One-step purification of cholesterol oxidase from culture broth of a Pseudomonas sp. using a novel affinity chromatography method

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Cholesterol oxidase from the culture broth of a Pseudomonas sp. was purified with a yield of more than 70% by a one-step procedure using a column of cholesterylglucine-carboxymethylcellulose; active enzyme was eluted by Triton X-100. The purified enzyme was homogeneous by SDS-PAGE.

Introduction

Cholesterol oxidase (EC 1.1.3.6) catalyses the oxidation of cholesterol (5-cholest-3-en-2-one) to 4-cholestene-3-one with the reduction of oxygen to hydrogen peroxide. This enzyme has been widely used for determination of cholesterol in clinical specimens by coupling with peroxidase (Richmond, 1973; Allain et al., 1974). We have previously described a Pseudomonas sp. that produces extracellular cholesterol oxidase (Lee et al., 1989). In this report we describe the synthesis of a novel cholesterol affinity adsorbent and a very simple purification procedure that gives a high yield of homogeneous cholesterol oxidase from culture broth of this Pseudomonas sp. This procedure eliminates laborious steps in the conventional purification procedures.

Methods

Organism and growth. The cholesterol-oxidase-producing microorganism Pseudomonas sp. COX629 was grown under the conditions described previously (Lee et al., 1989).

Synthesis of cholesterylglucine. The following procedure was used. A mixture of Cbz-glycine (2.1 g) and cholesterol (3.8 g) in chloroform (50 ml) was cooled to 0°C; dicyclohexylcarbodiimide (2.1 g) was then added and the mixture was stirred at 0°C. After 5 h, the solvent was evaporated under reduced pressure and the residue was dissolved in ethyl acetate. After filtration, the filtrate was successively washed with 500 ml ethyl acetate, 500 ml chloroform and 500 ml ethanol. About 10 pmol cholesterylglucine was bound per g of adsorbent as shown by alkaline hydrolysis (Fukuyama & Miyake, 1979).

Purification of cholesterol oxidase. Culture broth (500 ml) of the Pseudomonas sp. was centrifuged at 16000 g for 10 min and the clear supernatant obtained was used for purification of the enzyme. The supernatant was directly loaded on a column (2.5 x 16 cm) of cholesterylglucine-CM-cellulose, prepared as described above, previously equilibrated with 10 mM-potassium phosphate buffer (KPB; pH 7.5) at a flow rate of 1 ml min⁻¹. After washing the column with 200 ml KPB and 300 ml KPB containing 500 mM-KCl, cholesterol oxidase was eluted with a linear gradient of Triton X-100 (0-0.1% v/v, 600 ml).

Analytical methods. Protein was determined by the Lowry method. Cholesterol oxidase activity was assayed by the method of Allain et al. (1974): 1 unit of cholesterol oxidase activity was defined as the amount of enzyme that catalysed the formation of 1 μmol H₂O₂ min⁻¹ at 37°C in the presence of SDS was done by the method of Laemmli (1970).

Chemicals. Benzoylcarbonyl(Cbz)-glycine, cholesterol, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, dicyclohexylcarbodiimide, CM-cellulose, chloroform and dimethylformamide were purchased from Sigma.

Results and Discussion

Fig. 1 represents a typical elution profile from a cholesterylglucine-CM-cellulose column, showing protein concentration and cholesterol oxidase activity. Only
a very small amount of activity was detected in the flow-through fractions. After eluting with a linear gradient of Triton X-100, cholesterol oxidase from the Pseudomonas sp. culture broth was purified approximately 2600-fold with a yield of more than 70%. The purified enzyme was homogeneous by SDS-PAGE, and had the same properties as those of the enzyme purified by a conventional procedure (data not shown). By using a 78.5 ml cholesterylglycine-CM-cellulose column, about 500 ml of the culture broth can be treated. The column can be used at least twenty times without any significant loss of adsorbing activity. In coupling cholesterylglcine and CM-cellulose, glycine and the carboxymethyl group are condensed by peptide bond formation and play the role of spacer between matrix and ligand. In this system, carboxymethylglycine offers an effective spacer arm six atoms in length.

Various methods have been reported for purifying cholesterol oxidase from different species, based on enzyme-substrate specificity (Richmond, 1973; Uwajima et al., 1973; Tomioka et al., 1976; Kamei et al., 1978; Fukuyama & Miyake, 1979; Inoue et al., 1982). Richmond (1973), Kamei et al. (1978) and Inoue et al. (1982) reported the purification of cholesterol oxidase by cholesterol affinity chromatography. In their methods, a cholesterol slurry was directly packed into a column. These methods seem not to be appropriate to large-scale purification, since binding capacity was low and solubility of the adsorbent in detergent was high. Thus it is not easy to handle and preparation of the adsorbent is expensive. Fukuyama & Miyake (1979) purified cholesterol oxidase by affinity chromatography using 3-O-succinylcholesterol ethylenediamine-Sepharose gels. However, the main problem in large-scale purification systems is the compressibility of the matrix materials.

Affinity chromatography can provide the most elegant method for the purification of a protein from a complex mixture. However, affinity chromatography has not generally been used for large- or industrial-scale enzyme purification because in most cases matrices are expensive, have low capacity and frequently tend to be unstable. The novel affinity absorbent described here is advantageous in three respects: (i) CM-cellulose is cheaper even than agarose beads, and therefore financial costs are less for a large-scale enzyme purification process; (ii) the characteristic fast flow rate of cellulose solves the flow rate problems that occur upon scale-up; and (iii) it is stable. This purification procedure, therefore, provides a large amount of cholesterol oxidase with a great economy in labour required for purification.

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References


