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Chemically Modified Sepharose as Support for the Immobilization of Cholesterol Oxidase

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Because the cholesterol oxidase from *Brevibacterium* sp. M201008 was not as stable as the free enzyme form, it had been covalently immobilized onto chemically modified Sepharose particles *via* *N*-ethyl-*N'*-3-dimethylaminopropyl carbodiimide. The optimum immobilization conditions were determined, and the immobilized enzyme activity obtained was 12.01 U/g Sepharose-ethylenediamine. The immobilization of the enzyme was characterized by Fourier transform infrared spectroscopy. The immobilized enzyme exhibited the maximal activity at 35ºC and pH 7.5, which was unchanged compared with the free form. After being repeatedly used 20 times, the immobilized enzyme retained more than 40.43% of its original activity. The immobilized enzyme showed better operational stability, including wider thermal and pH ranges, and retained 62.87% activity after 20 days of storage at 4ºC, which was longer than the free enzyme.

Keywords: Chemical modification, sepharose, cholesterol oxidase, immobilization, enzyme stability

Introduction

Cholesterol oxidase (3β-hydroxysteroid oxidase, E.C.1.1.3.6) is a bacterial enzyme that catalyzes the first step in the degradation of cholesterol and is produced by several microorganisms [5, 14, 17, 21, 23]. It is found in two natural forms that catalyze identical reactions. In one form, the FAD cofactor is linked covalently to the protein backbone; in the other form, the FAD cofactor is bound tightly, but noncovalently, as in the majority of flavoprotein oxidases. Cholesterol oxidase has a number of important commercial applications. It catalyzes both the oxidation of cholesterol to 5-cholesten-3-one, with the reduction of molecular oxygen to hydrogen peroxide, and the isomerization of the double bond in the oxidized steroid ring system, to yield 4-cholesten-3-one as the final product. This enzyme is routinely used for the enzymatic transformation of cholesterol and for determining levels of total serum cholesterol in clinical and food specimens in a coupled system with cholesterol esterase and peroxidase. Additionally, it also exhibits potent insecticidal activity that is very important for pest control strategy in agriculture [1, 8, 13, 15].

Because the cholesterol oxidase from *Brevibacterium* sp. M201008 is not stable as a free enzyme form, we hoped to improve its stability *via* immobilization. Immobilized enzyme is a topic of active research in enzyme technology. In the past decade, several immobilization methods for cholesterol oxidase had been proposed. Yotova and Ivanov [28] reported the method for individual and simultaneous covalent immobilization of cholesterol oxidase to the copolymer of acrylonitrile with acrylamide. Gilles *et al*. [3] directly immobilized cholesterol oxidase onto Fe_3O_4 magnetic nanoparticles. The bound enzyme exhibited a better tolerance to pH, temperature, and substrate concentration. Suman *et al*. [18] immobilized cholesterol oxidase on polyaniline film for application to a cholesterol biosensor. Wang *et al*. [24] coated cholesterol oxidase on cellulose acetate membrane for free cholesterol biosensor development. At present, the method of cholesterol oxidase immobilization raises concerns on the reduced surface area for enzyme binding and pore-

diffusion resistance, and the key problem that should be solved urgently is the choice of appropriate support material and simple immobilization method.

In our previous work, cholesterol oxidase had been immobilized onto carboxyl-Sepharose particles [2]. In this work, the successful preparation of modified amino-Sepharose particles by chemical modification was illustrated. The modified Sepharose particles and *N*-ethyl-*N*'-3-dimethylaminopropyl carbodiimide (EDC) were then used as support and cross-linking agent, respectively, to immobilize cholesterol oxidase from *Brevibacterium* sp. M201008. The parameters controlling the immobilization process of cholesterol oxidase had been optimized. The pH, thermal, reusable, and storage stabilities of the immobilized enzyme were studied and compared with those of free enzymes.

Materials and Methods

Materials

Cholesterol oxidase from *Brevibacterium* sp. M201008 was obtained as described below. *N*-Ethyl-*N*'-3-dimethylaminopropylcarbodiimide was purchased from Sigma, USA. Sepharose CL 4B was from Pharmacia Biotech, Sweden. All the other reagents were of analytical grade from commercial suppliers.

Preparation of Different Modified Sepharose Particles

Sepharose CL 4B (90 g) was washed with water at a 10:1 ratio, drained, and suspended in 50 ml of activating solution (1 M NaOH, 2.5 g sodiumborohydride, and 10 ml epichlorohydrin). The mixture was tumbled for 2 h at 60°C. Then the activated gel was washed with 10:1 distilled water until the pH of the eluate was 7.0. After 90 g Sepharose CL 4B was activated by epichlorohydrin, the activated Sepharose CL 4B was suspended in 10% ammonia, ethylenediamine, and hexamethylenediamine, respectively. The gels were incubated for 12 h at 30°C on a rotary shaker (200 rpm), after which it was washed with distilled water on a sinter funnel [12]. The Sepharose-ammonia, -ethylenediamine, and -hexamethylenediamine were washed with distilled water to remove excess ammonia, ethylenediamine, and hexamethylenediamine not bound to the support.

Estimation of the density of amino groups on the Sepharose was performed in the following manner: 1 g of thoroughly washed and drained modified Sepharose was suspended in 10 ml of distilled water. The suspension was then titrated to pH 7.00 by the addition of 0.1 M HCl. The amino group density in µmol/g Sepharose was equal to the number of µmol acid required for the titration. Sepharose supports having amino group density in the 30 µmol/g of Sepharose were used for all experiments.

Expression and Purification of Cholesterol Oxidase in *E. coli*

Cholesterol oxidase cloned from *Brevibacterium* sp. M201008 was expressed in *E. coli* BL21 (DE3). Cells harboring pET28a-choBm were grown overnight at 37°C in an LB medium containing kanamycin (20 µg/ml). The culture was transferred into 100 ml of the LB medium, cultivated at 37°C until the OD_{600} reached 2, and induced with 10 g/l lactose for 10 h at 28°C. The cells were harvested *via* centrifugation (6,000 ×*g* for 20 min at 4°C), and resuspended in 20 mM sodium phosphate (pH 7.5). The suspended cells were disrupted by sonication and the resulting homogenate was centrifuged (30 min, 10,000 ×*g*, 4°C), in order to collect the supernatant.

Cholesterol oxidase was purified according to our previous work [26, 27]. The supernatant after centrifugation was loaded onto the equilibrated affinity column, washed with 20 mM sodium phosphate (pH 7.5), then washed with 20 mM sodium phosphate/0.02 M NaCl (pH 7.5), and finally eluted with 20 mM sodium phosphate/0.3 M NaCl (pH 7.5). The purification process was repeated more than three times.

Accessible Acidic Amino Acids on the Surface of Cholesterol Oxidase

Accessible acidic amino acids on the surface of cholesterol oxidase were analyzed by Discovery Studio 2.5 software, and the numbers of acidic amino acids available were calculated.

Choice of Different Immobilization Sepharose Supports

Three different Sepharose supports (1.0 g) were dissolved in 10 ml of 20 mM phosphate buffer solution, respectively. EDC and cholesterol oxidase (50:10:1 molar ratio of Sepharose-NH_2 to EDC to enzyme-COOH) were added. After being stirred gently at 4°C for 20 h, three different precipitates were collected by filtration, and noncovalently adsorbed enzyme molecules were removed by washing with 0.6 M NaCl. Three different immobilized enzymes were obtained by assaying their activity directly after immobilization and residual activity at 37°C after 24 h.

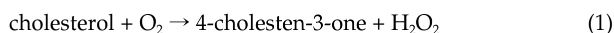
Optimization of Immobilization Parameters

The most efficient conditions of cholesterol oxidase immobilization on Sepharose-ethylenediamine were studied. Optimum parameters of immobilization were determined by changing individually the following conditions: immobilization pH (6, 6.5, 7.0, 7.5, and 8.0), molar ratio of enzyme-COOH to Sepharose-ethylenediamine (1:200, 1:150, 1:100, 1:50, and 1:10), and molar ratio of enzyme-COOH to EDC (1:5, 1:10, 1:20, 1:50, 1:100, and 1:200). Sepharose-ethylenediamine (1.0 g) was dissolved in 10 ml of 20 mM phosphate buffer solution. EDC and cholesterol oxidase were added to the above solution. After being stirred gently at 4°C for 20 h, the resulting precipitate was collected as the immobilized enzyme by filtration and was washed with 0.6 M NaCl to remove noncovalently adsorbed enzyme within the Sepharose supports; the supernatant was collected to determine the assay of cholesterol oxidase. All of the experiments were carried out at least in triplicate.

Cholesterol Oxidase Bioactivity Assay

Cholesterol oxidase catalyzed the conversion of cholesterol

into 4-cholesten-3-one and hydrogen peroxide (H_2O_2) according to the reaction (1).



$$\text{cholesterol} + O_2 \rightarrow \text{4-cholesten-3-one} + H_2O_2 \quad (1)$$

The amount of H_2O_2 that was produced by cholesterol oxidase during cholesterol oxidation was measured from the amount of quinoneimine formed by the oxidation of 4-aminoantipyrine and phenol by H_2O_2 in the presence of horseradish peroxidase. The reaction mixture (1 ml) contained 6 mM phenol, 1 mM 4-aminoantipyrine, 1 mM cholesterol, 5 U horseradish peroxidase, and 20 µl of diluted cholesterol oxidase. Under these assay conditions, the initial rate of formation of colored product was measured at 500 nm for a reaction of 15 min at 37°C. One unit of cholesterol oxidase was defined as the enzyme amount that formed 1 µmol H_2O_2 per minute at 37°C [16].

The relative enzyme activity of immobilized cholesterol oxidase (IC) was calculated as below:

$$IC = \frac{C_s}{C_i - C_r} \times 100\%$$

where C_s indicates the total enzyme activity of immobilized cholesterol oxidase; C_i and C_r denote the initial activity of cholesterol oxidase introduced and the enzyme activity in the supernatant, respectively.

Fourier-Transformed Infrared Spectroscopy

A FA-078 Fourier transform infrared spectrophotometer with a resolution of 1 cm^{-1} in the transmission mode in the region 400–4,000 cm^{-1} was used to confirm the expected amide bond formation in enzyme-immobilized Sepharose. The sample was mounted in a purged sample chamber at normal incidence. It (2 mg) was milled, mixed with potassium bromide (150 mg), and pressed into a solid disk of 1.2 cm diameter prior to the infrared measurement. Background spectra were obtained by using an untreated deoxidized flat silicon wafer mounted in the same geometry.

Effects of Temperature and pH on Enzyme Activity

The activity assays were carried out over the temperature range 25–65°C and pH range 5.5–9.5 to determine the temperature and pH profiles for the free and immobilized enzymes. The results of pH and temperature were presented in a normalized form, with the highest value of each set being assigned the value of 100% activity. All of the experiments were carried out at least in triplicate.

Stabilities of the Immobilized Enzyme

The pH stability of the free and immobilized cholesterol oxidases was studied by incubating the enzymes at 37°C in phosphate buffer (20 mM) of varying pH values in the range of 4.0–9.0 for 60 min.

The thermal stability of the free and immobilized cholesterol oxidases was tested by incubating the enzymes in phosphate buffer (20 mM) at varying temperatures in the range of 20–60°C for 60 min.

The reusability of the immobilized enzyme was evaluated by collecting the immobilized enzyme by centrifugation after one determination of the activity, washing the enzyme three times with phosphate buffer (20 mM, pH 7.5), and determining the enzyme activity again. The activity of the first run was defined as 100%, and the activity of the subsequent run was expressed as residual activity.

The storage stability of the free and immobilized enzymes was determined by assaying for their residual activity. They were stored at 4°C in phosphate buffer (20 mM, pH 7.5).

Results and Discussion

Accessible Acidic Amino Acids On the Surface of Cholesterol Oxidase

The gene from *Brevibacterium* sp. M201008 had been cloned, sequenced, and expressed in *E. coli* [25]. The cholesterol oxidase gene consisted of 1,653 base pairs and encoded 551 amino acid residues. Accessible acidic amino acids on the surface of cholesterol oxidase had been analyzed by DS2.5 (Fig. 1). Acidic amino acid residues consisting of -COOH groups in their side chain (*e.g.*, glutamate and aspartate) contributed to enzyme binding on Sepharose. The spheres and ball-sticks in Fig. 1 represented main and side chains of acidic amino acids, respectively. There were 45 -COOH groups available on the surface of cholesterol oxidase.

Choice of Different Immobilization Sepharose Supports

EDC was a popular reagent for activating carboxyl groups for reaction with other amine-containing molecules [9]. If the stable covalent linkages were produced, this method could claim the advantage of precluding depolymerization

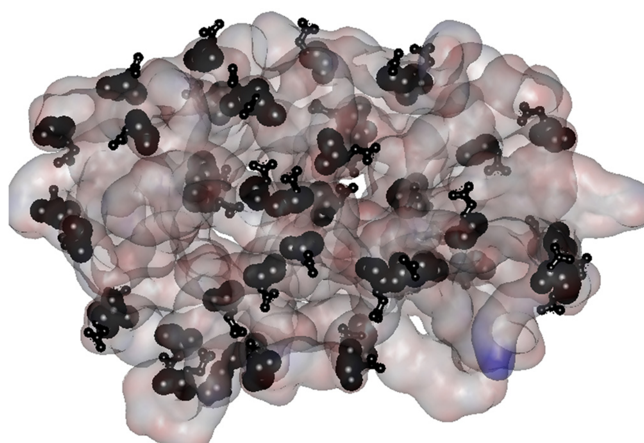


Fig. 1. Acidic amino acids (black) on the surface of cholesterol oxidase.

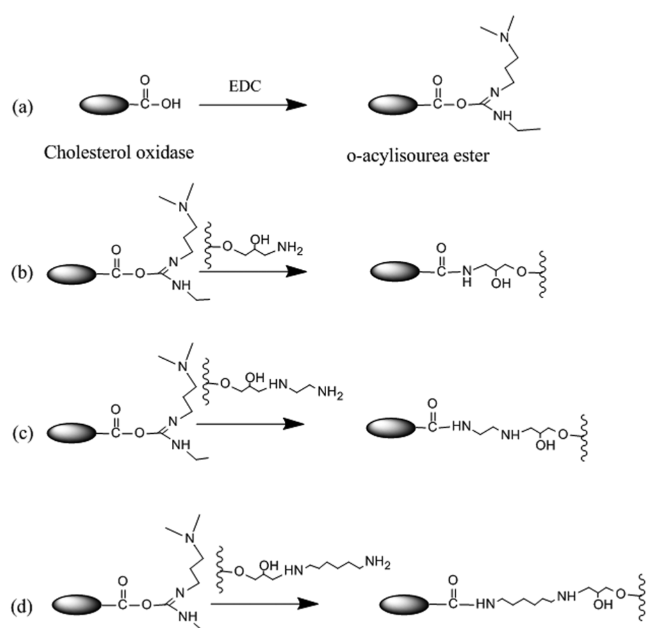


Fig. 2. Different immobilization pathways.
(**a**) Activation of free carboxylic groups with EDC. Immobilizing reactions *via* spacers like (**b**) ammonia; (**c**) ethylenediamine; and (**d**) hexamethylenediamine.

and release of residual reagent [10]. The conjugation reaction occurred in two sequential steps. The carboxylic acid group of enzyme surface was activated by EDC, forming an amine-reactive *o*-acylisourea ester intermediate. It was unstable in aqueous solution and susceptible to hydrolysis. The active ester underwent a nucleophilic substitution reaction with the amine group on the Sepharose, resulting in the formation of an amide bond between the Sepharose and enzyme. Cholesterol oxidase was immobilized to Sepharose-NH_2 *via* ammonia, ethylenediamine, and hexamethylenediamine (Figs. 2b, 2c, 2d).

The activity of enzyme was determined directly after immobilization and also after 24 h of storage at 37°C. Table 1 shows the strong dependence of activity and stability of the immobilized enzyme on different Sepharose supports. The Sepharose-ammonia immobilized enzyme showed the lowest enzyme activity (10.25 U/g), and 64.44% of this activity was lost by 24 h at 37°C. Relatively low enzyme activity (10.53 U/g) and residual activity (40.53%) were also observed when the enzyme was immobilized on Sepharose-hexamethylenediamine. The high values of the enzyme activity and residual activity of Sepharose-ethylenediamine immobilized enzyme were 12.25 U/g and 59.48%, respectively.

In this case, carboxylic acid groups of cholesterol oxidase did not easily react with the amino groups on the Sepharose-ammonia owing to steric hindrance of the short spacer. Although spacer-hexamethylenediamine could avoid steric hindrance between enzyme and Sepharose, the folding and twist between these soft long spacers would significantly increase the mobility of enzyme, which might bring about the conformational change and enzyme instability. Thus, Sepharose-ethylenediamine most efficiently enhanced the enzyme stability, and was chosen as the immobilization support.

Optimization Parameters of Cholesterol Oxidase Immobilization

To optimize the conditions of cholesterol oxidase immobilization, the effects of molar ratio of enzyme-COOH (the carboxyl group on the surface of cholesterol oxidase) to Sepharose-ethylenediamine, pH, and molar ratio of EDC to enzyme-COOH on the efficiency of immobilized enzyme were investigated.

The molar ratio of enzyme-COOH to Sepharose-ethylenediamine had an important effect on the enzyme activity and stability. It could be seen, from Fig. 3A, that with the increase of molar ratio from 1:200 to 1:100, the residual activity increased to a maximum value (68.89%) after 24 h at 37°C. With the ratio range from 1:100 to 1:10, the residual activity decreased to a minimum of 56.37%, but the enzyme activity was only slightly higher than that at ratio 1:100. Accordingly, the molar ratio 1:100 was accepted as the optimal ratio for immobilization to enhance the enzyme stability.

The pH had an important effect on enzyme activity. Results were presented in Fig. 3B, the maximum enzyme

Table 1. Dependence of the activity of cholesterol oxidase immobilized to Sepharose on different spacer lengths.

Spacer	Enzyme activity (U/g)	Relative activity (%)	Residual activity (%)
-ammonia	10.25 ± 0.74	59.35 ± 2.91	35.56 ± 2.97
-ethylenediamine	12.25 ± 0.49	66.81 ± 2.89	59.48 ± 3.04
-hexamethylenediamine	10.53 ± 0.76	53.35 ± 1.96	40.53 ± 1.94

Relative activity was determined by the percentage of the direct activity after immobilization in the activity of free enzyme used for binding. Residual activity was calculated after 24 h storage at 37°C and the initial activity of immobilized enzyme was used as 100%. Data represent the average ± standard deviation of three experiments.

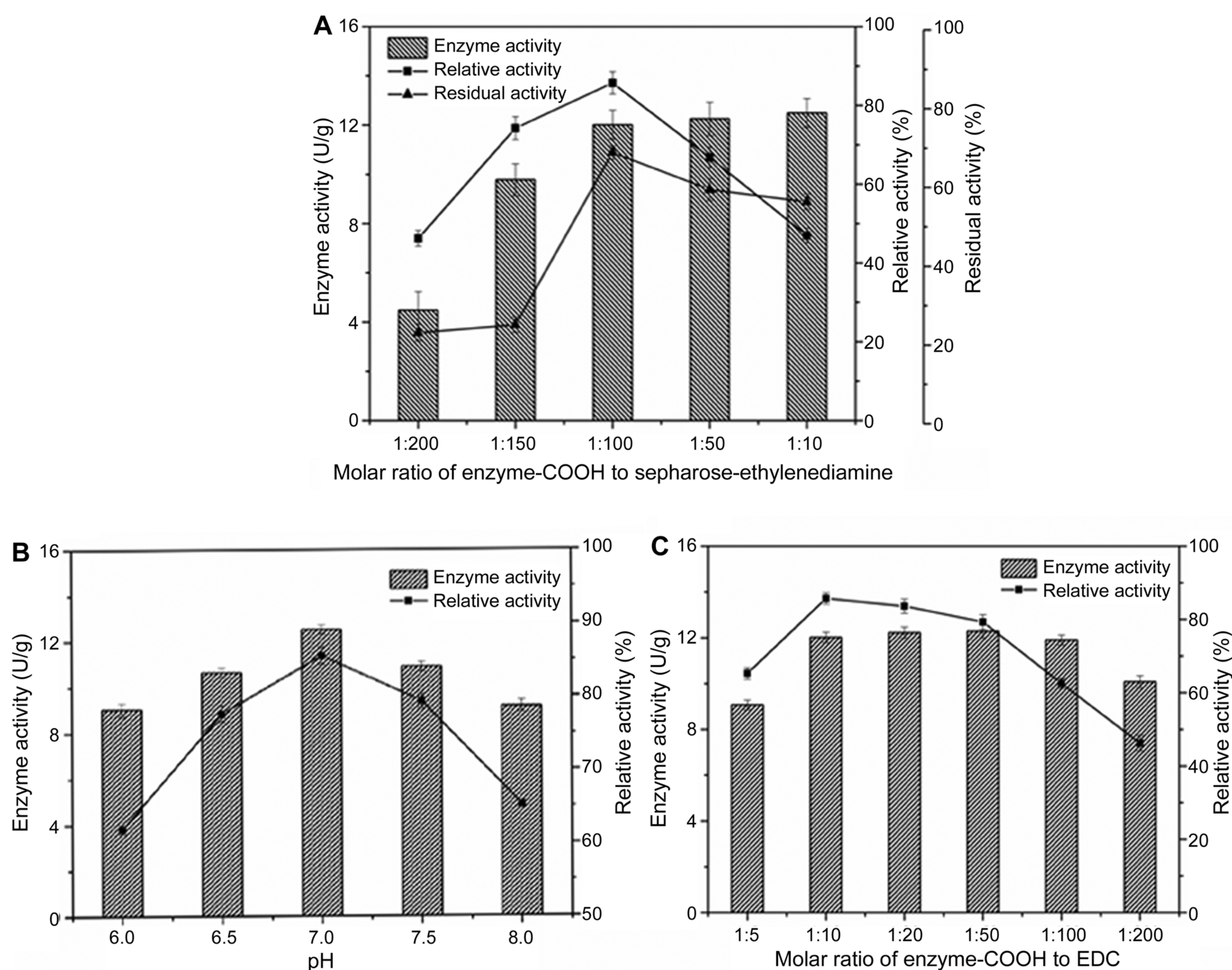


Fig. 3. Effects of molar ratio of enzyme-COOH to Sepharose-ethylenediamine (**A**), pH (**B**), and molar ratio of enzyme-COOH to EDC (**C**) on immobilized cholesterol oxidase.

Relative activity was determined by the percentage of the activity directly after immobilization in the activity of free enzyme used for binding. Residual activity was calculated after 24 h storage at 37°C and the initial activity of immobilized enzyme was used as 100%. Data represent the average ± standard deviation of three experiments.

activity and relative activity of the immobilized enzyme reached 12.01 U/g and 85.75%, respectively, at pH 7.0. It could be seen that the immobilization efficiency of both enzyme activity and relative enzyme activity increased with a pH range of 6.0 to 7.0. However, the enzyme activity drastically decreased with the increase of pH from 7.0 to 8.0, which may be due to the instability of EDC against the increase of pH. Therefore, the optimum pH for immobilization was 7.0.

The effect of the molar ratio of enzyme-COOH to EDC on immobilization is shown in Fig. 3C. When the molar ratio was low (<1:10), the enzyme activity and relative activity of immobilized enzyme increased significantly. The relative activity of immobilized enzyme reached the highest value when the molar ratio was 1:10. At low molar ratio, few amide bonds between Sepharose and cholesterol oxidase were formed. However, if an excessive molar ratio was introduced, the amide bonds could be formed not only between cholesterol oxidase and Sepharose, but also between cholesterol oxidase molecules. This cross-linking of enzyme restricted the conformation mobility of enzyme molecule, thereby leading to the loss in enzyme activity [6]. Hence, 1:10 was chosen as the optimum ratio of enzyme-COOH to EDC for immobilization.

Consequently, the optimal conditions of cholesterol oxidase immobilization were observed at a 100:10:1 ratio of

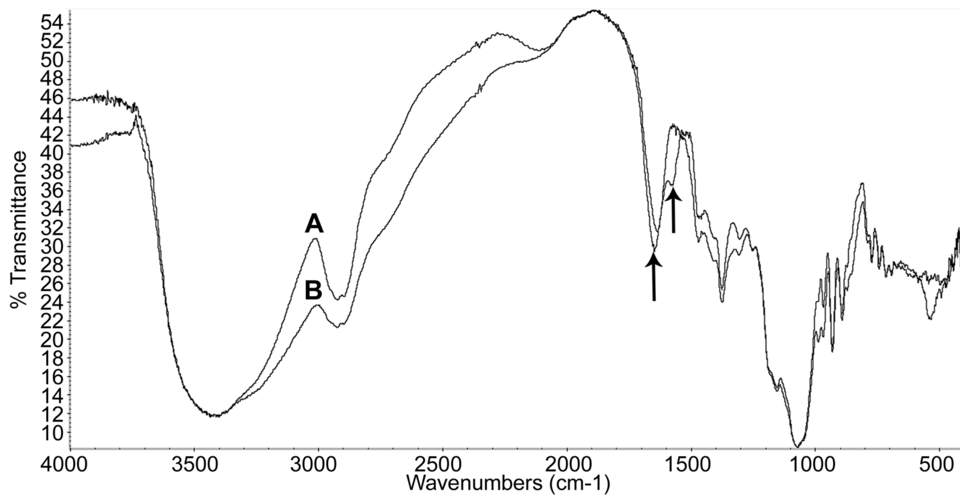


Fig. 4. FTIR spectra of support without (**A**) and with (**B**) immobilized cholesterol oxidase.

Sepharose-ethylenediamine to EDC to enzyme-COOH, and pH 7.0, with enzyme activity of 12.01 U/g Sepharose-ethylenediamine.

Fourier-Transformed Infrared Spectroscopy

The binding of cholesterol oxidase to Sepharose was confirmed by FTIR analysis. Fig. 4 exhibits the FTIR spectra of Sepharose without (A) and with (B) immobilized cholesterol oxidase. The peak absorbance of the -NH_2 group appearing at 1,579 cm^{-1} was assigned to N-H bending vibration. Clearly, this peak was not apparent in the spectra of immobilized enzyme, suggesting that immobilization was accomplished by involving the reaction between these amine groups on Sepharose and carboxyl groups of cholesterol oxidase after being activated by EDC. A new peak that appeared at 1,647 cm^{-1} in the spectra of immobilized enzyme confirmed the immobilization of enzyme to Sepharose. The weakness of this peak should be due to the low loading amount of enzyme immobilized to Sepharose.

Effects of pH and Temperature on the Response of the Immobilized Enzyme

The pH dependence of activity of free and immobilized enzymes is shown in Fig. 5A. The results showed that the activity increased with the increase of pH from 5.5 to 7.5 and then declined with the pH range from 7.5 to 9.5. After immobilization, the pH response of the enzyme did not

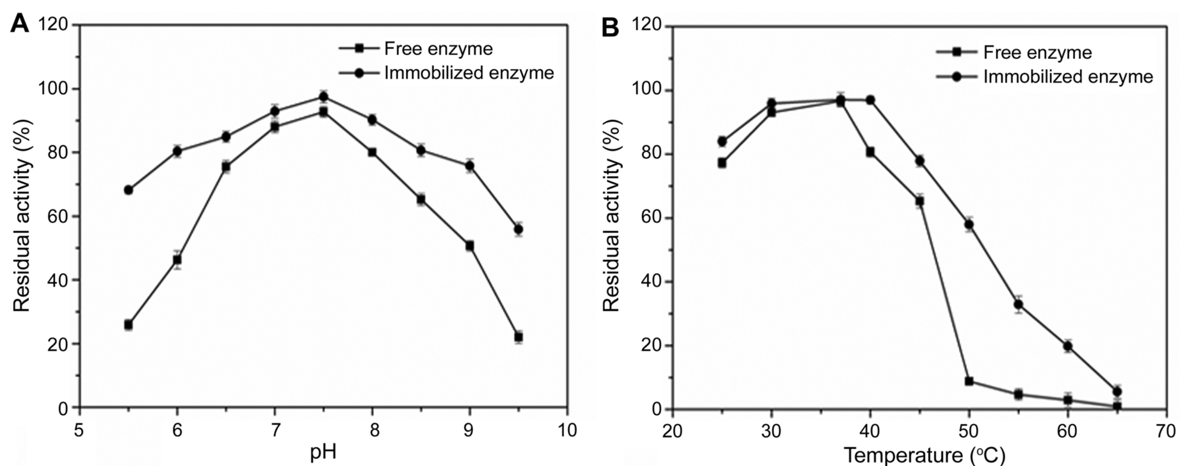


Fig. 5. Effects of pH (**A**) and temperature (**B**) on the activities of free and immobilized cholesterol oxidases.

Residual activity was calculated by using the highest activity of free and immobilized enzymes as 100%, respectively. Data represent the average ± standard deviation of three experiments.

change (optimal pH was 7.5). A similar observation for immobilized cholesterol oxidase had been reported by Gilles *et al*. [3]. They compared the activity of free and bound cholesterol oxidase as a function of pH and found that both had the same optimum pH value of 7.4.

The temperature dependence of enzyme activity was studied in the temperature range of 25–65°C, and the temperature profile of free and immobilized enzymes is shown in Fig. 5B. The maximum activity was observed at 35°C for both free and immobilized enzymes. Free enzyme was more sensitive to the increase of temperature, and the activity of free enzyme decreased sharply toward higher temperature. Meanwhile, the loss of the activity of immobilized enzyme was lower than that of the free one for high temperature.

Stabilities of the Immobilized Enzyme

The thermal stability of both the immobilized and free enzymes was studied by incubating the immobilized enzyme, prepared under the optimum conditions, at 20°C, 30°C, 40°C, 50°C, and 60°C for 60 min and then measuring the enzyme activity. As shown in Fig. 6A, the free enzyme was more sensitive to the increase of incubation temperature, and the residual activity of free enzyme decreased sharply toward high temperature. After incubation at 40°C, the free and immobilized enzymes retained 72.08% and 97.39%, respectively, of the original activity. The residual activity of immobilized enzyme was 7.5 times higher than that of free enzyme when the incubation temperature was at 50°C. However, at 60°C, the residual activity of immobilized enzyme only exhibited a little higher than that of the free one. Altogether, the thermal stability of cholesterol oxidase was improved after immobilization. This reason might be due to the conformational change and spatial rearrangement after being immobilized [3].

The pH stability of the free and immobilized enzymes was determined by dissolving each of the two enzymes in 20 mM of phosphate buffer with a pH of 4.0−9.0 for 60 min. The results are presented in Fig. 6B. At pH 4.0 and 9.0, the immobilized enzyme retained 62.19% and 91.23% of the

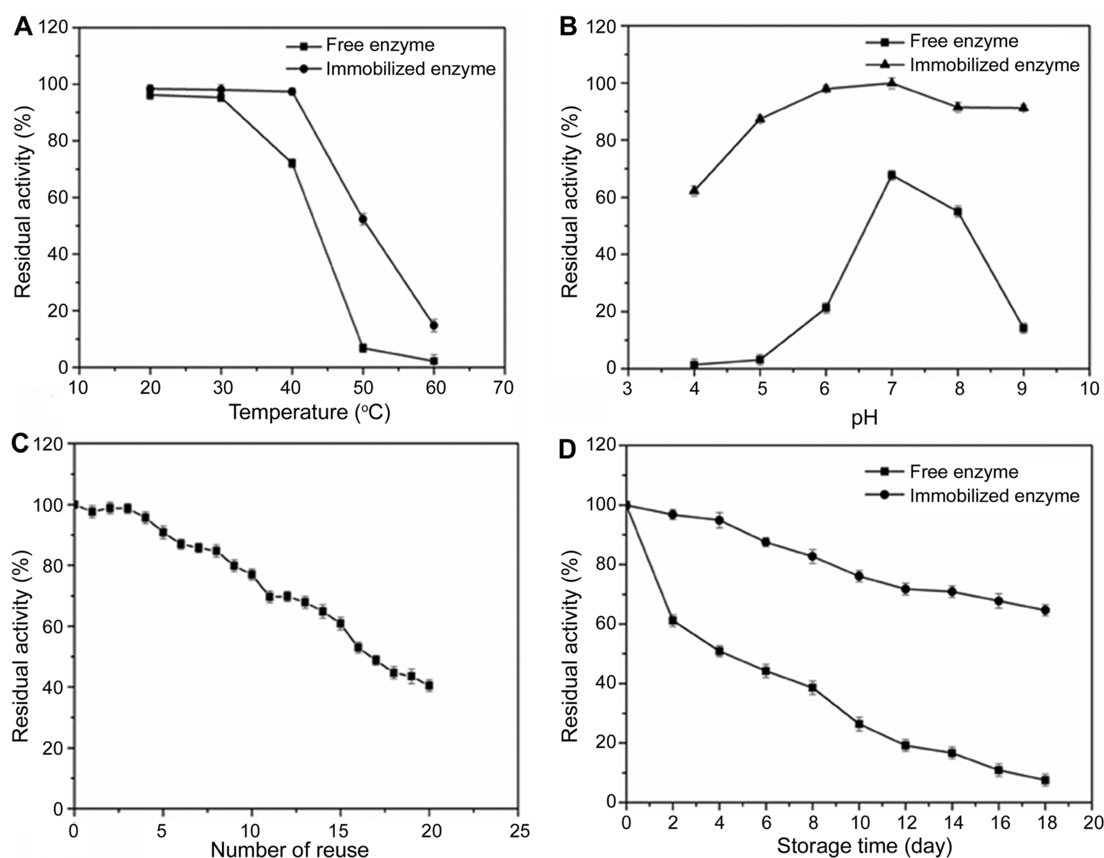


Fig. 6. Thermal (**A**), pH (**B**), reusability (**C**), and storage (**D**) stabilities of free and immobilized cholesterol oxidases.
Residual activity was calculated by using the highest activity of free and immobilized enzyme as 100%, respectively. Data represent the average ± standard deviation of three experiments.

original activity, respectively, whereas the free enzyme only retained about 1.38% and 14.22%, respectively. After immobilization, the resistance of cholesterol oxidase to pH was strengthened. This was because, upon immobilization, the active site became more exposed [11]. The immobilization clearly improved the thermal and pH stabilities of the cholesterol oxidase.

Reusability is a crucial feature of immobilized enzyme preparations in most practical applications. To understand the reusability of the immobilized enzyme, the enzyme activity of the particles was determined after repeated use. The activity of immobilized enzyme was determined after each of the 20 repeated recoveries and uses. As shown in Fig. 6C, the activity of immobilized enzyme decreased with the increase of the number of reuses. After 20 consecutive operations, the immobilized enzyme retained 40.43% of its initial activity. The gradual yet slight decrease in enzyme activity could be attributed to the denaturation and/or leakage of cholesterol oxidase from the supports [7]. With this excellent reusability and ease of recovery, the immobilized cholesterol oxidase would be of great use in industrial applications.

To characterize the storage stability of the enzyme for preparative or industrial uses is meaningful [19]. The storage stability of the immobilized enzyme prepared under the optimum conditions was also investigated. The free and immobilized enzymes in 20 mM phosphate buffers of pH 7.5 were stored at 4°C for 20 days, and the activities were determined. As shown in Fig. 6D, the free enzyme lost about 50% of its original activity within 4 days, whereas the immobilized enzyme only lost about 6.12% during the same period. In addition, the free enzyme lost all its activity within 20 days, whereas the immobilized enzyme was protected to about 62.87% under the same storage time. The decrease in activity was explained as a time-dependent natural loss in enzyme activity, and this was prevented to a significant degree by immobilization. It had been argued that the high storage stability of an immobilized enzyme was due to its fixation on the support, preventing autodegradation and thermal inactivity. These results are also in agreement with the literature [4, 20, 22].

To conclude, in this study, chemically modified Sepharose particles were prepared successfully and used for the covalent immobilization of cholesterol oxidase on the surface. Under these optimal conditions, the enzyme activity was found to be 12.01 U/g Sepharose-ethylenediamine. Cholesterol oxidase binding has been confirmed by FTIR analysis. From the FTIR spectra, the immobilization of cholesterol oxidase to Sepharose-NH_2 was confirmed. The immobilized enzyme exhibited the maximal activity at pH 7.5 and 35°C, respectively. Moreover, the immobilized enzyme exhibited remarkably improved stability properties to various parameters, such as pH, temperature, reuse, and storage time. The immobilized enzyme retained almost its full activity at the point of pH 6.0 and 7.0 after 60 min at 37°C. In addition, the immobilized enzyme showed 52.32% of the initial activity at 50°C, but the free enzyme exhibited only 6.83% at the same temperature. The great advantage of the immobilized enzyme was that it could be reused for more than 20 times while retaining 40.43% of its original activity. After the period of 20 days at 4°C, the free enzyme lost all its initial activity, whereas the immobilized enzyme was protected to about 62.87%. The stabilities of the immobilized enzyme were attributable to changing conformation and prevention of autodegradation.

Our past study focused on a method to immobilize cholesterol oxidase onto Sepharose particles by a reaction between amino groups of cholesterol oxidase and carbodiimide-activated carboxyl groups on Sepharose particles [2]. However, this research described an immobilization method by a reaction between carbodiimide-activated carboxyl groups of cholesterol oxidase and amino groups on Sepharose particles. The enzyme immobilized on a carboxyl-support resulted in better stability than that of amino-support-immobilized enzyme, but the method of preparation of the modified Sepharose particles was much more complicated. Taking the above results into consideration, Sepharose has been proven to be an efficient support for cholesterol oxidase immobilization.

Acknowledgments

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