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A NEW KIND OF IMMOBILIZED LIPASE IN ORGANIC SOLVENT AND ITS STRUCTURE MODEL*

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SUMMARY: In this paper, we used Ca-alginate gel beads coated with polyetheneimine and glutaraldehyde to adsorb Expansum penicillium lipase. The immobilized lipase catalyzed esterification of 1-dodecanol with dodecanoic acid in benzene. The results show that when the concentration of Ca-alginate, polyetheneimine (PEI) and glutaraldehyde is 1%, 6% and 1%, respectively, the activity of the immobilized lipase and the amount of adsorbed protein are the highest. The immobilized lipase is better than the SDS-immobilized lipase. The activity of the immobilized lipase connected by glutaraldehyde is higher than the activity of that without glutaraldehyde. The initial rate of the immobilized lipase and lyophilized lipase powder is 5.9×10^{2} nmol/min.mgpr and 2.8×10^{1} nmol/min.mgpr, respectively. After the immobilized lipase catalyzed the esterification reaction at 37℃ for about 12 hours, 93.3% of 1-dodecanol was converted to ester, but for lyophilized lipase powder, only 17.5% converted. Based on all above results, we have presumed and explained the structure of this kind of immobilized lipase.

Lipase is an important enzyme (1) that is applied widely in chemical industry. It can catalytically hydrolyze oils or fats (2) in aqueous media; whereas the same enzyme can catalyze esterification and transesterification (3,4) in organic media. By the use of lipase's enantioselectivity, we can obtain a lot of chiral compounds (5), which are difficult to be obtained by chemical method. In recent years, lipases have been immobilized on all kinds of carriers (6-8) in order to be applied widely in industry with higher enzyme activity and stability, but because of organic solvents, activities of immobilized lipases made by widely used methods are lower, when immobilized lipases catalyze esterification and transesterification etc. At present, lipases are mostly immobilized by the method of adsorption (7,8) in order that the enzymes can be used in organic media, and the carriers are often porous-glass (9), celite (7,8) ect. In order to solve all above problems, we made a new kind of immobilized lipase with higher activity in organic solvent. We used Ca-alginate gel beads coated with polyetheneimine and glutaraldehyde to adsorb lipase. The immobilized lipase catalyzed esterification of 1-dodecanol and dodecanoic acid in benzene. We have studied the effect of Ca-alginate gel, polyetheneimine, glutaraldehyde, SDS and reaction time on the immobilized lipase, Moreover, we have presumed and explained the structure of this kind of immobilized lipase.

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MATERIALS AND METHODS

Materials: Expansum penicillium lipase, alginate gel and PEI were made in China. Dodecanoic acid, 1-dodecanol and other chemicals were all of analytical grade.

Extract of Crude Lipase: 80g lipase was mixed with 500ml of 0.01mol/L Tris-HCl buffer (PH8.8) under high speed stirring. The solution was centrifuged at the speed of 6000rpm for 20min. The supernatant was remained. The precipitate was extracted again with 300ml buffer in the same way. Collected supernatant, then ultrafiltrated with PM30, lyophilized the concentrated solution.

Synthesis of the Carrier: After 10g alginate gel was dissolved thoroughly in 1000ml water at 100℃, 1% alginate gel was obtained. 180ml of 1% alginate gel was mixed with 250ml soybean oil under stirring. The mixture was added to 400ml of 0.5mol/L $CaCl_2$ aqueous solution under stirring for several minutes. After the sample was put aside for 12 hours, the upper oil was discarded, and 60~120 mesh beads were obtained from the lower aqueous solution. The beads were washed with water. 100ml of 6% PEI was mixed with 10g alginate gel beads under stirring for 20min. The mixture was filtrated to remove about 50ml aqueous solution. Next, added 100ml of 1% glutaraldehyde aqueous solution, then stirred for 10min. At last, they were stamped crushing, and the carrier was obtained.

Lipase Immobilization: 4.0ml of 0.1mol/L phosphate buffer (PH7.0) and 2ml lipase solution (7mgpr/ml) were added to 1g carrier (wet weight). The mixture was stirred at 4℃ for 2 hours, then washed with 0.1mol/L phosphate buffer (PH7.0) for 3 times. At last, filtrated to remove aqueous solution and the immobilized lipase was obtained. Added 10ml benzene containing 0.1mol/L SDS to the obtained immobilized lipase, then stirred for several minutes, washed thoroughly with benzene for several times. At last, removed benzene, and SDS-immobilized lipase was got.

Determination of Esterification Activity: 0.45ml of 0.8mol/L dodecanoic acid and 0.45ml of 0.8mol/L 1-dodecanol were mixed together, then the immobilized lipase (0.15g), SDS-immobilized lipase (0.15g) or lyophilized lipase powder (15mg) was added to the mixture, and incubated at 37℃ for 12 hours under stir. The reaction was stopped by removing the enzyme. Esterification activity was calculated from the increase of area of product peak, which was determined by HPLC. The analysis was performed using C_{18} column in 80% tetrahydrofuran, and the flow rate was 1.0ml/min. One unit of enzyme activity is defined as the amount of enzyme that liberates 1 nmol of product or that will decrease 1 nmol of substrate/min.

Removal of Water from Organic Phase: Removal of water from organic phase was performed with 4A molecular sieve.

Determination of Protein Concentration: Protein concentration was determined according to the method of Bradford(10).

RESULTS AND DISCUSSION

Effect of Alginate Gel Concentration on the Immobilization of Lipase: According to the above method, we made SDS-immobilized lipase, whose carrier is made up of 6% PEI, 1% glutaraldehyde and different concentration of alginate gel. The SDS-immobilized lipase catalyzed the esterification of 0.45 ml of 0.8 mol/L dodecanoic acid with 0.45ml of 0.8mol/L 1-dodecanol at 37℃ for 12 hours in benzene under stir. The esterification activity was determined according the above method. The results (Fig.1) show that the enzyme activity and the amount of adsorbed protein are dependent on the carrier's Ca-alginate concentration. When the concentration of PEI is constant, the higher the concentration of Ca-alginate gel is, the lower the amount of adsorbed protein and the enzyme activity are. When the concentration of alginate gel is 1%, the amount of adsorbed protein and the enzyme activity are the highest.

Effect of PEI Concentration on the Immobilization of Lipase: According to the above method, we made SDS-immobilized lipase, whose carrier is made up of 2% alginate gel,

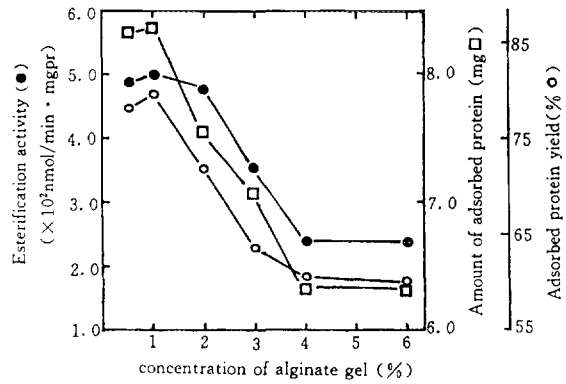


Fig. 1. Effect of Ca-alginate gel concentration on the immobilization of lipase. The carrier's weight (wet): 1g.

1% glutaraldehyde and different concentration of PEI. The SDS-immobilized lipase catalyzed the same reaction as Fig. 1 under the same condition. The esterification activity was determined according to the above method. The results (Fig. 2) show that the activity of the SDS-immobilized lipase and the amount of adsorbed protein are dependent on the carrier's PEI concentration. When the concentration of PEI is 6%, the amount of adsorbed protein and the esterification activity are the highest. No matter the concentration of PEI is lower or higher, the amount of adsorbed protein and the esterification activity will decrease.

Effect of SDS on the Immobilization of Lipase: Lipase is an unsoluble powder in organic solvents. In order to disperse the immobilized lipase very well in organic solvents, we dealt the immobilized lipase with SDS. SDS is a kind of amphipathics, its polar heads can be close to the enzyme, and its hydrophobic tails can point toward the organic solvent, thus forming reverse micelles. We think SDS will be able to increase the dispersion of enzyme in benzene and the likelihood of encounters between enzyme and

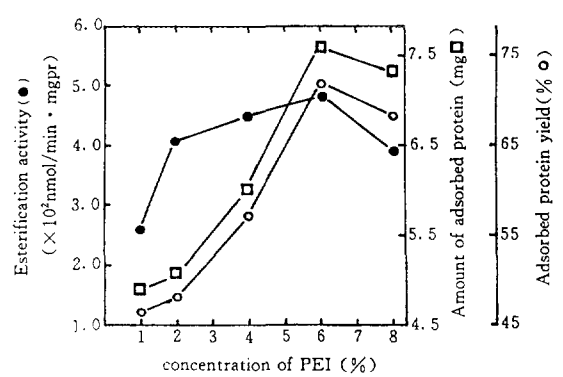


Fig. 2. Effect of PEI concentration on the immobilization of lipase. The reaction condition is the same as Fig. 1.

Table 1. Effect of SDS on the Immobilization of Lipase[1]

Concentration of Ca-alginate Gel (%)	SDS Concentration (mol/L)	Adsorbed Protein Yield (%)	Esterification Activity ($\times 10^2$nmol/min.mgpr)
1	0.0	88.2	7.6
1	0.1	80.0	5.0
2	0.0	75.0	4.9
2	0.1	72.0	4.8

1. The carrier's weight (wet): 1g.

substrate, so we compared the immobilized lipase with SDS-immobilized lipase. The results (Table 1) show that the esterification activity of the immobilized lipase and the amount of adsorbed protein are higher than that of SDS-immobilized lipase. This result is opposite to the one that we thought before our experiment. It is probably because that PEI has already served as amphipathics without SDS.

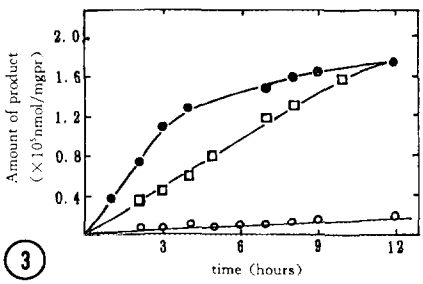
Effect of Glutaraldehyde on the Immobilization of Lipase: Under all above better conditions, we compared the immobilized lipase connected by glutaraldehyde with that without glutaraldehyde. The results (Table2) show that the enzyme activity and the amount of adsorbed protein of the immobilized lipase connected by glutaraldehyde is higher than that of the immobilized lipase without glutaraldehyde.

The Time-Course of Esterification Catalyzed by the Immobilized Lipase, SDS-Immobilized Lipase and Lyophilized Lipase Powder: In benzene, 15mg lyophilized lipase powder, the immobilized lipase and SDS-immobilized lipase, which contained the same amount of protein as lyophilized lipase powder did, catalyzed separately the esterification of 0.45ml of 0.8mol/L dodecanoic acid with 0.45 ml of 0.8mol/L 1-dodecanol at 37℃ for different time under stir. The results (Fig. 3) show that the immobilized lipase and SDS-immobilized lipase not only exhibited higher activity but also came to equilibrium fast. The initial rate of the immobilized lipase, SDS-immobilized lipase and lyophilized lipase powder is respectively 5.9×10^2nmol/min.mgpr, 2.5×10^2nmol/min.mgpr

Table 2. Effect of Glutaraldehyde on the Immobilization of Lipase[1]

Concentration of Glutaraldehyde (%)	Adsorbed Protein Yield (%)	Esterification Activity ($\times 10^2$nmol/min.mgpr)
1	72.0	4.8
0	38.3	2.1

1. The reaction condition is the same as Table 1.



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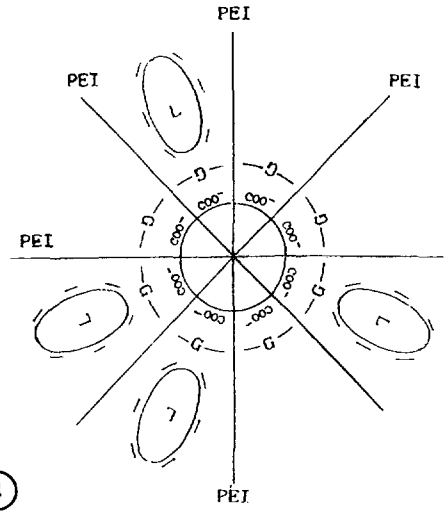

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Fig. 3. The time-course of esterification catalyzed by the immobilized lipase (●), SDS-immobilized lipase (□) and lyophilized lipase powder (○).

Fig. 4. The structure model of the immobilized lipase.
G: glutaraldehyde; L: lipase.

and 2.8×10^{1}nmol/min.mgpr. The initial rate of the immobilized lipase and SDS-immobilized lipase is respectively 21.1 and 8.9 fold greater than that of lyophilized lipase powder. It is more surprising that within 12 hours, the immobilized lipase and SDS-immobilized lipase make 93.3% 1-dodecanol converse to ester, but for lipase powder, only conversion of 17.5%.

Presumption of the Immobilized Lipase's Structure: From all above results, we presumed the structure (Fig.4) of this kind of immobilized lipase. In the structure, Ca-alginate gel works as beads. PEI connected by glutaraldehyde is most probably around the beads in the state of radiant star, instead of closing around the beads with several thin layers, because when the concentration of PEI is higher (6%), we can obtain better immobilized lipase. The lipase (PH8.8) has negative electricity, and PEI has positive electricity. They can be adsorbed together, so the lipase molecules are in the middle of PEI molecules.

In the structure, PEI has positive electricity and many hydrophobic radicals of $-(CH_2-CH_2)-$. Its polar heads are close to the lipase, and its hydrophobic tails point toward the organic solvent; thus forming reverse micelles. This kind of structure increases the dispersion of lipase in organic solvent and the likelihood of encounters between lipase and substrates. Therefore, the enzyme activity, the reaction rate and the conversion of the substrates are enhanced evidently. We are the first to make this kind of immobilized lipase. Experiment basis has been provided for lipase to be industrially applied in organic solvent.

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