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Reverse Micellar Extraction of ß-Galactosidase from *E.coli*

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Forward and back extraction of *E.coli* ß–galactosidase(ß－gal) were carried out using AOT–isooctane reverse micellar systems, in which an injection method and a phase transfer method were employed. ß-Gal is a large oligomeric protein with molecular weight of 540kDa. Unexpectedly, ß-gal was easily solubilized with the former method and back extracted into 0.1M KCl aqueous solution without much loss of enzyme activity, though ß-gal in the micellar solution did not show enzyme activity. In the latter method, ß-gal was extracted into the reverse micellar solution up to 40% of the feed protein depending on both salt type and salt concentration. A novel solubilization mechanism was suggested for the large oligomeric proteins.

1. Introduction

A reverse micelle forms a micro water-pool, surrounded by surfactant molecules such as sodium di-2-ethylhexylsulfosuccinate(AOT) in organic media. This has the ability to solubilize protein and other biopolymers in an organic solvent. Extraction behavior and mechanism of some proteins and enzymes into reverse micelles have been investigated[1,2,5,6]. Extraction of proteins is dependent on electrostatic, steric and hydrophobic interactions between protein and micelles. Selective extraction of the desired protein from protein mixture is possible by the combination of these factors[3,4]. Solubilization mechanism and controlling factor to achieve good activity yield are, however, not well understood. Some proteins, especially large proteins are difficult to dissolve into reverse micelles and certain enzymes are irreversibly denaturated by surfactants and/or organic solvents.

ß-Galactosidase(ß-gal) is one of the important enzymes in the various

fields, and it has been widely used for the production of lactose-free milk, whey syrup and garactooligosuccarides. However no work has been reported on the extraction of ß-gal using reverse micellar systems. ß-Gal from *E.coli* is a very large oligomeric protein with molecular weight of 540kDa with isoelectric point(pI) of about 5. It has very hydrophilic surface, though hydrophobicity is drastically increased by the addition of salt.

In this work, extraction and back extraction of ß-gal was attempted using AOT-isooctane reverse micellar systems both with an injection method and a phase transfer method.

2. Experimental

ß-Galactosidase from *E.coli* was purchased from Sigma Chemical Co. AOT was obtained from Wako Chemical Co. A 200mM AOT solution in isooctane was used in all experiments. Phosphate(pH 6.0-8.0), Tris-HCl(pH 7.2-9.0) and glycine-NaOH(pH 8.5-11.0) buffers were used in 10mM in all cases.

Solubilization of ß-gal by an injection method was carried out by adding microliter quantities of a ß-gal solution at Wo=20 to a solution of AOT in isooctane. Back extraction was carried out by contacting reverse micellar solution containing ß–gal(60μg/ml,Wo=20), prepared by injection method, with aqueous solution of given pH and salt concentration for 4hrs. Forward extraction of ß-gal was carried out by a phase transfer method in such a way that equal volume of AOT-isooctane solution was contacted with feed aqueous solution containing ß–gal(100μg/ml) of given pH, salt concentration for 3 hrs.

The concentration of ß-gal was determined by UV spectrometer at 280nm. Water content in organic micellar solution was measured by Karl–Fischer titration. Reverse micelle size was measured by the dynamic light scattering method by using DLS–700Ar(Otsuka Elector.). The activity of ß-gal in aqueous phase was analyzed by using o-nitrophenyl-ß-D-galactopyranoside(ONPG) as its substrate[7].

3.Results and discussion

3.1 Solubilization of ß-gal by an injection method

Clear and stable micellar solution containing ß-gal was obtained with an injection method. Fig.1 shows the relation between ß-gal concentration in the micellar solution and the micelle diameter. The micelle diameter was increased with an increase in ß-gal concentration in the micellar solution. Such a large increase in the average micellar diameter containing ß-gal was not explained on the basis of the previous protein uptake model[5,6].

Solubilized ß-gal did not show any enzyme activity in the micellar solution where another portion of micellar solution containing ONPG was added as a

substrate. This suggests that the active site of solubilized ß-gal is exposed to the organic solvent or blocked by the surfactant molecules.

3.2 Back extraction of ß-gal from reverse micellar solution

ß-Gal solubilized in the reverse micellar solution using the injection method was back extracted into the aqueous phase without much loss of the enzyme activity. The effect of salt type and concentration of the aqueous phase on the back extraction and the resulting Wo are shown in Fig.2. Back extraction of ß-gal was influenced by NaCl concentration, the maximum recovery obtained at 0.5M. In the case of KCl, however, back extraction of ß-gal was very efficient, nearly complete at 0.1M and not influenced by the further increase in KCl concentrations. This suggests that effective separation of ß-gal from other proteins can be achieved in this back extraction step, since in this low salt concentration most protein remain in the reverse micelles. At pH 7.0 and 9.0 Tris-HCl buffer, the recovery of ß-gal was almost independent on the pH value.

When high salt concentration and acidic aqueous solution of pH less than 6 were employed for the back extraction, significant amount of precipitates was observed at the interface between the organic micellar phase and the aqueous phase.

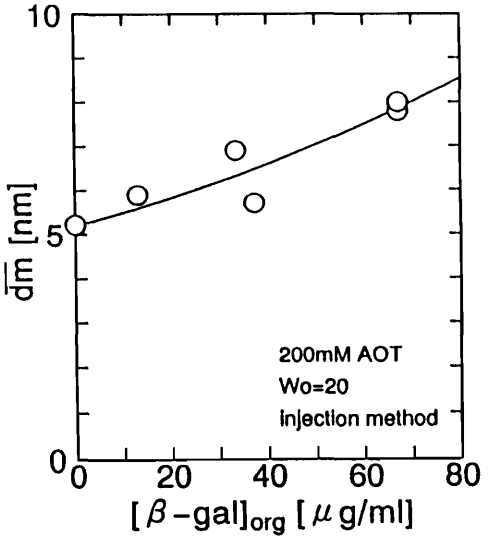

Fig.1 The relation between β-gal concentration in the water pool and the average micelle diameter

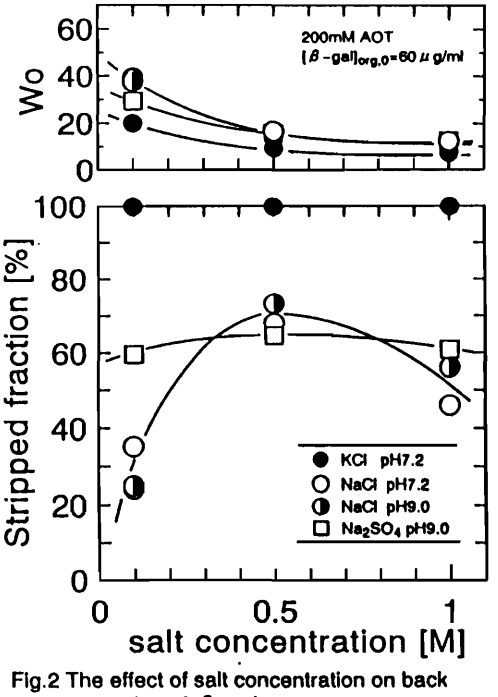

Fig.2 The effect of salt concentration on back extraction of β-gal

3.3 Extraction of ß-gal by phase transfer method

1) Effect of salt

The influence of salt type and concentration on forward extraction was studied at pH 7.2 with Tris-HCl buffer. Fig. 3 shows the effect of salt type and aqueous phase salt concentration on the extracted fraction, E, and on the water content of the resulting organic micellar solution Wo. The extraction was influenced both by salt type and by salt concentration. In the cases of KCl and sodium salts, the extracted fraction E decreased with an increase in salt concentration as expected from the previous works[3], the maximum extracted fraction of ß-gal was about 30% at 0.05M NaCl. On the other hand, in the case of the addition of divalent metal salts such as $CaCl_2$, $MgCl_2$ and $MnCl_2$, E increased with salt concentration up to 0.5M, and then decreased slightly.

When divalent metal salts were used, it was also observed that aggregates formed and precipitated at the interface between the organic and the aqueous phase and also in the aqueous phase. This may be caused by the increase in surface hydrophobicity due to the electrostatic binding of divalent cation on ß-gal, resulting in strong hydrophobic interaction among them. The water content in the organic micellar solution Wo decreased with an increase in monovalent metal salts concentration. However, when divalent metal salts were used, Wo was not dependent on salt concentration.

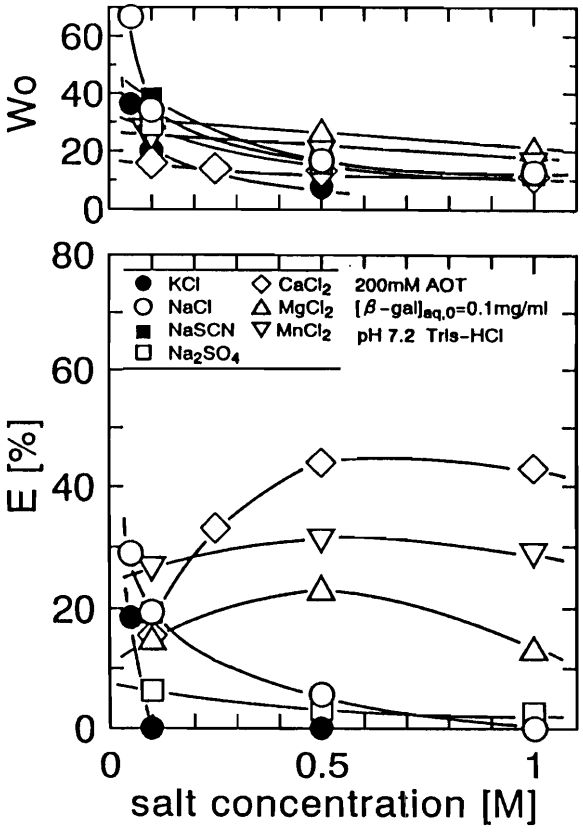

Fig.3 The effect of salt concentration on extraction of β-gal

2) Effect of pH of the aqueous phase

The influence of pH on the extraction of ß-gal was studied at 0.1M NaCl and shown in Fig. 4. Although pH dependence was not clear, a maximum of ß–gal extraction was obtained at pH 9.0 with the extracted fraction of 40%. The

Wo of the resulting micellar solution was in the range of 35-40 at the pH range examined.

In the range of pH less than 6, no extraction of ß-gal was observed, and a large amount of aggregates precipitated. In this acidic range, surface net charge of ß-gal was changed from negative to positive (pI=5). The electrostatic interaction between ß-gal and anionic surfactant AOT might have enhanced denaturation and precipitation of ß-gal.

3)Solubilization mechanism of ß-gal

Different dependency of the extracted fraction E on salt concentration between monovalent and divalent metal salts may suggest a different solubilization mechanism from the previous protein uptake models[5,6]. This was further studied by comparing the micellar concentration, $C_{m,tot}$ and $C_{m,eff}$ with the solubilized ß–gal concentration in the reverse micellar organic solution, $C_{ß\text{-}gal}$. Where $C_{m,tot}$ is the total micellar concentration calculated by the measured reverse micellar size distribution and Wo, and $C_{m,eff}$ is the micellar concentration which is effective for the uptake of the target protein (ß-gal), i.e. the concentration of micelles with larger micro water pool than the size of the protein. Observed ß-gal concentration, $C_{ß\text{-}gal}$, was plotted against Wo in Fig. 5,

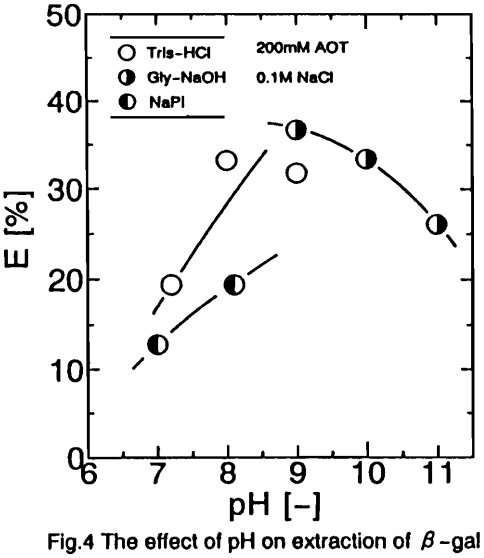

Fig.4 The effect of pH on extraction of β-gal

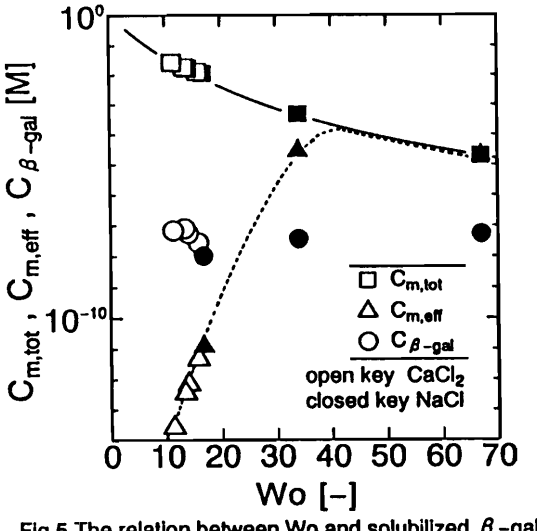

Fig.5 The relation between Wo and solubilized β-gal concebtration, total micelle concentration and effective micelle concentration

together with $C_{m,tot}$ and $C_{m,eff}$ at the identical extraction conditions. When NaCl was used at low concentrations (larger Wo range), $C_{m,eff}$ was far larger than the observed $C_{ß\text{-}gal}$ as expected from previous study. While, in the case of $CaCl_2$ or NaCl at high concentrations, $C_{ß\text{-}gal}$ was much larger than $C_{m,eff}$. This result can not be explained by the previous protein uptake models in which the proteins are assumed to be solubilized in the water pool of the micelles. Large proteins such as oligomeric ß-gal are not solubilized in the micro water pool of the reverse micelles but likely to be solubilized by coagulation or adsorption of small micelles on the protein.

4. Conclusion

ß-Gal was easily solubilized into reverse micellar solution by the injection method, and an increase in micellar diameter was observed. Solubilized ß-gal in the micellar solution was back extracted into aqueous phase without much loss of enzyme activity, most effectively using KCl aqueous solution. Extraction of ß–gal by phase transfer was also possible but strongly influenced by salt type, salt concentration in the range of pH 7–11. Electrostatic and hydrophobic interactions among ß-gal, AOT, reverse micelles, and divalent metal salt seem to play a key role for the effective solubilization of ß-gal by the phase transfer method. Large oligomeric proteins may possibly be solubilized by coagulation or adsorption of the micelles on the proteins. But enhancement of the interaction by high salt concentration and low pH was not in favor of forward and back extraction of ß-gal, because of the formation of precipitates.

5.Reference

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