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Enhancing Enzymatic Properties by the Immobilization Method

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ABSTRACT

Effects of some immobilized carriers on enzymatic properties have been studied. The following results were obtained: (1) When cholinesterase was immobilized on the hydrophobic carrier with either α-naphthylamine, benzylamine, or *p*-methylbenzylamine groups, the affinities of immobilized cholinesterase for toxic organophosphors, GB (Isopnopy l-methylphophonofluoridate) and Vx [o-ethyl-S-(2-di-isopnoylomino-thyl) methyl phosphonothiolate], were enhanced 60–90 times and 700–1200 times, respectively, whereas the thermal stability of the immobilized cholinesterase increased to 110%. Approximately 82–88% activity of the immobilized cholinesterase remained after continuously operating for 8 h; and (2) Lipase was immobilized on the carrier that was made up of 6% polyethylenimine, 1% alginate gel, and 1% glutaraldehyde. The initial reaction rate of the esterification of lauric acid with lauric alcohol catalyzed by this kind of immobilized lipase was increased 21 times, as compared to lipase powder. About 72% esterification activity of lipase remained after continuous operating for 10 d.

Index Entries: Cholinesterase; lipase; immobilization.

INTRODUCTION

Like many enzymes *in vitro*, native cholinesterase is neither stable nor sensitive to toxic organophosphors. The low affinity of native cholinesterase is not satisfactory for application to food examination, environment

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protection, and military detection. Some scientists have attempted to enhance the affinity of cholinesterase for toxic organophosphors by the methods of immunology and changing the source of cholinesterase. We thought the ability for organophosphors to inhibit cholinesterase depended not only on charge effects, but also on the hydrophobic interaction between the hydrophobic chains of organophosphors and the hydrophobic regions of cholinesterase. Therefore, we selected hydrophobic carriers to immobilize cholinesterase. By this method, we greatly enhanced the affinity of cholinesterase for toxic organophosphors.

Lipase is an important enzyme (*1*) that is widely applied in chemical industry. In recent years, it has been used to catalyze reactions in organic solvent. In order to enhance lipases' activity in organic solvent, they have been immobilized on all kinds of carriers (*2–4*), such as porous-glass (*5*) and celite (*3,4*). However, owing to the presence of organic solvents. the esterification and transesterification activities of immobilized lipases prepared by widely used methods are low. In order to solve this problem, we made a new kind of immobilized lipase with high esterification activity in organic solvent.

In this paper, we describe the enhanced properties of cholinesterase and lipase by the immobilization method.

MATERIALS AND METHODS

Materials

1. Penicillium expansum lipase was purchased from Nantong Biochemical Pharmaceutical Factory, Nantong, China.
2. Cholinesterase from duck serum was prepared in Beijing, China.
3. Other chemicals were of analytical grade prepared in China.

Methods

Method 1: Determination of the Activity of Cholinesterase and the Affinity of Cholinesterase for Toxic Organophosphors

The determination of the activity of cholinesterase was carried out according to Ellman's method (*6*). One unit of cholinesterase activity (1 U) is defined as the amount of enzyme that hydrolyzes 1μmol of DTNB (5,5′-dithiohis-2,2′-nitrobenzote)/min. The affinity of cholinesterase for toxic organophosphors was determined according to the formula: the affinity of of cholinesterase = total activity of cholinesterase − remaining activity of cholinesterase after contact with the organophosphors.

Method 2: Preparation of Cholinesterase

Cholinesterase from duck serum was partially purified by $(NH_4)_2SO_4$ fractionation. The specific activity of cholinesterase was 2.7 U/mg protein.

Method 3: Preparation of the Hydrophobic Carriers for Immobilized Cholinesterase

The preparation of the hydrophobic carriers for immobilized cholinesterase were prepared as previously described (*7*). Agar powder (6 g) was dissolved in 100 mL water with heat, then cooled to 55°C. Soybean oil (300 mL) was added to the agar powder solution with stirring, then cooled to 0°C. The mixture was not stirred until the agar beads became solid. After the sample was put aside for 12 h, 40–60 mesh beads were obtained from the lower aqueous solution.

The wet beads (100 g) were washed two times with 0.5 mol/L NaOH, then suspended in acetone (120 mL). To the suspension was added 30 mL acetone and 30 mL dioxane containing 4 g cyanuric chloride. The reaction was carried out for 2–5 min with stirring, then stopped by 20% acetic acid aqueous solution. The reaction mixture was suspended in acetone again. To the suspension were added α-naphthylamine (10 g), benzylamine (8 mL), and *p*-methylbenzylamine (8 mL), respectively. The reactions were carried out for 4 h with stirring, then filtered, and washed four times with acetone and water. The carriers were stored until used.

Method 4: Determination of the Quantities of the Hydrophobic Groups on the Carriers

The quantities of the hydrophobic groups on the carriers were determined by the method established by Perterson E. A. (*8*).

Method 5: Preparation of Immobilized Cholinesterase

Cholinesterase (8 mg) was dissolved in 0.05 mol/L of Na_2HPO_4-citric acid buffer (pH 5.5) and 0.25 g hydrophobic carrier with either α-naphthylamine, benzylamine, or *p*-methylbenzylamine groups prepared according to Method 3, then 0.075 g KCl was added to the mixture. Afterward, mixture was stirred at 4°C for 4 h, then filtered.

Method 6: Preparation of Penicillium Expansum Lipase Powder

Penicillium expansum lipase (80 g) was extracted with 500 mL of 0.01 mol/L Tris-HCl buffer (pH 8.8). The supernatant was lyophilized into enzyme powder.

Method 7: Synthesis of the Carrier for Immobilized Lipase

One hundred eighty milliliters of 1% alginate gel, 250 mL soybean oil, and 400 mL of 0.5 mol/L $CaCl_2$ aqueous solution were mixed together with stirring for several minutes. After the sample was put aside for 12 h, 60–120 mesh beads were obtained from the lower aqueous solution. One hundred milliliters of 6% polyethylenimine (PEI) were mixed with 10 g alginate gel beads with stirring for 20 min, then added to 100 mL of 1% glutaraldehyde aqueous solution and stirred for 10 min. At last, they were pounded to pieces, and the carrier was obtained.

Method 8: Lipase Immobilization

Four milliliters of 0.1 mol/L phosphate buffer (ph 7.0) and 2 mL lipase solution (7 mg protein/mL) were mixed with 1 g carrier (wet weight). The mixture was stirred at 4°C for 2 h, then washed three times with 0.1 mol/L phosphate buffer (pH 7.0). At last, the mixture filtered to remove the aqueous solution. The immobilized lipase was stored until used.

Method 9: Determination of Esterification Activity of Lipase

To 0.45 mL of 0.8 mol/L dodecanoic acid were added 0.45 mL of 0.8 mol/L 1-dodecanol. The immobilized lipase (0.15 g) or lyophilized lipase powder (15 mg) was added to the mixture, and incubated at 37°C for 12 h with stirring. The reaction was stopped by removing the enzyme. The esterification activity was calculated from the increased area of product peak, as determined by HPLC. The analysis was performed using C_{18} column [4.6 mm (Φ) × 50 mm (length)] in 80% tetrahydrofuran, and the flowrate was 1.0 mL/min. One unit of enzyme activity is defined as the amount of enzyme that liberates 1 nmol (10^{-9} mol) of product or that will decrease 1 nmol of substrate/min.

RESULTS AND DISCUSSION

The Properties of Immobilized Cholinesterase

The Affinity of Immobilized Cholinesterase for Toxic Organophosphors

The cholinesterase was immobilized on the hydrophobic carriers according to Methods 3 and 5. The content of α-naphthylamine, benzylamine, and *p*-methylbenzylamine on the wet carrier was 0.077 mmol/g, 0.082 mmol/g, and 0.079 mmol/g, respectively, as determined according to Method 4. We have reported the effects of the content of the hydrophobic groups and the effects of varying the hydrophobicity of the carriers on the activity of immobilized aspartase previously (*7*). In this paper, different concentrations of toxic organophosphors (GB and Vx) were reacted with native or immobilized cholinesterase at 37°C for 5 min. The immobilized cholinesterase contained the same amount of protein as did the native cholinesterase. The relative affinity and remaining activity of cholinesterase were determined according to Method 1. The results (Table 1 and Fig. 1) showed that the affinity of immobilized cholinesterase for GB and Vx was enhanced 60–90-fold and 700–1200-fold, respectively, as compared to the native enzyme. With low concentrations of toxic organophosphors, the affinity of the immobilized cholinesterase on the α-naphthylamine carrier is evidently poorer than that of the two other types of immobilized enzymes. The reasons for these results may be that: (1) The hydrophobic interaction between the hydrophobic carrier and the hydrophobic region of enzyme modified the conformation and hydrophobic properties of

Table 1
Comparison of Affinity of Native and Immobilized Cholinesterase for Toxins (Vx and GB)

		Native enzyme	Immobilized enzyme with		
			α-naphthylamine	benzylamine	*p*-methylbenzylamine
Relative affinity	GB	1.0	58.8	91.0	81.2
	Vx	1.0	757.3	1286.3	1071.2

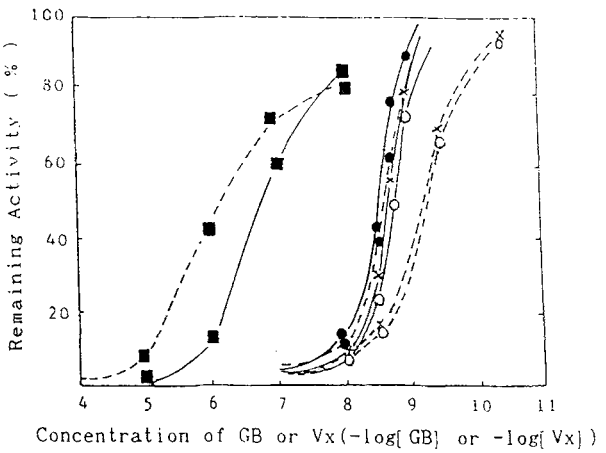

Fig. 1. Inhibition-curves for GB(—) and Vx (-------) on immobilized and native cholinesterase. ■, native; ●, α-naphthylamine; ○, benzylamine; ×, *p*-methylamine.

enzyme so that the organophosphor (GB or Vx) made contacted with enzyme easily; and/or (2) The hydrophobic group in the immobilized cholinesterase made the hydrophobic organophosphor (GB ox Vx) concentrate around the enzyme.

The Thermal Stability of Immobilized Cholinesterase

The native enzyme and the immobilized enzyme, which contained the same amount of protein as did the native enzyme in 0.5 mL of 0.1 mol/L phosphate buffer (pH 7.8) was incubated at 45°C for 1 h, respectively. The remained activity was determined according to Method 1. We have repeated this experiment three times. The results (Fig. 2) showed that the immobilized enzyme on the α-naphthylamine carrier possessed higher thermal stability than native enzyme did. The thermal stability of the two other types of immobilized enzymes was almost the same as that of native enzyme.

The Operating Stability of Immobilized Cholinesterase

The substrate solution was passed through a column [0.8 cm (ϕ) × 4.8 cm (length)] containing the wet immobilized cholinesterase (2 g) at room temperature. The remaining enzyme activity was determined at different time. The results (Fig. 3) showed that the immobilized cholinesterase maintained over 80% activity after continually operating for 8 h.

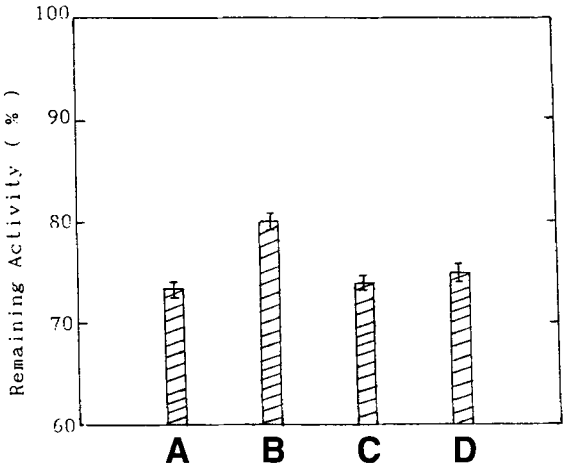

Fig. 2. Comparison of thermal stability of native and immobilized enzymes. **A,** native enzyme; **B,** α-naphthylamine: **C,** benzylamine; **D,** *p*-methylbenzylamine.

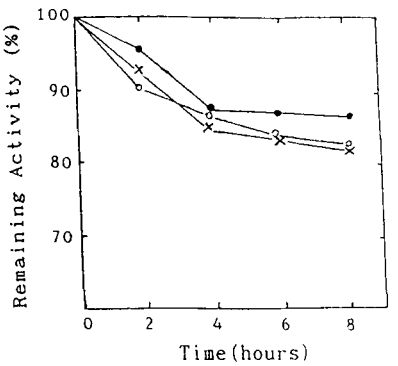

Fig. 3. The operating stability of immobilized cholinesterrase with α-naphthylamine (●), benzylamine (○), and *p*-methylamine (×).

In this paper, we are the first to immobilize cholinesterase on these hydrophobic carriers to greatly enhance the affinity of cholinesterase for toxic organophosphors (GB and Vx). The immobilized cholinesterase is sensitive to 10^{-9} mol/L toxic organophosphors (GB and Vx). The results have reached and exceeded the requirements of detecting trace toxicant. It is very significant for food examination, environmental protection, and military detection.

The Properties of Immobilized Lipase

The Time Course of Esterification Catalyzed by the Immobilized Lipase and Lyophilized Lipase Powder

In benzene, 15 mg of lyophilized lipase powder and the immobilized lipase, which contained the same amount of protein as did lyophilized

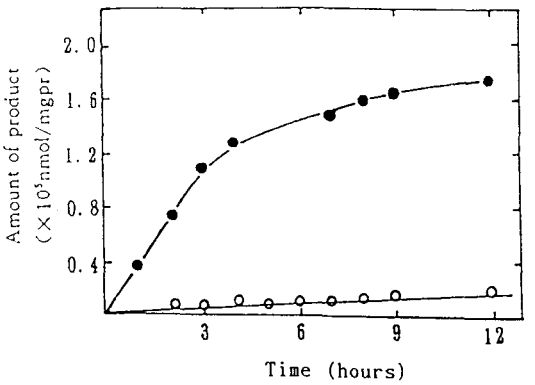

Fig. 4. The time-course of esterification catalyzed by the immobilized lipase (●) and lyophilized lipase powder (○).

lipase powder, separately catalyzed the esterification of 0.45 mL of 0.8 mol/L dodecanoic acid with 0.45 mL of 0.8 mol/L 1-dodecanol at 37°C for different reaction time with stirring. The results (Fig. 4) showed that the immobilized lipase not only exhibited higher activity but also made the reaction come to equilibrium fast. The initial reaction rate of the immobilized lipase and lyophilized lipase powder was, respectively, 5.9×10^2 nmol/min·mg protein and 2.8×10^1 nmol/min·mg protein. The initial reaction rate of the immobilized lipase was 21.1-fold greater than that of lyophilized lipase powder. It is surprising that, within 12 h, the immobilized lipase converts 93.3% 1-dodecanol to the ester, while the lipase powder converts only of 17.5%. The reasons for these results may be that polyethylenimine is positively charged and contains hydrophobic radicals of (CH_2-CH_2). Its polar heads are close to the lipase, whereas its hydrophobic tails point toward the organic solvent. This kind of structure increases the dispersion of lipase in the organic solvent and the likelihood of encounters between lipase and hydrophobic substrates. Therefore, the enzyme activity, the reaction rate, and the conversion of the substrates are evidently enhanced.

The Operating Stability of Immobilized Lipase

Lauric acid (3.15 mL of 0.8 mol/L), lauric alcohol (3.15 mL of 0.8 mol/L), and 1.4 g immobilized lipase were added to benzene with stirring and incubated at 37°C for 10 d. The remaining activity of the immobilized lipase was determined at different times according to Method 9. The results (Fig. 5) showed that the immobilized lipase maintained over 72% esterification activity after continuously operating for 10 d.

We are the first to use this type of immobilized lipase in an organic solvent. An experiment basis has been provided for lipase to be industrially applied in organic solvent.

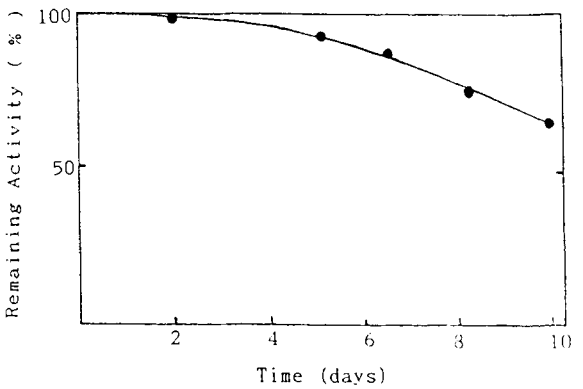

Fig. 5. The operating stability of the immobilized lipase (●).

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