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Immobilization and stabilization of cholesterol oxidase on modified sepharose particles

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ABSTRACT

For the cholesterol oxidase from *Brevibacterium* sp. M201008 was not stable as free enzyme form, it had been covalently immobilized onto functionalized sepharose particles that were activated with N-ethyl-N′-3-dimethylaminopropyl carbodiimide (EDC).The optimal conditions of enzyme immobilization were determined, and the immobilized enzyme activity was 18.03 U/g of support. The surface morphology and thermal behavior of the immobilized enzyme were observed by scanning electron microscopy and differential scanning calorimetry, respectively. The binding of the enzyme to support was confirmed using Fourier transform infrared spectroscopy. The results demonstrated that the thermal, pH, and storage stabilities of cholesterol oxidase were increased by immobilization. More than 90% of the initial activity of the immobilized enzyme was remained at the points of pH 4.0 and 9.0, and that was much more stable than it was free enzyme form. Under same storage conditions, the free enzyme lost 97.8% of its initial activity after 45 h, whereas the immobilized enzyme only lost its 35.8% activity.

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1. Introduction

Cholesterol oxidase (3β-hydroxysterol oxidase, EC1.1.3.6) from the bacterial is a specific flavoenzyme involved in the initial step of cholesterol catabolism. It catalyzes the oxidation and isomerization of cholesterol containing a 3β hydroxyl group to 4-cholesten-3-one. This enzyme is a member of a large family of flavin oxidoreductases and is found in two different forms: one where the flavin adenine dinucleotide (FAD) cofactor is covalently linked to the enzyme and one where the cofactor is non-covalently bound to the enzyme. It is secreted by a number of microorganisms [1–4] that are capable of utilizing cholesterol as their sole source of carbon and energy. The enzyme has potent insecticidal activity in agriculture, in addition to a number of important industrial and commercial applications, such as the determination of cholesterol concentrations in serum and other clinical samples in a coupled system with cholesterol esterase and peroxidase [5,6].

For the cholesterol oxidase from *Brevibacterium* sp. M201008 was not stable as free enzyme form, we hoped to improve stability via immobilization. Different methods had been proposed to immobilize enzyme: adsorption, covalent binding, polymer entrapment, and cross-linking [7]. Among them, covalent attachment was the most effective in improving enzyme stabilization. Numerous support materials for the covalent immobilization of cholesterol oxidase were available, including magnetic nanoparticles, hollow fiber dialyzers, perlite, silk mats, polyaniline films, and so on [8–13]. Gilles et al. directly immobilized cholesterol oxidase onto Fe_3O_4 magnetic nanoparticles [8]. The bound enzyme exhibited a better tolerance to pH, temperature and substrate concentration. The polyacrylonitrile hollow fibers were covalently bonded with cholesterol oxidase via glutaraldehyde by Lin and Yang [9].In addition, cholesterol oxidase was covalently immobilized onto the woven silk mat via using N-ethyl-N′-3-dimethylaminopropyl carbodiimide (EDC) by Saxena and Goswami [11]. Sepharose was a spherical mesh polysaccharide polymer extracted from seaweed. It, combined with some forms of activation chemistry, was also used to immobilize enzymes, proteins and peptides through covalent attachment. Overall, sepharose became an appropriate support material that reduced surface area for enzyme binding and pore-diffusion resistance.

To covalently immobilize cholesterol oxidase to sepharose, a simple immobilization method is the use of carbodiimide chemistry. The water-soluble N-ethyl-N′-3-dimethylaminopropyl carbodiimide (EDC) is a popular reagent for activating carboxyl groups for reaction with other amine-containing molecules [14]. The chemical reactions of the covalent immobilization are summarized in Fig. 1. EDC first reacts with the carboxyl group of the support to form an amine-reactive o-acylisourea intermediate. It reacts with the amine group of the enzyme surface to produce a stable isopeptide bond. In the reaction, EDC does not result in the incorporation of crosslinking agent. If the stable covalent linkages are produced, this method can claim the advantage of precluding depolymerization and release of residual reagent [15].

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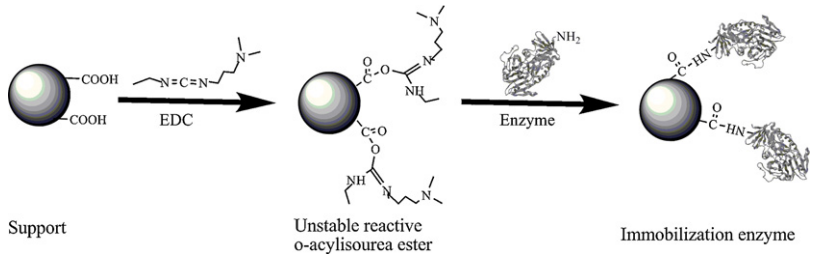

Fig. 1. Schematic of enzyme immobilization on support by cross-linking with N-ethyl-N′-3-dimethylaminopropyl carbodiimide (EDC).

In this work, cholesterol oxidase was immobilized onto the modified sepharose by water-soluble carbodiimide (EDC). An investigation was carried out to determine the conditions for the immobilization and the characteristics of the immobilized enzyme. The properties of the immobilized enzyme, including enzyme activity as well as thermal, pH and storage stabilities, were examined. The results were compared with those for the free enzyme. We hoped the results of this work could not only further improve the stability performance but also expand the clinical diagnostic application of cholesterol oxidase.

2. Methods

2.1. Materials

N-ethyl-N′-3-dimethylaminopropyl carbodiimide (EDC) was supplied by Sigma. Sepharose CL 4B obtained from Pharmacia Biotech, Sweden. Cholesterol oxidase from *Brevibacterium* sp. M201008 was obtained as described below. All other chemicals and reagents used were of analytical grade and sourced from local companies.

2.2. Expression and purification of cholesterol oxidase gene in *E. coli*

Cholesterol oxidase 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). Then the culture was transferred into 100 ml of the LB medium with the same antibiotic concentration as above and cultivated at 37 °C until the OD_{600} reached 2. After that it was induced with 10 g/l lactose for 10 h at 28 °C. The cells were harvested via centrifugation at 6000 × *g* for 20 min at 4 °C and were re-suspended in 20 mM sodium phosphate (pH 7.5). The cells were disrupted by sonication and centrifuged at 10,000 × *g* for 30 min at 4 °C.

The supernatant enzyme was then purified according to our previous work [16,17]. The supernatant after centrifugation was loaded onto the equilibrated affinity column and washed with 20 mM sodium phosphate (pH 7.5) then washed with 20 mM sodium phosphate/0.02 M NaCl (pH 7.5), finally eluted with 20 mM sodium phosphate/0.3 M NaCl (pH 7.5).

2.3. Accessible basic amino acids on the surface of cholesterol oxidase

The gene from *Brevibacterium* sp. M201008 had been cloned, sequenced and expressed in *E. coli* [18]. The cholesterol oxidase gene consisted of 1653 base pairs and encoded 551 amino acid residues. Accessible basic amino acids on the surface of cholesterol oxidase were analyzed by Discovery Studio 2.5 software, and the numbers of basic amino acids available on cholesterol oxidase surface were calculated.

2.4. Preparation and choice of different immobilization supports

Sepharose CL 4B (100 g) was washed with water and suspended in 50 ml activating solution (1 M NaOH, 2.5 g sodiumborohydride, and 10 ml epichlorohydrin). The mixture tumbled for 2 h at 60 °C. Then the activated gel was washed with water until the pH of eluate was 7.0. After 100 g sepharose CL 4B was activated by epichlorohydrin, the activated sepharose CL 4B was suspended in 350 ml water, and 150 ml 35% ammonia was added. The gel was incubated for 12 h at 30 °C on a rotary shaker, after which it was washed with water. Aminated sepharose CL 4B was suspended in 350 ml 50% (v/v) acetone solution, maintained in an ice bath and 8 g cyanuric chloride dissolved in 70 ml acetone was added over a period of 2 h with shaking. The gel was reacted with a two fold molar excess of Asp, Glu, Ser and Pro at pH 7.0 for 12 h [19]. The support-Asp, -Glu, -Ser, and -Pro were washed with distilled water to remove the excess of Asp, Glu, Ser and Pro not bound to the support.

Estimation of the density of carboxy groups on the support was performed in the following manner: 1 g of washed support was suspended in 10 ml distilled water. The suspension was then titrated to pH 7.00 by the addition of 0.1 M NaOH. The carboxy group density in μmol/g support is equal to the number of μmol base required for the titration. Support-Asp and -Glu had carboxyl group density in the 40 μmol/g, and support-Ser and -Pro had 20 μmol/g.

Four different supports (1.0 g) were dissolved in 10 mL of 20 mM phosphate buffer solution, respectively. EDC and cholesterol oxidase (1:25 ratio of enzyme-NH_2 to support —COOH, pH 7.0 and 1:10 ratio of carboxyl group to EDC) were added. After being stirred gently at 4 °C for 16 h, the four different precipitates were collected by filtration, and non-covalently adsorbed enzyme molecules were removed by washing with 0.6 M NaCl. Four different immobilized enzymes were performed by assaying their directly activity after immobilization and residual activity at 37 °C after 24 h.

2.5. Optimization of the reaction conditions of the immobilized enzyme

The most efficient conditions of cholesterol oxidase immobilization on support-Asp were investigated. The factors were immobilization pH (5.0, 5.5, 6.0, 6.5, 7.0, and 7.5), molar ratio of enzyme-NH_2 to support-Asp (1:200, 1:80, 1:40, 1:20, 1:10, and 1:5), and molar ratio of carboxyl group to EDC (1:1, 1:2, 1:5, 1:10, 1:20, and 1:40) for 16 h at 4 °C. Support-Asp (1.0 g) was dissolved in 10 mL of 20 mM phosphate buffer solution. Different amounts of EDC and cholesterol oxidase were added to the above solution. After being stirred gently at 4 °C for 16 h, the resulting precipitate was collected as the immobilized enzyme by filtration, and non-covalently adsorbed enzyme molecules were removed by thorough washing of the precipitate with 0.6 M NaCl. The supernatant was collected to determine the activity of cholesterol oxidase.