse micellar solution at $W_o = 20$ and $pH_{inj} = 7.2$, and then the micellar solution was contacted with 0.1 M KCl solution at various pH_{aq} . The effect of pH_{aq} on E_b based on enzyme activity and total soluble protein are shown in Fig. 8 together with RSA of the back-extracted enzyme. About 80% of β -galactosidase solubilized in the micelles was back-extracted to the aqueous phase almost independently of the pH_{aq} value. In contrast, the recovery of total soluble protein increased with pH_{aq} . As a result, RSA decreased with increasing pH_{aq} . By such manipula-

tions of pH_{aq} , contaminant proteins in crude extracts, where the pI may be higher than that of β -galactosidase, were back-extracted by the electrostatic repulsion with AOT. Reduction of the back-extraction of contaminant proteins at lower pH_{aq} enabled β -galactosidase to be purified four-fold over the crude cell extracts.

Conclusion

Catalase, β -galactosidase, BSA and hemoglobin were solubilized into AOT reverse micelles by the injection method, and then back-extracted to a new aqueous phase. The back-extraction of the protein is affected by the pH value, and the salt concentration in the feed solution and the aqueous solution used for the back-extraction. The pH values of these solutions, especially of the feed solution, should be greater than their isoelectric points, pI , since bulky proteins are easily denatured by electrostatic interaction with AOT. High KCl concentration tends to enhance the inactivation and precipitation of the enzymes and proteins in the back-extraction step. The protein solubilized in reverse micelles at pH_{inj} higher than its pI is rather stable over a long period. The interaction between proteins and micelles, such as steric, electrostatic and hydrophobic interaction, plays an important role in the back-extraction of the protein solubilized in reverse micelles by the injection method. By controlling these interactions, effective separation of the protein can be achieved by the present method.

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Nomenclature

[AOT]	=	concentration of AOT	[mM]
$C_{m,eff}$	=	effective micellar concentration for protein solubilization	[mol/m ³]
dp	=	average hydrodynamic diameter of the protein	[nm]
E_b	=	fraction of back-extraction	
	=	$100[\text{Protein}]_{aq}/[\text{Protein}]_{org,0}$	[%]
E_t	=	fraction of extraction	
	=	$100[\text{Protein}]_{org}/[\text{Protein}]_{aq,0}$	[%]
HFS	=	surface hydrophobicity of protein	[kJ/mol]
P	=	fraction of precipitates	
	=	$100\{([\text{Protein}]_{org,0}-[\text{Protein}]_{org})/[\text{Protein}]_{aq}\}/[\text{Protein}]_{org,0}$	[%]

[Protein]	=	concentration of protein	[mol/m ³]
RSA	=	relative specific activity based on feed specific activity	[%]
W_o	=	$[\text{H}_2\text{O}]/[\text{AOT}]$	
	=	water content, molar ratio of H ₂ O to AOT	[-]

<subscript>

0	=	initial state
inj	=	condition of solubilization by the injection method
aq	=	aqueous phase used for back-extraction
org	=	organic phase

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