Separation Science and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713708471

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Shau-Wei Tsai[a]; Jun-Lone Chen[a]
[a] DEPARTMENT OF CHEMICAL ENGINEERING, NATIONAL CHENG KUNG UNIVERSITY, TAINAN, TAIWAN, REPUBLIC OF CHINA

To cite this Article Tsai, Shau-Wei and Chen, Jun-Lone(1995) 'Enzyme Separation Using Supported Liquid Membrane Filled with Reversed Micelles', Separation Science and Technology, 30: 12, 2551 — 2563
To link to this Article: DOI: 10.1080/01496399508021401
URL: http://dx.doi.org/10.1080/01496399508021401

SEPARATION SCIENCE AND TECHNOLOGY, **30**(12), pp. 2551–2563, 1995

Enzyme Separation Using Supported Liquid Membrane Filled with Reversed Micelles

SHAU-WEI TSAI* and JUN-LONE CHEN
DEPARTMENT OF CHEMICAL ENGINEERING
NATIONAL CHENG KUNG UNIVERSITY
TAINAN, TAIWAN, REPUBLIC OF CHINA

ABSTRACT

A phenomenological model describing the transfer of lysozyme between a bulk aqueous phase and a reversed micellar phase in a stirred membrane cell has been confirmed. Transport of the enzyme at the interface at low surfactant concentrations is dominant, while that through the membrane is the rate-determining step at high surfactant concentrations. Complete separation of α-chymotrypsin from lysozyme using a supported liquid membrane filled with reversed micelles demonstrates the feasibility of the present process for enzyme separation.

Key Words. Reversed micelles; Enzyme separation; Supported liquid membrane

INTRODUCTION

Reversed micelles are the aggregates of surfactant molecules dispersed in an organic medium. By providing an aqueous microenvironment in these water-in-oil microemulsions, they have been applied to extract hydrophilic molecules such as amino acids, proteins, and metal ions from a bulk aqueous phase (1–5). Protein recovery from an aqueous phase by extraction using reversed micelles consists of two steps: the desired pro-

* To whom correspondence should be addressed.

tein is transferred from the aqueous phase to the reversed micellar phase (forward extraction) and then it is recovered from the reversed micellar phase to a second aqueous phase (backward extraction). These two extractions can be performed in a continuous mode using conventional mixer/settler or membrane extractor equipment (6–8). Recently, some possible mechanisms have been suggested to elucidate how a protein transfers into and out of the reversed micelle (9, 10).

Reversed micelles entrapped in a membrane phase have been proposed to shuttle proteins across the immiscible liquid barrier (11, 12). With this concept Armstrong and Li (13) performed continuous extraction of cytochrome c, lysozyme, bovine serum albumin, and myoglobin between the feed and receiving sides of the liquid membrane filled with reversed micelles as carriers. Yet improvement of the mass transfer rate by reducing the membrane thickness remains necessary. Supported liquid membrane (SLM), in which a thin layer of organic solvent containing reversed micelles is immobilized in a microporous inert support interposed between two aqueous solutions, represents a possible solution to the above shortcoming.

In a previous paper (14) we used a stirred membrane cell (SMC) where an aqueous phase and a reversed micellar phase were separated by a hydrophobic membrane to solubilize or desolubilize α-chymotrypsin between these two phases. A phenomenological model was proposed to interpret enzyme transport through the membrane. The results were then applied to predict enzyme transport through an SLM. In the present report we extend the previous analysis to another protein, lysozyme. Also, the feasibility of using the process for enzyme separation from an enzyme mixture is illustrated.

MATERIALS AND METHODS

Materials

Lysozyme (EC 3.2.1.17) from chicken egg white, Type II α-chymotrypsin (EC 3.4.21.1) from bovine pancreas, and sodium di(2-ethylhexyl)sulfosuccinate (AOT) were obtained from Sigma (St. Louis, Missouri, USA). Isooctane and other chemicals of analytical grade were commercially available and were used without further purification. Polysulfone asymmetric membrane (PTHK, with a nominal molecular weight cutoff of 10^5) was obtained from Millipore (Japan).

Equilibrium Transfers in Stirred Diffusion Cell (SDC)

The reversed micellar phase was prepared by dissolving the required amounts of AOT in isooctane. Buffer solutions of different pH values were

obtained by mixing the following solutions in suitable ratios: disodium hydrogen phosphate dihydrate (66.7 mM) and potassium dihydrogen phosphate (66.7 mM) solutions for pH 7; sodium hydroxide (100 mM), glycine (100 mM), and sodium chloride (100 mM) solutions for pH 12. The experimental temperature was kept at 25°C. No additional salt was added to control the ionic strength of the aqueous phase. By using a Baird and Tatlock AF-5 Karl-Fischer Titrator, the saturated water contents (in v/v %) of the reversed micellar phase, which varied with the aqueous phase pH and AOT concentration, were measured and illustrated in Fig. 3 of a previous paper (14).

The forward transfer was carried out in an SDC, where 10 cm^3 of the pH 7 aqueous phase with a lysozyme concentration of 0.6 mg/cm^3 was contacted with 10 cm^3 of the reversed micellar phase at various AOT concentrations. The equilibrium enzyme concentration in the organic phase was determined by UV spectroscopy. The enzyme concentration in the aqueous phase was calculated from an enzyme balance on the whole system, where the effect of water partitioning on the volume change of each phase has been considered (14). Then the forward partition coefficient of the enzyme, P_f, defined as the ratio of enzyme concentration in the organic phase to that in the aqueous phase, was determined.

In the backward transfer, a pH 7 aqueous solution containing the enzyme was prepared. It was injected into the reversed micellar phase, giving a lysozyme concentration of 0.3 mg/cm^3 in this phase. Experiments were carried out as in the forward extraction except that a pH 12 aqueous phase was used. Thus the backward partition coefficient of the enzyme, P_b, as defined above, was calculated after the protein concentration in each phase was determined.

Forward and Backward Extractions in an SMC

An SMC is composed of two glass cells connected by two Teflon gaskets where a membrane is inserted to separate the two phases. Each cell has a volume of 150 mL. The stirring speed of the magnetic stirrer was kept at 200 rpm in all experiments. In order that the reversed micelles could fill the pores of the membrane, all membranes were placed in an ultrasonic cleaner containing the organic phase of the required AOT concentration for 500 minutes before the extraction.

Depending on the AOT concentration applied in the forward extraction, a saturated amount of the pH 7 buffer solution was added to the reversed micellar phase, which was then introduced to the cell with the above membrane. The same buffer solution containing a lysozyme concentration of 0.6 mg/cm^3 was carefully introduced into the cell, giving a clear solution in each phase. UV absorbance of the organic phase was monitored with

time. A blank run without enzyme was then carried out at the same conditions. Next the enzyme concentration in the organic phase was determined from the difference of both measurements.

The experimental conditions in the backward extraction were the same as in the forward extraction except that the lysozyme concentration in the reversed micellar phase was kept at 0.3 mg/cm^3 and a pH 12 aqueous phase was used. The difference of UV absorbance in the aqueous phase for both test and blank runs was monitored. The same experiment was performed, however, with concentrations of AOT, α-chymotrypsin, and lysozyme in the reversed micellar phase as 100 mM, 0.3 mg/cm^3, and 0.3 mg/cm^3, respectively.

Overall Extraction in an SLM

The experimental conditions were the same as in the forward extraction except that a pH 7 aqueous phase with an initial enzyme concentration of 0.6 mg/cm^3 and a pH 12 aqueous phase without enzyme were used as the feed and receiving phases, respectively. The membrane was treated in the reversed micellar phase at an AOT concentration of 100 mM. The difference of UV absorbances in the receiving phase for both test and blank runs was recorded with time. Similar experiments were performed, however, in which the initial concentration for each enzyme was fixed at 0.6 mg/cm^3 in the feed phase.

Protein Concentration Assay

Using a Shimadazu UV-160 spectrophotometer at 280 nm and extinction coefficients of 2.34 cm^3/mg·cm, the lysozyme concentration in the aqueous phase was determined. The difference of the absorbances at 280 and 310 nm was used to prepare a calibration curve to correct for turbidity effects in the reversed micellar phase. An extinction coefficient of 2.19 cm^3/mg·cm was used to determined the lysozyme concentration in this phase.

MASS TRANSFER THEORY

Overall Extraction in an SLM

The enzyme transfer rate for the overall extraction in an SLM is affected by several mass transfer resistances. An enzyme molecule has to diffuse from the bulk feed phase to the interface. At the interface the enzyme is encapsulated to form a protein-filled reversed micelle. The filled reversed micelle then diffuses through the membrane to the interface at which the enzyme molecular is released and diffuses into the bulk receiving phase.

In a previous analysis (14) we assumed that the interfacial enzyme fluxes for the forward and backward extractions are proportional to the nth and the mth order of AOT concentration, respectively. Moreover, based on the pseudosteady-state assumption for enzyme transport through the membrane, the overall mass transfer coefficient K_o, defined from the equivalent enzyme concentration difference in the organic phase [i.e., $P_f(E_3) - P_b(E_O)$], was expressed as follows:

$$\frac{1}{K_o} = \frac{P_f}{k_3} + \frac{P_f}{k_f((S)N^{-1})^n} + \frac{1}{k_2} + \frac{P_b}{k_1} + \frac{P_b}{k_b((S)N^{-1})^m} \tag{1}$$

where (E_O) and (E_3) are the enzyme concentrations in the receiving and feed phases, respectively. All symbols are defined in the Nomenclature Section. In general, the mass transfer coefficient in the receiving phase, k_1, and that in the feed phase, k_3, depend on the stirring rate and the enzyme diffusivity in the aqueous phase. The mass transfer coefficient in the membrane, k_2, depends on the membrane characteristics, the diffusivity of protein-filled micelle, and the surfactant concentration in the organic phase. The rate constants k_f and k_b for the encapsulation and release of enzyme at the interface, as well as the parameters m, n, N, P_f, and P_b, depend on the pH and ionic strength of the aqueous phase or the surfactant concentration.

By taking an enzyme balance for each phase, the time-dependent enzyme concentration in the receiving phase was predicted from the following equation (14):

$$-\ln\left[1 - \left(1 + \frac{V_O P_f}{V_3 P_b}\right)\left(\frac{P_b(E_O)}{P_f(E_t)}\right)\right] = ItK_o\left(\frac{P_f}{V_3} + \frac{P_b}{V_O}\right) \tag{2}$$

where (E_t) is the initial enzyme concentration in the feed phase. The parameters I, t, V_O, and V_3 are for the membrane area for enzyme transfer, time, and volumes for the receiving and feed phases, respectively.

Forward Extraction in an SMC

For forward extraction, Eqs. (1) and (2) are still valid except that $k_b = \infty$ and $P_b = 1$. By comparing the order of magnitude for k_3 or k_1 (i.e., 10^{-2} to 10^{-1} cm/min) with that of k_2 (i.e., 10^{-4} to 10^{-3} cm/min) at the experimental conditions, Eq. (1) could be further simplified to

$$\frac{1}{K_o} = \frac{P_f}{k_f((S)N^{-1})^n} + \frac{1}{k_2} \tag{3}$$

When Stokes' law is assumed for the dilute solution, the diffusivity of the

protein-filled reversed micelle, and hence k_2, is inversely proportional to the viscosity of the organic phase. However, the organic phase containing the surfactant and empty micelles does not satisfy the dilute assumption, so k_2 is assumed to be inversely proportional to $(S)^r$. Thus Eq. (3) is rewritten as

$$\frac{1}{K_o} = \frac{AP_f}{(S)^n} + B(S)^r \tag{4}$$

where A was $N^n k_f^{-1}$. A and B are constants that depend on physical parameters excluding the surfactant concentration.

Backward Extraction in an SMC

Using a similar derivation for the initial enzyme concentration in the reversed micellar phase, the time-dependent enzyme concentration in the aqueous phase is predicted as follows (14):

$$-\ln\left[1 - \left(1 + \frac{V_3}{V_O P_b}\right)\left(\frac{(E_3)P_b}{(E_t)}\right)\right] = ItK_o\left(\frac{P_b}{V_3} + \frac{1}{V_O}\right) \tag{5}$$

where the overall mass transfer coefficient is calculated from Eq. (4) in which n and P_f are replaced by m and P_b, respectively, and A is defined as $N^m k_b^{-1}$.

RESULTS AND DISCUSSION

Equilibrium Transfer in an SDC

The partition coefficients defined above are important parameters in modeling and optimizing enzyme recovery processes. The variation of the forward partition coefficient with AOT concentration for α-chymotrypsin and lysozyme is presented in Table 1. Comparison of the results reveals that lysozyme solubilization is more dependent on surfactant concentration. This implies that enzyme transfer is not only driven by electrostatics but is also affected by the entropic effect associated with surfactant redistribution over the empty and enzyme-filled micelles. For a given AOT concentration, the value for this enzyme is an order of magnitude more than that for α-chymotrypsin. This might be due to the larger electrostatic effect between the lysozyme molecule (with the index of $pI - pH = 4.1$, compared with that of 1.6 for α-chymotrypsin) and the surfactant head group to stabilize the solubilization in the water pool of the reversed micelle.

In order to maintain the enzyme activity in the desolubilization operation, the aqueous phase pH is usually kept at a value slightly below the

TABLE 1
Variation of Parameters and Their Combinations with AOT Concentration for α-Chymotrypsin (a) and Lysozyme (b) Using a pH 7 Aqueous Phase at 25°C

		AOT concentration, mM[a]					
		25	50	100	200	300	400
(a)[b]	P_f	1.92	1.97	2.10	2.32	2.66	2.71
	K_o	0.608	4.43	1.63	1.08		
	($\times 10^4$)	(0.294)	(4.50)	(2.49)	(0.884)		
	P_fK_o	1.16	8.72	3.42	2.51		
	($\times 10^4$)	(0.566)	(8.86)	(5.23)	(2.05)		
	$(S)^{5.5}A^{-1}$ ($\times 10^4$)	(0.574)	(26.0)	(1180)	(53,200)		
(b)	P_f	31.4	38.0	45.8	48.8	50.0	50.5
	K_o	3.23	12.7	5.98	3.57		
	($\times 10^6$)	(0.700)	(12.2)	(7.93)	(2.83)		
	P_fK_o	1.00	4.68	2.51	1.71		
	($\times 10^4$)	(0.220)	(4.64)	(3.63)	(1.38)		
	$(S)^{5.5}A^{-1}$ ($\times 10^4$)	(0.222)	(10.0)	(454)	(20,600)		

[a] Values in parentheses are theoretical results.
[b] Obtained previously (14) with $A = 8.5 \times 10^{11}$ min·mM$^{5.5}$/cm, $B = 4.0$ min/cm·mM$^{1.5}$.

isoelectric point of the enzyme. Therefore, high salt concentrations are added to increase the ionic strength of the aqueous phase, and hence decrease the size of reversed micelles to remove the enzyme from the reversed micellar phase. In the present study, only the aqueous phase pH of 12 was controlled. Desolubilization of lysozyme into the aqueous phase at various AOT concentrations was not found under the present experimental conditions. Obviously, this result might be attributed to the weak electrostatic repulsion between the charged lysozyme molecule and the surfactant head groups (with pH − pI = 0.9) with no additional salt being added, so the enzyme still remains in the reversed micellar phase.

Forward Extraction in an SMC

Figure 1 gives the time-dependent variation of enzyme concentration in the reversed micellar phase at various AOT concentrations for forward extraction. Maximum protein concentration, and hence solubilization, occurred when AOT concentration was around 50 mM. An asymptotic enzyme concentration for each system might be expected from the figure. However, it was far below the equilibrium concentration (more than 0.59

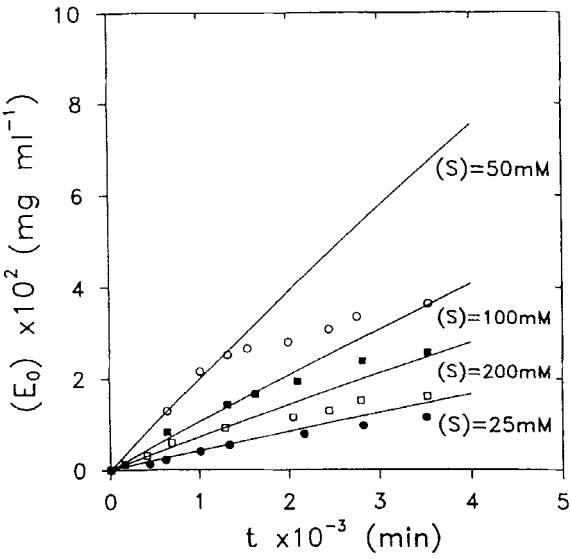

FIG. 1 Time-course enzyme concentrations in the reversed micellar phase at various AOT concentrations in forward extraction. Theoretical results: (——); experimental results: (●) for (S) = 25 mM, (○) for (S) = 50 mM, (■) for (S) = 100 mM, (□) for (S) = 200 mM.

mg/cm^3, calculated by using the forward partition coefficients in Table 1). This was due to enzyme adsorption on the membrane surface. Therefore, an additional resistance for enzyme transport was induced in the forward extraction. Similar behaviors have been found for α-chymotrypsin and were illustrated in Fig. 4 of a previous report (14).

By substituting the data for 1000 minutes in Fig. 1 and P_f in Table 1 into Eq. (1) with P_b equal to 1, the overall mass transfer coefficient was determined and is shown in Table 1. The time-course enzyme concentration in the reversed micellar phase was recalculated and is plotted in Fig. 1. As expected, deviation between the theoretical and experimental results occurred. This implies that the order of magnitude of the mass transfer resistance due to protein adsorption is comparable to that obtained from Eq. (1) at times greater than 1000 minutes. Consequently, a more rigorous model is necessary to consider this resistance.

Substituting the above coefficients into Eq. (4) resulted in the following constants: $A = 2.2 \times 10^{12}$ min·mM$^{5.5}$/cm, $B = 125.0$ min/cm·mM$^{1.5}$, $n = 5.5$, and $r = 1.5$. Using these constants, the theoretical overall mass transfer coefficient at each AOT concentration was calculated and is presented in Table 1. Based on the enzyme concentration difference in the

aqueous phase, $[(E_3) - (E_O)P_f^{-1}]$, we also calculated the overall and interfacial mass transfer coefficients [i.e., P_fK_o and $(S)^{5.5}A^{-1}$, respectively]. The results are shown in the same table. Thus, at low AOT concentrations, the interfacial transfer process was concluded to be the rate-determining step for forward extraction. When the surfactant concentration was increased to 200 mM, only the mass transfer resistance through the membrane needed to be considered. Consequently, a maximum enzyme solubilization for the system of $(S) = 50$ mM can be obtained. Aside from the strong dependence of the interfacial mass transfer coefficient on the pH and salt concentration of the aqueous phase previously observed (10), the dominant role of surfactant concentration on the interfacial forward transport kinetics was demonstrated.

Similar behaviors were observed for α-chymotrypsin in the forward extraction (14). We have elucidated these results from a net balance of the electrostatic forces between a charged protein particle and an oppositely charged interface and the surface tension due to interface deformation. At a given AOT concentration, α-chymotrypsin has a larger overall or interfacial mass transfer coefficient than does lysozyme, yet with the same order of magnitude regardless of the widely different physical properties between these enzymes. Therefore, a further study on the possible mechanism for protein encapsulation into the reversed micellar phase is necessary in order to quantitatively predict the solubilization rate under the influence of various physiochemical parameters.

Backward Extraction in an SMC

As expected from the results of the above equilibrium transfers, desolubilization of lysozyme into the aqueous phase in backward extraction was not found at a time greater than 4000 minutes for various AOT concentrations. Therefore, we anticipate using the present experimental conditions for separating an enzyme mixture composed of both enzymes. Figure 2 gives the time-course adsorption intensity of the aqueous phase in the backward extraction for systems containing lysozyme, α-chymotrypsin, and the enzyme mixture, respectively. Complete separation of α-chymotrypsin from the enzyme mixture is possible because the latter two systems have the same adsorption intensity.

Overall Extraction in an SLM

Figure 3 gives the time-course adsorption intensity of enzyme in the receiving phase for systems containing lysozyme, α-chymotrypsin, and the enzyme mixture, respectively. As lysozyme could not release into the receiving phase, a complete separation of α-chymotrypsin from the en-

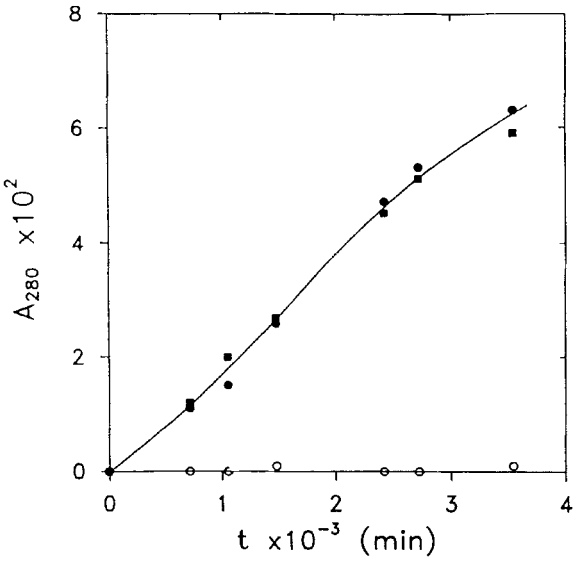

FIG. 2 Variation of adsorption intensity of enzyme in the aqueous phase with time for backward extraction at (S) = 100 mM. Experimental results: (○) for the system containing lysozyme, (●) for the system containing α-chymotrypsin, (■) for the system containing the enzymes mixture.

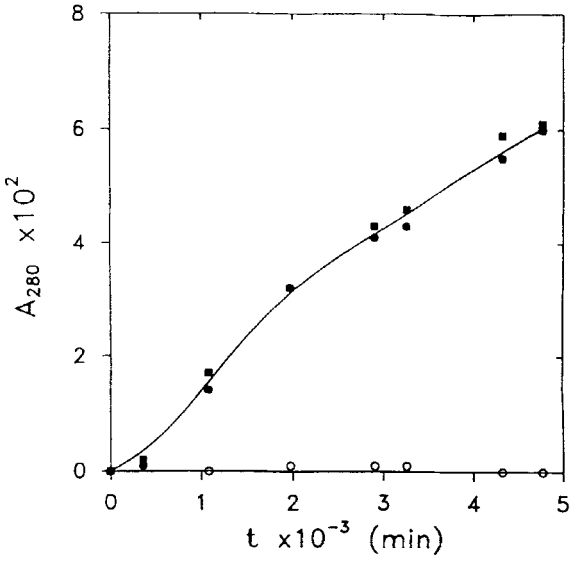

FIG. 3 Variation of adsorption intensity of enzyme in the receiving phase with time for overall extraction at (S) = 100 mM. The symbols are the same as those in Fig. 2.

zyme mixture was possible. It was obtained when the latter two systems had the same adsorption intensity in the receiving phase.

Based on the above preliminary experiments, we have demonstrated the feasibility of using an SLM for enzyme separation. However, more research is necessary to provide a more rigorous model that includes the effect of protein adsorption. In addition, a suitable membrane to reduce enzyme adsorption on the membrane surface and enhance the entrapment of reversed micelles should be investigated.

CONCLUSIONS

Results on the equilibrium transfer of lysozyme into reversed micelles demonstrated that the entropic effects associated with surfactant redistribution over the empty and enzyme-filled micelles are important in elucidating the variation of the forward partition coefficient with the surfactant concentration. By measuring the overall mass transfer coefficient in an SMC, we have confirmed the phenomenological model previously proposed for the extraction of α-chymotrypsin. At low surfactant concentrations the transfer process at the interface is the rate-determining step, and at high surfactant concentrations passage through the membrane is dominant. With the proposed model we might also predict the surfactant concentration effect on the backward extraction of lysozyme. However, desolubilization of this enzyme was not found with the present experimental conditions. Preliminary experiments on the backward extraction of the enzyme mixture have shown the possibility of complete separation of α-chymotrypsin from the mixture. Further experiments on the overall extraction of the enzyme mixture in an SLM with reversed micelles as carriers also illustrated the feasibility of using the present process for enzyme separation.

NOMENCLATURE

A	constant defined as $N^n k_f^{-1}$ in forward extraction, as $N^m k_b^{-1}$ in backward extraction (min·mM$^{5.5}$/cm)
B	constant defined in Eq. (4) (min/cm·mM$^{1.5}$)
(E_t)	initial enzyme concentration of aqueous phase in forward or overall extraction; that of organic phase in backward extraction (mg/cm^3)
(E_O)	enzyme concentration of organic phase in forward or backward extraction; that of receiving aqueous phase in overall extraction (mg/cm^3)
I	membrane area for enzyme transfer (cm^2)

K_o overall mass transfer coefficient (cm/min)
k_b rate constant for release of enzyme in backward extraction (cm/min·mM$^{5.5}$)
k_f rate constant for encapsulation of enzyme in forward extraction (cm/min·mM$^{5.5}$)
k_1 mass transfer coefficient in receiving phase (cm/min)
k_2 mass transfer coefficient through the membrane (cm/min)
k_3 mass transfer coefficient in feed phase (cm/min)
m, n parameters defined in Eq. (1)
N aggregation number of a reversed micelle
P_b partition coefficient defined as the ratio of enzyme concentration in organic phase to that in aqueous phase for backward extraction
P_f partition coefficient with the same definition as P_b but for forward extraction
r parameter defined in Eq. (4)
(S) surfactant concentration (mM)
t time (min)
V_O volume of organic phase (or receiving phase) in forward and backward extractions (or overall extraction) (cm^3)
V_3 volume of aqueous phase (or feed phase) in forward and backward extractions (or overall extraction) (cm^3)

ACKNOWLEDGMENT

The authors express their thanks to the National Science Council of the Republic of China for financial aid (Grant NSC 81-0402-E006-541).

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Received by editor October 3, 1994