ULTRAFILTRATION OF REVERSE MICELLES CONTAINING ENZYME

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The cross-flow ultrafiltration of reverse micelles was investigated to examine the applicability of a membrane reactor for continuous enzymatic reaction. An ultrafiltration ceramic membrane module of tubular type was used to separate the Aerosol OT reverse micelles from the continuous phase of *iso*-octane. The rejection of reverse micelles as well as the permeation flux were measured at different concentrations of surfactant, water, enzyme and molar ratio of water to surfactant ω_o .

The permeation flux decreased and the rejection of water increased with increase in the concentration of lipase, probably as a result of the higher enzyme mass adsorbed on the membrane. The rejection of reverse micelles also increased with increase in ω_o , which influences the diameter of reverse micelle. The permeation flux, however, could not be correlated solely with the parameter ω_o , and it depended on the concentration of surfactant itself when ω_o was less than five. This direct influence of surfactant is supposed to be due to the adsorption of surfactant in the membrane which can change the hydrophobic property of the membrane.

Introduction

Reverse micelles are nanometer-sized droplets of W/O emulsion consisting of the water pool and the surrounding surfactant molecules. The size of the water pool crucially influences the inclusion of enzyme molecules, and it can be controlled by the parameter, ω_o , defined as the molar ratio of water to surfactant. The other physical properties of reverse micelles, such as dielectric constant, microviscosity and acidity, can also be continuously modulated with change of ω_o . These characteristics make it possible to use reverse micelles for extraction of a particular protein from aqueous protein solution, enzymatic reaction in organic solvent and refolding of denatured proteins^{7, 9, 12, 13}. Reverse micelles have such a large specific surface area that an efficient reaction can be expected if an appropriate condition is chosen for better contact between the enzyme in the water pool and the substrate in organic solvent.

Reverse micelles containing enzyme molecules should be separated from the organic solvent and reused in continuous reaction. The back extraction, which is combined with the forward extraction in the case of separation of proteins dissolved in aqueous solution, will not be applicable to the continuous reaction because the properties of reverse micelles will change significantly and the extraction efficiency is low³⁻⁵. Lüthi and Luisi¹⁴ proposed an enzyme reactor for reverse micelles where the enzyme-containing micelles are entrapped in the bore side of semipermeable hollow fibers. They reported that a large volume ratio of the inside to outside compartments of the hollow fiber modules should be chosen to obtain a high product yield because the total volume of the water pool,

the site of enzymatic reaction, was very small. Another disadvantage of this membrane reactor is mass transfer resistance to solute permeation that may hamper an efficient reaction. Membrane separation is, however, a useful method of recovery or retention of enzymes as reported recently by Chang *et al.*² and Prazeres *et al.*¹⁷ who operated the reactor by a mode of filtration and a batch mode with recycling of the permeate stream, respectively.

In this study, cross-flow ultrafiltration of the reverse micelles hosting enzyme molecules was investigated to examine the possibility of continuous reaction¹⁵. The effect of concentrations of lipase, water and AOT on flux and rejection was measured using an ultrafiltration ceramic membrane of tubular type.

1. Experiments

1.1 Materials

The surfactant AOT (di-2-ethylhexyl sodium sulfo-succinate) was a reagent from Nacalai Tesque, INC., Japan (purity 98.8%) and was used without further purification. The lipase (600 U/mg, *Rhizopus delemar*, MW 44,000) was purchased from Seikagaku Kogyo Co., Ltd., and the organic solvent, *iso*-octane, was purchased from Wako Pure Chemical Industries Ltd.

1.2 Apparatus

The experimental apparatus is schematically shown in Fig. 1. The main parts consist of an ultrafiltration module, a feed tank and a feed pump, which was a single plunger pump (NP-S-321, Nihon Seimitsu Kagaku Co., Ltd.) or a HPLC pump with double plungers (880-PU, Japan Spectroscopic Co., Ltd.). The HPLC pump could circulate the fluid more smoothly than the other pump. The reverse micellar solution was fed using either of these

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Fig.1 Schematic diagram of experimental apparatus

pumps into the bottom of the membrane module at a constant flow rate of 7.9×10^{-3} m/s, which corresponded to a Reynolds number ($= D_m(1-\kappa)\rho u/\mu$) from 25 to 32 (the viscosity of the micellar solution, μ , was estimated to change from 0.516 to 0.659 cp)¹⁶. A valve was used to control the trans-membrane pressure. The structure and dimension of the tubular membrane module is shown in Fig. 2. The Carbosep M5 membrane (cutoff MW 20,000, Sumitomo Heavy Industries Ltd., Japan) is made of carbon substrate with an inner skin layer of zirconium oxide. The module was 6 mm in inner diameter, 2 mm in thickness and 392 mm in length. A stainless steel rod (diameter 3 mm) was inserted coaxially. This membrane module was chosen because it was stable in organic solvents and it was made of an electro-conductive carbon substrate. The membrane itself and the stainless steel rod will be used as an electrode and a counter electrode respectively in the future study of electro-ultrafiltration of reverse micelles.

1.3 Procedure

300 cm³ of *iso*-octane was put into a 500 cm³ flask together with AOT and then mixed with the enzyme solution. The concentration of water was changed from 0.02 to 0.38 mol/l-*iso*-octane, which corresponded to the volume ratio of 3.6×10^{-4} to 6.84×10^{-3} water to organic solvent, while the concentration of AOT ranged from 0.02 to 0.15 mol/l-*iso*-octane. The lipase concentration ranged from 1.4 to 4.7 μ mol-enzyme/l-*iso*-octane which corresponded to the concentration in the water pool, 0.32 to 1.05 mmol-enzyme/l-water. The maximum enzyme concentration is equal to the solubility of enzyme in water.

The flask was vigorously shaken by hand to prepare a clear reverse micelle solution. All experiments were carried out by the total circulation method under conditions of constant temperature (310 K) and constant trans-membrane pressure (50 kPa). The concentrations of water and AOT in the solution were measured with a coulometric moisture analyzer (VA-05, Mitsubishi Chemical Ind. Ltd.) and a spectrophotometer (100-60, Hitachi, Ltd.) using a wavelength of 320 nm. The rejection, R , is defined as follows;

Fig.2 Schematic diagram of vertical tubular cross-flow ultra-filtration module

$$R = 1 - \frac{C_p}{C_b} \quad (1)$$

where C_p and C_b are the concentrations of solute in the permeate solution and in the retentate solution, respectively.

2. Results and Discussion

2.1 Estimation of molecular weight of one reverse micelle

The diameter of reverse micelles is important information for interpreting the experimental results and the method proposed by F.J.Bonner *et al.*¹⁾ was used to estimate it. Assuming each spherical micelle to host one protein molecule, the inner core volume can be expressed as;

$$\frac{4\pi}{3} r_o^3 = \frac{4\pi}{f_s} r_o^2 \omega_0 V_w + V_p \quad (2)$$

where r_o , f_s , V_w and V_p are the inner core radius, the molar surface area of surfactant head group, the molar volume of water and the molar volume of protein, respectively. Since the surfactant and the water are almost insoluble in the continuous phase of *iso*-octane, the value of ω_0 can be calculated directly from the initial amount of surfactant and water added.

Denoting the thickness of the surfactant shell, l_s , the radius of a protein-containing micelle (filled micelle), r_t , is expressed by the equation:

$$r_t = r_o + l_s \quad (3)$$

The thickness of surfactant shell, l_s , is assumed to be the length of the surfactant molecule.

The apparent molecular weight of a reverse micelle is estimated as follows;

Fig. 3 Relationship between ω_0 and calculated size and molecular weight of one reverse micelle¹⁾

$$M = \frac{4\pi r_o^2}{f_s} (\omega_0 M_w + M_s) + M_p \quad (4)$$

where M_w , M_s and M_p are the molecular weight of water, surfactant and protein, respectively.

Several researchers^{8, 11, 18)} measured the diameter of reverse micelles by the dynamic light scattering method and discussed the applicability of Eq.(2) to estimate it. Zulauf *et al.*¹⁸⁾ and Kuboi *et al.*¹¹⁾ showed that the average diameter of reverse micelles increased with increase in ω_0 . The diameter of either empty or unfilled reverse micelles measured by both of the researchers was, however, smaller than the one estimated with Eq.(2) ($V_p = 0$). There seemed to be a room for further study on the applicability of Bonner's equation, although it was used in this study to estimate the diameter of reverse micelles.

Fig. 3 was the result of diameter and molecular weight calculated using the equations (2), (3) and (4) with the data of $f_s (= 1/1.9 \text{ nm}^2)$ and $l_s (= 1.2 \text{ nm})$ for AOT. Although the molecular weight and the diameter of either a filled or an empty reverse micelle increased with increase in ω_0 , the molecular weight of an empty reverse micelle was estimated to be smaller than the cut-off molecular weight of the membrane (20,000), when ω_0 was less than five. The population of the filled reverse micelles was calculated using the number of enzyme molecules and that of reverse micelles and it accounted for from 0.39% to 1.3% of the total number of reverse micelles.

2.2 Effect of solute concentration

The rejection of water and the permeation flux, R_w and J_v , were measured by changing the concentration of either AOT, water or enzyme, while the concentrations of the other two components were kept constant.

1) Effect of AOT and water concentration The effect of AOT and water concentrations on R_w and J_v were rearranged using the molar ratio, ω_0 , and shown in **Fig. 4**. Rejection data, R_w , could be correlated with ω_0 and increased in proportion to ω_0 , while the permeation flux, J_v , was significantly influenced by the AOT concentration even at the same ω_0 value when ω_0 was less than 5. Prazeres *et al.*¹⁷⁾ also reported the influence of ω_0 in the

Fig. 4 Effect of ω_0 on rejection of water and permeation flux

Fig. 5 Effect of lipase concentration on permeation flux and rejection of water

mass transfer process of AOT/*iso*-octane reverse micellar solution through a ceramic membrane. The membrane of Carbosep, with 10,000 molecular weight cutoff, was tighter than the membrane used in this study. Their experimental results of rejection and permeation flux measured without loading of lipase partly differed from those shown in Fig. 4. The rejections of AOT and water increased up to the ω_0 value of 4, but they decreased with further increase in ω_0 . These results differed from what we observed and expected from the size sieving mechanism of membrane separation.

Prazeres *et al.*¹⁷⁾ supposed that molecular interactions and chemical compatibility of the permeating components with the membrane material could be partially responsible for separation of the other species, probably together with a molecular size exclusion effect in the case of micellar solutions. They reported that the ceramic membrane, which was made of a zirconium oxide layer over a porous carbon substrate, showed a strong hydrophobic character. They described a lamellar-like structure formed in the internal pore surface of the hydrophobic membrane by referring to the work of Kawashima *et al.*¹⁰⁾; the hydrophobic tails of surfactant molecules become oriented toward the hydrophobic micropore wall surface, while the polar groups pointed toward solubilized water molecules. This lamellar-like structure will influence the passage of water and organic solvent through the membrane. It is said¹⁰⁾ that the water is tightly bound to the surfactant shell up to the ω_0 value of 4 and starts to gain mobility with an increase

Fig.6 Effect of reverse micelles concentration on rejection of water and permeation flux

in ω_0 . The state of water, free or bound, is also supposed to influence the rejection of water. The increase of viscosity in reverse micellar solutions with increase in ω_0 may partly account for the flux reduction. The mechanism of membrane separation in reverse micellar systems is so complicated that it cannot seem to be explained only with a size sieving effect.

2) Effect of enzyme concentration The effect of lipase concentration on the rejection of water and the permeation flux is shown in **Fig. 5**. The rejection of water increased with increase in the concentration of lipase, while the permeation flux decreased. The results were similar to those observed in ultrafiltration of an aqueous high polymer solution although the concentration of lipase in reverse micellar system was very low, that is, 0.015 wt% at most. The population of lipase-containing reverse micelles, which could induce fouling of the membrane and reduce the flux, was supposed to increase with an increase in enzyme concentration.

2.3 Effect of concentration of reverse micelles

Figure 6 shows the effect of reverse micelle concentration on the rejection of water and the permeation flux which were measured at a constant molar ratio of AOT, water and lipase. The reverse micelle concentration was calculated with the total mass of AOT, water and lipase in *iso*-octane. The rejection of water decreased with the increase in concentration of reverse micelles, while the permeation flux increased. This rather unexpected result of permeation flux can probably be attributed to the effect of AOT adsorption in the membrane pore as previously mentioned in the results of **Fig. 4**.

Results obtained using the single plunger pump has been reported up to this point, but this pump produces a pulsation flow of feed solution, which seemed to influence the permeation of reverse micelles. Additional experiments were carried out using a HPLC pump with double plungers, which could deliver the feed solution smoothly.

2.4 Effect of trans-membrane pressure

Figure 7 shows the relationship between the rejection of water and trans-membrane pressure when the double plunger pump was used. When the enzyme was not added, the rejection of water decreased with the increase in trans-membrane pressure, or correspondingly with the

Fig.7 Relationship between rejection of water and trans-membrane pressure

Fig.8 Relationship between permeation flux and trans-membrane pressure

increase in flux (see **Fig.8**). The rejection of water, however, shifted to a constant higher value with addition of the enzyme, probably because the enzyme molecules accumulated on the membrane and resisted permeation of reverse micelles. Rejection measured using the single plunger pump is included in **Fig. 7** and it is supposed from its smaller value that the pulsation flow of the single plunger pump may force reverse micelles to permeate through the membrane.

Figure 8 shows the effect of trans-membrane pressure on the permeation flux which increased with the increase in trans-membrane pressure irrespective of loading of enzyme. The permeation flux was larger in the case of the single plunger pump when the enzyme was added, the reason for which could be the same one as that for the reduced value of rejection shown in **Fig. 7**.

Conclusion

Ultrafiltration of reverse micelles was studied to examine the effects of ω_0 and several parameters such as enzyme concentration and mass concentration of reverse micelles on the rejection of reverse micelles and the perme-

ation flux. The conclusions are summarized as follows;

- 1) Permeation flux decreased with the increase in enzyme concentration in a manner similar to that observed in the ultrafiltration of aqueous high polymer solution.
- 2) Permeation flux increased with the increase in AOT concentration or the decrease in water concentration, while the rejection of reversed micelles decreased.
- 3) The rejection of reverse micelles could be correlated with the molar ratio of water to surfactant, ω_0 , the parameter influencing the size of reverse micelles, while the permeation flux was influenced by AOT concentration as well as ω_0 .
- 4) Membrane separation of the reverse micelles containing enzyme was influenced by the smoothness of feed flow and the use of a HPLC pump without pulsation flow was preferable for rejecting the reverse micelles, compared use of a single plunger pump.

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Nomenclature

C_b	= solute concentration in retentate	[kg/m ³]
C_p	= solute concentration in permeate	[kg/m ³]
D_m	= inner diameter of tubular membrane	[m]
D_s	= diameter of stainless steel rod	[m]
d_i	= diameter of reverse micelle	[m]
f_s	= molar surface area of surfactant head group	[m ²]
J_v	= permeation flux	[m ³ /(m ² s)]
l_s	= thickness of surfactant shell	[m]
R	= rejection	[-]
R_e	= Reynolds number	[-]
r_0	= radius of inner core of reverse micelle	[m]
r_f	= radius of protein-containing micelle	[m]
u	= flow velocity of micellar solution	[m/s]

V	= molar volume	[m ³]
κ	= ratio of diameter (= D_f/D_m)	[-]
ρ	= density of micellar solution	[kg/m ³]
μ	= viscosity of micellar solution	[kg/(ms)]
ω_0	= molar ratio of water to surfactant	[-]

<Subscripts>

p	: protein
w	: water

Literature Cited

- 1) Bonner F.J., R. Wolf, P.L. Luisi : *J. Solid-Phase Biochem.*, **5**, 255-268 (1980)
- 2) Chang P.S., J.S. Phee and J.J. Kim : *Biotech. and Bioeng.*, **38**, 1159-1165 (1991)
- 3) Dekker M., R.Hilhorst, C.Laane:*Anal. Biochem.*, **178**, 217-226 (1989)
- 4) Dekker M., K.Van't Riet, B.H. Bijsterbosch, P.Fijneman, R.Hilhorst: *Chem. Eng. Sci.*, **45**, 2949-2957 (1990)
- 5) Dungan S.R., T.Bausch, T.A. Hatton, P.Plucinski, W. Nitsch:*J. Colloid Interface Sci.*, **145**, 33-50 (1991)
- 6) Gierash L.M., K.F.Thompson, J.E. Lacy, A.L.Rockwell : "Reverse micelles",p.265-277,Plenum, New York (1984)
- 7) Hatton T.A.:"Surfactant-based separation processes", p.59-90, Marcel Dekker, New York (1989)
- 8) Ishikawa H., K. Noda and T. Oka :*J.Ferment. Bioeng.*, **70**, 381 (1990)
- 9) Kadam K.L.:*Enzyme Microb. Technol.*, **8**, 266-273 (1986)
- 10) Kawashima Y., T. Hino, H. Takeuchi, T. Niwa, K. Horibe:*J.Coll.Interface Sci.*, **145**, 512-523 (1991)
- 11) Kuboi R., Y. Mori and I. Komasa : *Kagaku Kogaku Ronbunshu*, **16**, 763-771 (1990)
- 12) Leser M.E., G. Wei, P.L. Luisi, M. Maestro : *Biochem. Biophys. Res. Commun.*, **135**, 629-635 (1986)
- 13) Luisi P.L., M. Giomini, M.P. Pileni, B.H. Robinson:*Biochem. Biophys. Acta*, **947**, 209-246 (1988)
- 14) Lüthi P. and P.L. Luisi : *J. Am. Chem. Soc.* **106**, 7285-7286 (1984)
- 15) Nakamura K. and M. Hakoda: "Biochemical Engineering for 2001", p.433-436, Springer-Verlag, Tokyo (1992)
- 16) Onori G. and A. Santucci: *J. Colloid and Interface Sci.*, **150**, 195-199 (1992)
- 17) Prazeres D.M.F., F.A.P. Garcia, J.M.S. Cabral : *Biotech. Bioeng.*, **41**, 761-770 (1993)
- 18) Zulauf M. and H.-F. Eicke : *J. Phys. Chem.*, **83**, 480-486 (1979)