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키틴을 이용한 효소막

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Chitin-Based Enzyme Membrane

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요약 : 키틴 막에 trypsin을 immobilize 해서 효소막을 제조하고 ultrafiltration 기구를 사용하여 이 효소막의 activity를 측정하였다. Benzoylarginine ethyl ester (BAEE)와 casein이 기질로 사용되었다. BAEE를 기질로 해서 flow condition 하에서 pH 대한 영향을 조사하였다. 그 결과 최대의 activity를 보여주는 pH가 자연상태의 trypsin의 최적 pH 7.8보다 훨씬 염기성 쪽인 pH 9.0으로 옮겨갔다. 이러한 최적 pH의 이동은 효소가 이 효소막 내부에 높은 농도로 모여있고 또 분해 반응중에 생성되는 carboxylic acid에 의한 것이다. "Figure of merit"를 측정하기 위해서 이 효소막을 이용해서 flow와 batch condition의 두 조건하에서 각각 activity를 측정하였다. 이 두조건하에서 conversion이 같게 되었을 때를 찾아 그때의 반응속도를 비교하는 방법으로 "figure of merit"를 결정하였다. BAEE를 기질로 사용하였을 때 figure of merit는 5.67이었다. 이것은 flow system이 batch system보다 다섯배 이상의 높은 activity를 보여준다는 것을 뜻한다. 이 결과로부터 flow condition하에서 diffusion limitation이 많이 제거 되어졌음이 밝혀졌다. Casein을 기질로 사용 하였을때는 figure of merit가 10.3으로 나타나 큰 분자량을 가진 물질을 기질로 하였을 경우 diffusion limitation 문제가 더 심각함을 알 수 있었다.

ABSTRACT : An enzyme membrane system was constructed by immobilizing trypsin onto chitin and ultrafiltration set-up was employed to investigate the activity of thus prepared enzyme membranes. Benzoylarginine ethyl ester (BAEE) and casein were used as substrates. Using BAEE as a substrate, the dependence of conversion on pH was examined under flow condition (ultrafiltration). The results showed that the optimum pH for the enzyme membrane was shifted from 7.8 for native enzyme toward more alkaline pH 9.0. The shift was assumed to be caused by the localized high concentration of enzyme molecules and of the released carboxylic acids during the hydrolysis of substrate. In order to measure the "figure of merit" the reaction rates with a membrane were measured under the flow and batch conditions. The rates were calcul-

ated from the conversions of substrate, when the conversions for both flow and batch conditions were equal. The results obtained with BAEE as a substrate gave the figure of merit 5.67, indicating that reaction rate for flow system was five times larger than that for batch system. This result implies that in the forced flow of substrate solution through an enzyme membrane, diffusion limitations could be eliminated by exposing all the enzyme molecules in the membrane to incoming substrate molecules. When casein, a high molecular weight substrate, was used this tendency was expected to be more pronounced. In the case of casein conversion was expressed by total uv absorption ($\triangle E$) and the figure of merit was determined by comparing the time periods required to reach the same uv absorption, i.e., the same extent of reaction. The results showed that the figure of merit was 10.3, and then figure of merit for casein was two times larger than that for BAEE.

INTRODUCTION

Chitin is a naturally occurring polysaccharide constituted of N-acetylglucosamine units, and also known is that when isolated, about one out of every six acetylamino groups in chitin is in free amine form[1]. In the course of our study on chitin membrane[2] we saw the possibility in that the free amine groups could be utilized to immobilize natural enzymes, thus enabling us to construct a semipermeable enzymatic membrane system. One of the problems associated with the enzymatic reactions occuring within insoluble matrices is the slow diffusion of substrates through solid matrices. The reaction rates observed with insolubilized enzyme systems are the consequence of three stepwise processes; the diffusion of substrate molecules into insoluble matrices, the enzyme reaction, and the emergence of reaction procucts from the insoluble matrices. When substrate diffusion is much slower than enzyme reaction the reaction should occur essentially at the outer periphery of insoluble matrices and the enzyme immobilized in the interior contributes little to the reaction.

In the present study an effort was also made to look into the problem of diffusion limitations. The work was based on the assumption that under forced flow of substrate solution through an enzyme membrane, diffusion limitations are essentially eliminated as all the enzyme molecules in the membrane are exposed to substrate.

EXPERIMENTAL

Materials.

Clean chitin samples were prepared by the procedure of Cho and Lee[2]. Among the various natural chitin sources, the internal skeletal bones of squid (sepia esculents, sepia subaculeata or sepia hercules) were found to provide chitin samples in the cleanest form when properly treated. Crystalline trypsin and benzoyl-arginine ethyl ester (BAEE) were obtained from Tokyo Kasei Ltd. Casein was obtained from Difco Chemicals.

Determination of Free Amine Groups in Chitin Sample.

The treated chitin samples were thoroughly washed with distilled water. The chitin samples were then dried overnight in vacuum oven at 100°C. A 0.5g sample was soaked with stirring for 1 hr in 100ml of 1.03×10^{-2} N HCl. A 10ml aliquot was withdrawn and back titrated to determine the milliequivalents of acid taken up

Table I. Determination of Free Amine Group in Chitin*

	Chitin sample (%)	NaOH(ml) used in back titration	Glucosamine (%)
Exp. 1	0.5437	12.6	16.1
Exp. 2	0.4639	13.9	16.1
Exp. 3	0.5218	13.0	16.0

*1.08×10^{-2} N hydrochloric acid solution and 5.03×10^{-3}N sodium hydroxide solution were used in this experiment.

by the chitin. The results are summarized in Table I. The titrations showed that these chitin samples posses about 16% of the total acetylamino groups in free amine form (D-glucosamine).

Preparation of Membrane.

About 1g of the chitin sample was dissolved in 100ml of anhydrous formic acid with stirring for 7-8 hrs at room temperature. The solution was filtered to remove the insoluble residue. And then the solution was poured onto a clean glass plate. Side edge of the plate was lined with cellophane tape of certain thickness. After standing in the air for 5 hrs, thus cast film was taken off the glass plate under distilled water. In order to prepare the membrane of larger flux, drying time was shortened. To remove the formic acid remaining in the membrane, the film was immersed in 0.4% aqueous sodium hydroxide solution for a few hours. The membrane was then stored under distilled water more than 5 days befere use for the experiments. Thickness of thus cast membrane was usually about 26 μm of dry and 73 μm of wet thickness as measured by micrometer (Model 54 p, Testing Machine Inc.). Water content(S) was determined from the dry and wet weight. Water content was about 64% and dry weight was 1.26×10^{-3}g/cm².

$$S=\frac{W_w-W_d}{W_w}$$

W_w: Weight of wet membrane

W_d: Weight of dry membrane

Immobilization of Enzyme on Membrane.

A representative procedure for the immobilization of enzyme on membrane was as follows: A chitin-based membrane prepared as described above was mounted on the support disc of the ultrafiltration cell (Amicon Model 12) and then the cell chamber was filled with 10ml of the glutaraldehyde solution (2.5%, w/v in 0.05M phosphate buffer solution of pH 7). The exposed membrane area was 3.6cm². As pressure was applied, the glutaraldehyde solution started to flow through the membrane with the concurrent reaction between the free amine group of the chitin and glutaraldehyde. It took 1 hr to deplete the glutaraldehyde solution. The membrane was then washed 5 times with the buffer solution to remove residual glutaraldehyde. Immobilization of enzyme was conducted by passing enzyme solution of proper concentration through thus prepared chitin membrane. The membrane was then washed with buffer solution containing 1 M NaCl to remove non-bonded residual enzyme. The amount of enzyme bonded was determined from the difference between the 280 nm absorbance of the trypsin solution added and that of the filtrate and washings. Very small amount of trypsin was detected in the filtrate and washings. We could control the amount of trypsin bonded to chitin membrane by means of controlling the amount of added trypsin solution. Finally a $NaHSO_3$ solution (10%) was added to deactivate the unreacted aldehyde groups on the matrix.

Determination of Enzyme Membrane Activity

The enzyme activity was examined by follo-

wing the hydrolysis of BAEE. All the hydrolysis reactions of BAEE were performed in the solution buffered with 0.01M tris(hydroxymethyl)aminomethane and hydrochloric acid with sufficient sodium chloride to adjust the ionic strength to 0.2. Below pH 7.0, KH_2PO_4-NaOH system was used as the buffer and above pH 9.0, $Na_2B_4O_7$-NaOH system was used. All the experiments were carried out at 35°C. Determination of enzyme activity was done spectrophotometrically by comparison the absorbance of substrate solution at 251 nm before and after the hydrolysis reaction. Enzyme activity toward casein substrate was determined by the method of Kunitz[3], except that 2% stock casein solution and 10% trichloroacetic acid were used. The reaction rates were measured under two sets of conditions as follows:

Batch conditions:

The membrane was placed in certain volume of substrate solution and the solution was kept stirred. The change in substrate concentration was measured at certain time intervals.

Flow conditions:

The membrane was mounted on a ultrafiltration set up, and substrate solutions were forced to flow through the membrane under various pressure. Flow rates and product concentrations in the filtrate were measured.

RESULTS AND DISCUSSION

In the first phase of the work a proper reaction conditions for the study were determined.

Effect of Enzyme Loading on Conversion. To find proper level of loaded enzyme for the rate studies the dependence of conversion upon the level of loaded enzyme was first examined The results are summarized in Table Ⅱ. As indicated in the Table Ⅱ, the conversions were almost directly proportional to the trypsin loading. We took the loading level of 10-20mg /g for the further studies.

Effect of Flux.

To find the proper ultrafiltration conditions the conversions of substrate hydrolysis were determined under the different applied pressures. The results are summarized in Table Ⅲ.

Table Ⅱ. Dependence of Conversion on the Level of Enzyme Loading in Hydrolysis Reactions of Benzoylarginine Ethyl Ester by Chitin-Based Trypsin Membrane*

Trypsin loading(mg/g of chitin)	Conversion
7.10mg/g	31.8%
11.8mg/g	52.7%
16.5mg/g	73.6%
20.9mg/g	83.4%

*pH: 7.97
Temp.: 35°C
BAEE: 2.00×10^{-3} mole/l
Pressure: 3 atm

Table Ⅲ. Dependence of Conversion on Pressure in Hydrolysis Reactions of Benzoylarginine Ethyl Ester by Chitin-Based Trypsin Membrane*

Pressure (atm)	Flux (ml/hr)	Conversion (%)
1	3.10	85.1
1.5	4.65	73.9
2	6.30	55.7
2.5	7.75	45.8
3	9.30	38.5
3.5	10.85	33.7

*pH: 7.13
Trypsin loading: 16.5mg/g(chitin)
BAEE: 2.00×10^{-3} mole/l
Temp.: 35°C

As the pressure was raised, flux was also increased. The increase in flux shortened the resident time of substrates in membrane, thus

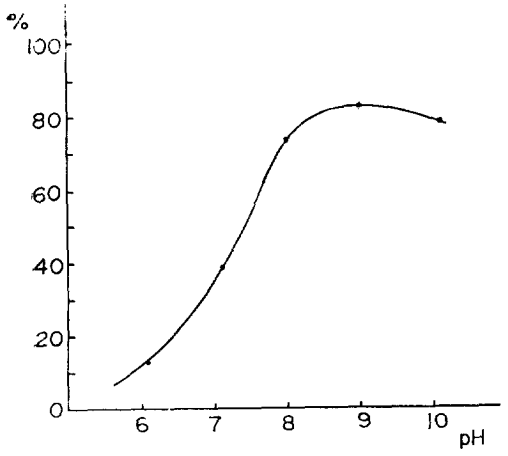

Figure 1. Dependence of Conversion on pH in Hydrolysis of BAEE by Chitin-Based Trypsin Membrane.
BAEE ; 2.00×10^{-3}mole/l
Trypsin loading: 16.5mg/g(chitin)
Temp.: 35°C
Pressure: 3 atm

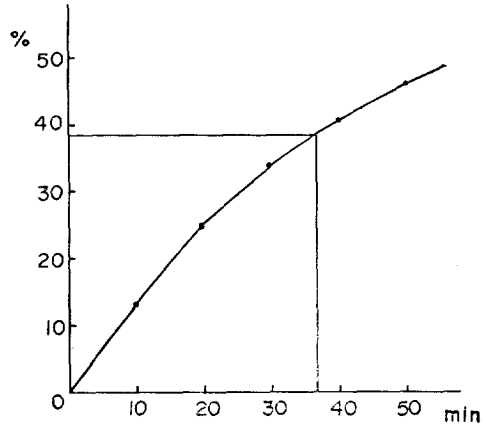

Figure 2. Dependence of Conversion on Time in Hydrolysis of BAEE by Chitin-Based Trypsin Membrane under Batch Condition.
pH:7.13
Trypsin loading: 16.5mg/g(chitin)
BAEE:2.00×10^{-4} mole/l
Temp.: 35°C

resulting in the decrease of conversion.

Effect of pH.

Dependence of conversion of pH was examined. The results were shown in Figure 1.

The optimum pH for the highest activity of enzyme membrane was shifted from pH 7.8 for native enzyme toward more alkaline pH 9.0. This shift toward alkaline pH was caused probably by the localized high concentration of enzyme molecules and of the released carboxylic acid groups during hydrolysis of substrate[5].

"Figure of Merit".

It was difficult to compare the reaction rates obtained under two different conditions, batch and flow, and the extent to which the diffusion limitations can be eliminated by forced flow through the membrane was determined by an empirical "figure of merit"[4]. This is defined as the ratio of the reaction rate under the forced flow conditions to the rate under batch conditions.

Table Ⅳ. Analysis of the Hydrolysis Reactions of Benzoylarginine Ethyl Ester by Chitin-Based Trypsin Membrane under Flow and Batch Conditions*

	Flow	Batch
Substrate(BAEE)	2.00×10^{-3}mole/l	2.00×10^{-4}mole/l
Pressure	3 atm	—
Volume	3.1 ml	10 ml
Conversion	38.5%	38.5%
Time	20 min	36.8 min
Reaction rate, V	1.19×10^{-7} mole/min	2.10×10^{-8} mole/min

Figure of merit $=\frac{V_{flow}}{V_{batch}}=5.67$

*pH: 7.13
Temp.: 35°C
Trypsin loading: 16.5mg/g(chitin)

The reaction rates of substrate hydrolysis by enzyme membrane were measured under flow and batch conditions. The rates were calculated for substrate conversions when both conversions for flow condition and batch condition are

equal. The results for using BAEE as a substrate were shown in Figure 2 and Table Ⅳ.

Table Ⅳ showed that reaction rate for flow system was about five times larger than that for batch system. This results showed that under forced flow of substrate solution through an enzyme membrane, diffusion limitations were eliminated, assuming that all enzyme molecules in the membrane were exposed to substrate. In the case of hydrolysis of high molecular weight compound these behavior will be more pronounced and casein was selected as the next substrate. Using casein as a substrate, we expressed conversion in terms of uv absorption (△E) and the figure of merit was determined, comparing the time periods which required to reach the same uv absorption. The results were shown in Figure 3 and Table Ⅴ.

Table Ⅴ. Analysis of the Hydrolysis Reactions of Casein by Chitin-Based Trypsin Membrane under Flow and Batch Conditions*

	Flow	Batch
Substrate(casein)	1% solution	1% solution
Pressure	3 atm	—
Volume	2.5 ml	2.5 ml
Conversion(△E)	0.207	0.207
Time, T	20 min	206 min

Figure of merit $= \frac{T_{batch}}{T_{flow}} = 10.3$

*pH: 8.00
Trypsin loading: 20.9mg/g (chitin)
Temp.: 35°C

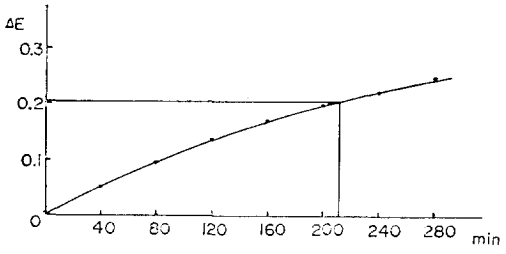

Figure 3. Dependence of Conversion on Time in Hydrolysis of Casein by Chitin-Based Trypsin Membrane under Batch Condition.
pH:8.00
Trypsin loading : 20.9mg/g(chitin)
Substrate (casein): 1% solution
Temp: 35°C

Table Ⅵ. Stability of Chitin-Based Trypsin Membrane for Hydrolysis Reactions of Benzoylarginine Ethyl Ester*

Extent of hydrolysis reaction of benzolyarginine ethyl ester by trypsin membrane		
When freshly prepared	After storing at 4°C for 7 days	After storing in buffer solution of pH 7.97 at room temp. for 24hrs.
73.6%	70.4%	68.9%

*pH: 7.97
BAEE: 2×10^{-3} mole/l
Temp.: 35°C
Trypsin loading: 16.5mg/g(chitin)

Table Ⅴ showed that the figure of merit for casein was about 10 and two times larger than that for BAEE. However in the case casein as a substrate, ultrafiltration reaction suffer a small loss of flux because of its large molecular size. Interesting to note is that in spite of loss of flux, figure of merit was enhanced. This means that in the case of high molecular weight compound as a substrate diffusion limitation phenomena become more severe as expected.

Stability

The chitin-based enzyme membrane could be used repeatedly without loss of activity and could be left standing in pH 8 buffer at room temperature for 24 hrs with only a 5% loss of activity. This membranes could be stored wet at 4°C for 7 days with only 3% loss of activity.

The results were summarized in Table Ⅵ.

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