Journal of Zhejiang University SCIENCE
ISSN 1009-3095
http://www.zju.edu.cn/jzus
E-mail: jzus@zju.edu.cn

Characterization of cellulose acetate micropore membrane immobilized acylase I*

GUO Yong-sheng (郭永胜)†, WANG Jie (王　杰), SONG Xi-jin (宋锡谨)

(*Department of Chemistry, Zhejiang University, Hangzhou 310027, China*)

†E-mail: wjjw@zju.edu.cn

Received Nov. 21, 2003; revision accepted July 17, 2004

Abstract: This paper describes an innovative method for the immobilization of acylase I, which was entrapped into the CA-CTA micropore membrane. The most suitable casting solutions proportion for immobilizing the enzyme was obtained through orthogonal experiment. Properties of the enzyme membrane were investigated and compared with those of free enzyme and blank membrane. The thermal stability and pH stability of the enzyme inside the membrane were changed by immobilization. The optimum pH was found to be 6.0, which changes 1.0 unit compared with that of free acylase I. The optimum temperature was found to be about 90 °C, which is higher than that of free acylase I (60 °C). Experimental results showed that immobilization had effects on the kinetic parameters of acylase I.

Key words: Acylase I, CA-CTA micropore membrane, Enzyme immobilization, Orthogonal experiment
doi:10.1631/jzus.2004.1608 **Document code:** A **CLC number:** Q55

INTRODUCTION

The immobilization of enzyme has become a subject of interest for industry. General operational advantages of immobilized enzymes are reusability, possibility of batch or continuous operational modes, rapid termination of reaction, controlled product formation and easy separation of the formed product, greater variety of engineering design for continuous processes and possible greater efficiency in consecutive multistep reactions.

A number of enzyme immobilization methods had been proposed for making enzyme sensors or enzyme reactors (Ling *et al.*, 2000; Deniz *et al.*, 1998; Caras and Janata, 1980; Baran *et al.*, 1997; Shen and Tu, 1999). Study of membrane immobilization is currently an active research area and the entrapment method plays an important role in enzyme immobilization. Inside the enzyme membrane there are uncountable intersecting micropores which expose the enzyme molecules, producing a relatively large effective surface area. When the substrate is pressed to filter through the enzyme membrane, the enzyme molecules entrapped in the membrane can catalyze it to the maximum. At the same time the enzyme membrane possesses of separation function. So it is becoming a very important method for enzyme immobilization (Shin *et al.*, 1998; Chen *et al.*, 1998).

In the present work based on study of the entrapment of urease in cellulose acetate (Wang *et al.*, 1998; Guo *et al.*, 2001), acylase I was immobilized into cellulose acetate micropore membrane. Acylase is widely employed in industry for producing optically pure amino acid. Orthogonal ex-

* Project (No. 20276067) supported by the National Natural Science Foundation of China

periment was used to study the influence of casting solutions proportion on the resulting activity of CA-CTA enzyme membrane. Studies were also carried out on the properties of this kind of enzyme membrane as compare with those of free acylase I. Some useful information had also been obtained through SEM.

MATERIALS AND METHODS

Reagents and apparatus

All chemicals used were commercially available and of A.R. grade. Freshly redistilled de-ionized water was used to prepare the solutions. Acylase I (EC 3.5.1. 14) was purchased from SIGMA Company (USA). Bovine serum albumin, Acetyl-DL-methionine, L-methionine, cellulose diacetate and cellulose triacetate were obtained from the Department of Biochemical Reagents (Shanghai, China). The biological activity of the enzyme was measured by 722 spectrophotometer (GUANGHENG Co., China), and the configuration of the membrane was studied by S-570 SEM (HITACHI Co., Japan).

Preparation of immobilized acylase I membrane

A definite quantity of cellulose acetate (CA) and cellulose triacetate (CTA) was put into a three-neck flask; dichloromethane and butanol with fix mass in certain proportion were then also added. Continuous stirring made the mixture dissolve completely; afterwards suitable amount of iso-butanol was added into the mixture, which was stirred for about 30 min to get a homogeneous mixture, and then 1 ml acylase I buffer solution (15.6 IU/ml) was injected into the mixture, which was stirred for 1 minute to remove vapour bubbles inside the mixture by reducing pressure. The prepared micropore enzyme membrane (Wang *et al.*, 1998) immobilized with acylase I was then washed with distilled de-ionized water, stored at 4 °C for future use.

Enzyme assay

Acylase I activity was measured as described by Zhang *et al.*(1981). Five ml phosphate buffer (0.1 mol/L, pH=7) and 5 ml Acetyl-DL-methionine solution (0.04 mol/L) were put into a test tube and shaken till a uniform intermixture was obtained, which was kept at 37 °C for about 5 min. Then 1 ml acylase I buffer solution preincubated at 37 °C for 5 min was added into it with shaking. The mixture was kept at 37 °C for 5 min, during which time, 0.5 ml reaction solution was taken out every one minute and rapidly mixed with 0.5 ml 5% trichloroacetic acid and shaken sharply to deactivize the enzyme, so that the enzyme catalyzing reaction could be stopped immediately. In this work, ninhydrin was used to determine the acyase I activity.

Membrane immobilized acylase I activity was also measured under the same conditions as that of the free enzyme. A piece of enzyme membrane was installed in a super-filter, then mixture of 10 ml acetyl-DL-methionine solution and 10 ml phosphate buffer was added into it. The pressure in the system was increased to filter the substrate solution through the enzyme membrane, and the filtrate was collected into a beaker containing 20 ml trichloroacetic acid (5%). The time it took the substrate to filter through the enzyme membrane was recorded to determine the membrane activity just as a free enzyme. In this study, one enzyme activity unit (IU) is defined as the needed quantity of enzyme that can catalyze 1 μmol L-methionine for one minute.

RESULTS AND DISCUSSION

Effect of casting proportion on enzyme activity

Orthogonal experiment was used as certain which was the most suitable casting solution proportion for immobilizing acylase I. The quantity of CA was kept constant (1.8 g), whereas quantities of the other constituents were changed in turn. All the different kinds of compounding constituents were analyzed carefully, and results are listed in Table 1 and Table 2.

The two tables show that activity of enzyme membrane was greatly affected by the proportion

Table 1 Results of orthogonal experiment

No.	CTA (g)	Butanol (ml)	Iso-butanol (ml)	Dichloromethane (ml)	Resulting activity (%)
1	0.8	3.0	24.0	28.0	96.8
2	0.8	4.0	15.0	32.0	96.6
3	0.8	5.0	18.0	36.0	74.5
4	0.8	6.0	21.0	40.0	85.5
5	1.0	3.0	18.0	32.0	80.0
6	1.0	4.0	21.0	36.0	78.8
7	1.0	5.0	24.0	40.0	85.8
8	1.0	6.0	15.0	28.0	70.0
9	1.2	3.0	18.0	40.0	56.2
10	1.2	4.0	21.0	28.0	78.6
11	1.2	5.0	24.0	32.0	54.0
12	1.2	6.0	15.0	36.0	74.9
13	1.4	3.0	24.0	36.0	41.9
14	1.4	4.0	15.0	40.0	73.3
15	1.4	5.0	18.0	28.0	53.0
16	1.4	6.0	21.0	32.0	77.3

of every component in the casting solution, and that the content of CTA played an important role. Its content could also affect some of the enzyme membrane's other properties such as aperture and rate of dankness [rate of water infiltration (Guo *et al*., 2001)], which were correlated directly with the yield of activity. Fig.1 shows the influence of CTA content in the casting solution on the yield of enzyme membrane activity. There were more hydrophobic bonds in CTA molecules than in CA molecules, which was of disadvantage for the enzyme membrane in adsorbing water. Since water is indispensable for enzyme reaction, increase of CTA content was not of advantage for the enzyme membrane reaction with substrate. On the other hand, the existence of the hydrophobic bond could also affect the enzyme membrane's rate of dankness (Fig.1), which was directly related to the reaction rate. But the CTA molecules could increase the strength of the membrane. All these factors have pivotal roles in selecting suitable quantity of CTA for the preparation of the enzyme membrane. The above two tables show the most suitable content of CTA and that of other compounding constituents for getting maximum yield of activity. It should be No. 1. But since some other properties, such as porosity and rate of dankness were also important to membrane reaction, these should be taken into account when preparing the membrane. The most suitable proportion of the casting solution was: CA, 1.8 g; CTA, 0.8 g; dichloromethane,

Table 2 Results of analysis of extreme difference (Δ) of enzyme membrane activity (%)

No.	CTA	Butanol	Iso-butanol	Dichloro-methane
1	88.4	68.7	78.7	74.6
2	79.4	82.6	65.9	76.9
3	65.9	66.8	80.8	68.3
4	61.4	77.0	69.6	75.2
Δ	27.0	15.8	14.9	8.6

All the membranes immobilized acylase I were analyzed under the same conditions as that of the free enzyme, the activity of which is defined as 100%

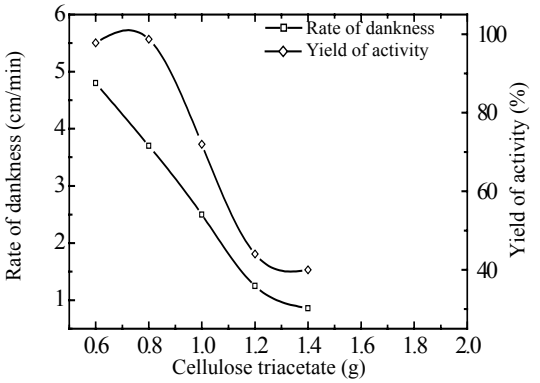
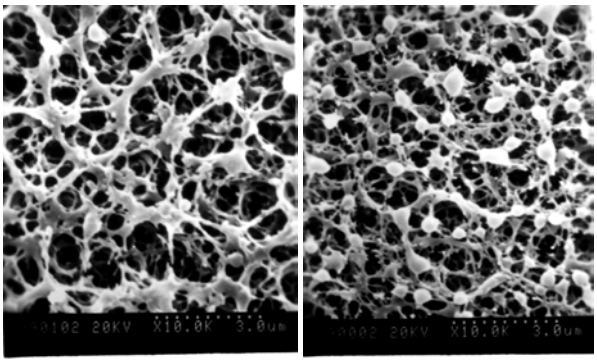


Fig.1 Influence of CTA on rate of dankness and yield of activity

32 ml; butanol, 4 ml and iso-butanol, 21 ml (Guo *et al.*, 2001).

Properties of membrane

A piece of membrane immobilized acylase I was made according to study of the most suitable proportion casting solution and some properties of this enzyme membrane were investigated. The average aperture was investigated by bubble pressure method. The most possible aperture was 0.67 μm (Guo *et al.*, 2001) and the distribution of aperture was even from SEM pictures as shown in Fig.2.



Blank membrane Enzyme membrane

Fig.2 SEM photograph of acylase I membrane

The SEM photograph showed that in the enzyme membrane some conglobation was combined with the crossing of membrane, in which the enzyme molecules might be just entrapped, and while being pressed to filter through the enzyme membrane the substrate could be effectively catalyzed. The photograph also showed that immobilization of acylase I barely changes the CA-CTA membrane structures, which proved that it was suitable for immobilizing acylase I.

Optimum pH

The effect of pH on the yield of activity of free acylase I and of the enzyme membrane was studied in the pH range of 3.0 to 11.0 at 37 °C. The maximum activity for free enzyme was observed at pH 7.0, which shifted to the acidic region about one unit upon immobilization (Fig.3).

In this study, the shift of optimum pH to acidic region for the enzyme immobilization can be explained by the acidic gradients between the bulk phase and the microenvironment of enzyme molecule entrapped in CA-CTA micropore membrane.

Optimum temperature

The activity of free and immobilized acylase I at pH 7.0 was examined at the temperature range from 20 °C to 100 °C by the same method as that before (Fig.4).

Free enzyme has optimum temperature of approximately 60 °C, while it is shifted to 90 °C for the immobilized system. For acylase I, shifts towards both lower and higher temperature were observed in this study. Research also shows that immobilization increased the thermal stability of acylase I (Jiang *et al.*, 1999).

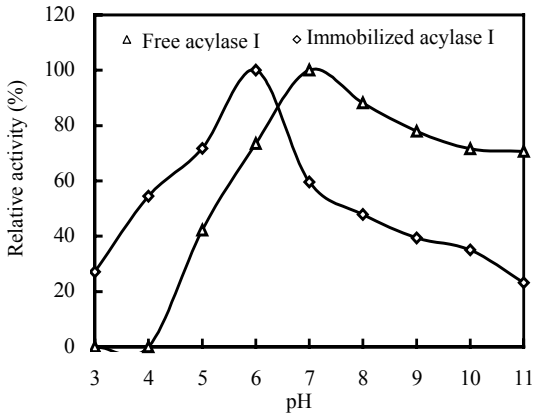


Fig.3 Optimum pH of enzyme membrane and that of free one

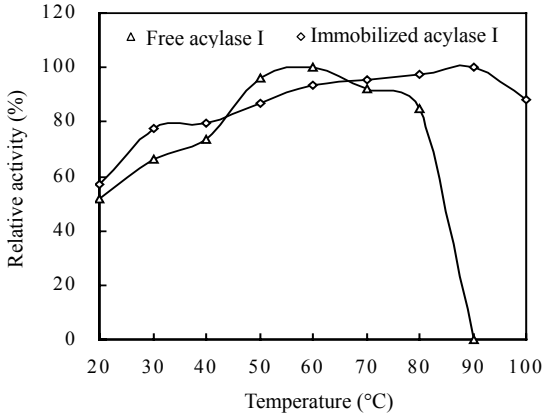


Fig.4 Optimum temperature of enzyme membrane and that of free one

Kinetic parameters

The activities of free and immobilized enzymes for various substrate concentrations are plotted in Fig.5, from which V_{max} and K_m values are calculated. V_{max} defines the highest possible velocity when all the enzymes are saturated with substrate, therefore, this parameter reflects the intrinsic characteristics of the immobilized enzyme, but may be affected by diffusional constraints. K_m is defined as the substrate concentration that gives a reaction velocity of $1/2V_{max}$.

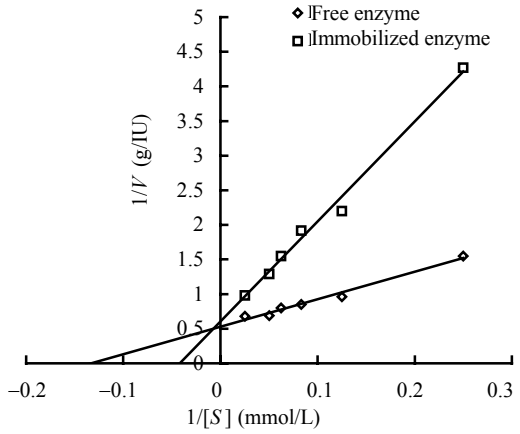


Fig.5 Michaelis constants of immobilized acylase I and free enzyme

In the present work, V_{max} values were estimated as 1.89 IU/g and 1.66 IU/g for free and immobilized enzymes; K_m values for free and immobilized enzymes were 7.52 mmol/L and 24.03 mmol/L, which can be explained as resulting from the diffusional limitations of the substrate.

Storage stability

The storage stability of the membrane immobilized acylase I was also investigated. After being stored in refrigerator at 4 °C for 60 days, the enzyme membrane activity was still more than seventy-five percent of its initial activity. Studies also showed that the enzyme membrane activity was still high after the membrane was used repeatedly. It was found that the membrane immobilized acylase I activity remained at eighty percent of its initial activity after being repeatedly used 10 times.

CONCLUSION

Acylase I was immobilized into the CA-CTA membrane. Upon immobilization in the micropore membrane, the enzyme achieved very high storage stability and reusability. These properties prove the usefulness of the micropore membrane in the immobilization of enzyme.

References

Baran, T., Arica, M.Y., Denizli, A., 1997. Comparison of β-galactosidase immobilization by entrapment in and adsorption on poly (2-hydroxyethylmethacrylate) membranes. *Polym. Int.*, **44**:530-536.

Caras, S., Janata, J., 1980. Field effect transitor sensitive to penicillin. *Anal. Chem.*, **52**:1935-1937.

Chen, J., Sun, Y., Chu, D., 1998. Immobilization of α-amylase to a composite temperature-sensitive membrane for starch hydrolysis. *Biotechnol. Prog.*, **14**:473-478.

Deniz, T., Belma, I.Y., Ahmet, R.O., 1998. Immobilization of a thermostable α-amylase, Termamyl®, onto nitrocellulose membrane by Cibacron Blue F3GA dye binding. *Biochem. Eng.*, **2**(3):179-186.

Guo, Y.S., Wang, J., Dong, J., Song, X.J., 2001. Study on immobilization of acylase with cellulose acetate membrane. *Prog. Biochem. Biophys.*, **28**(5):748-751 (in Chinese).

Jiang, X.R., Yang, J., Ren, K.Q., 1999. Production of 7-amidocephalosporanic acid from glutaryl-7-amidocephalosporanic acid by immobilized recombinant Escherichia coli cells. *Zhongguo Kangshengsu Zazhi*, **24**(4):273-276 (in Chinese).

Ling, D., Wu, G., Wang, C., Wang, F., Song, G., 2000. The preparation and characterization of an immobilized L-glutamic decarboxylase and its application for determination of L-glutamic acid. *Enzyme and Microbial Technol.*, **27**(7):516-521.

Shen, S.Y., Tu, S.I., 1999. Immobilization of horseradish peroxidase in cross-linked phyllosilicates: conditions and characterizations. *Biotechnol. Appl. Biochem.*, **29**:185-189.

Shin, I.S., Yu, J.K., Akira, I., 1998. Immobilization of glucose oxidase on poly-(L-lysine)-modified polycarbonate membrane. *Biotechnol. Appl. Biochem.*, **27**:245-248.

Wang, J., Song, X.J., Wu, M., 1998. Studies on immobilization of urease on cellulose acetate membrane. *Chem. J. Chinese Universities*, **19**(7):1104-1106 (in Chinese).

Zhang, L.X., Zhang, T.F., Li, L.Y., 1981. Method and Technology of Biochemical Experiment. Higher Education Press, Beijing, p.155 (in Chinese).