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

HAE-IK RHEE,¹* KYUNG-JIN JEONG,² BOO-KIL PARK,² YONG-SOON CHOI¹ and SANG-YOUNG LEE²

¹Department of Applied Biology and Technology and ²Department of Food Science and Technology, Kangwon National University, Chuncheon 200-701, Republic of Korea

(Received 24 September 1990; revised 31 January 1991; accepted 13 February 1991)

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 cholesteryl-glycine-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-cholesten-3 β -ol) to 4-cholesten-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 micro-organism *Pseudomonas* sp. COX629 was grown under the conditions described previously (Lee *et al.*, 1989).

Synthesis of cholesteryl-glycine. 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 1 M-HCl, saturated NaHCO₃ solution and saturated NaCl solution, and dried over Na₂SO₄. It was then purified by preparative silica gel thin layer chromatography using n-hexane/ethyl acetate (2:1, v/v) as the solvent. For deprotection of the benzyloxycarbonyl group, the product was dissolved in ethyl acetate and hydrogenated in the presence of palladium charcoal at room temperature for 3 h. After

filtration, the solvent was removed under reduced pressure. Cholesteryl-glycine was prepared as a syrup, and used for the next step without further purification. About 500 mg cholesteryl-glycine was added to 250 ml dimethylformamide and mixed with 15 g of CM-cellulose. The pH of this suspension was adjusted to 4.6 with 2 M-HCl. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (5 g) in 5 ml of water was added to the suspension and the reaction was allowed to proceed at room temperature overnight. Then the substituted cellulose was successively washed with 500 ml ethyl acetate, 500 ml chloroform and 500 ml ethanol. About 10 μ mol cholesteryl-glycine 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 onto a column (2.5 $\times$ 16 cm) of cholesteryl-glycine-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%, 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 μ mol H₂O₂ min⁻¹ at 37 °C. PAGE in the presence of SDS was done by the method of Laemmli (1970).

Chemicals. Benzyloxycarbonyl(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 cholesteryl-glycine-CM-cellulose column, showing protein concentration and cholesterol oxidase activity. Only

Abbreviation: KPB, potassium phosphate buffer.

Fig. 1. Elution profile from the cholesterol oxidase affinity column. ●, Cholesterol oxidase activity; ○, protein. At the point indicated by the arrow, the column was washed with 200 ml KPB (I) followed by 300 ml KPB + 500 mM-KCl (II); then the enzyme was eluted with a linear gradient of Triton X-100 (III; 0–0.1%, 600 ml). (For I, II and III the flow rate was 1 ml min⁻¹ and 10 ml fractions were collected.)

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 cholesteryl-glycine-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 cholesteryl-glycine 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, carboxymethyl-glycine 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 adsorbent 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.

The authors thanks J. Hiratake, the Chemical Institute, Kyoto University, for helpful discussions and advice during the organic synthesis. This work was supported in part by a Grant-in-Aid for Genetic Engineering Research from the Ministry of Education of Korea.

References

- ALLAIN, C. C., POON, L. S., CHAN, C. S. G., RICHMOND, W. & FU, P. C. (1974). Enzymatic determination of total serum cholesterol. *Clinical Chemistry* **20**, 470–475.
- FUKUYAMA, M. & MIYAKE, Y. (1979). Purification and some properties of cholesterol oxidase from *Schizophyllum commune* with covalently bound flavin. *Journal of Biochemistry* **85**, 1183–1193.
- INOUE, Y., TAGUCHI, K., FUJII, A., ISHIMARU, K., NAKAMURA, S. & NOMI, R. (1982). Purification and characterization of extracellular 3 β -hydroxy steroid oxidase produced by *Streptomyces violaceus*. *Chemical Pharmacology Bulletin* **30**, 951–958.
- KAMEI, T., TAKIGUCHI, Y., SUZUKI, H., MATSUZAKI, M. & NAKAMURA, S. (1978). Purification of 3 β -hydroxysteroid oxidase of *Streptomyces violaceus* origin by affinity chromatography on cholesterol. *Chemical Pharmacology Bulletin* **26**, 2799–2804.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- LEE, S. Y., RHEE, H. I., TAE, W. C., SHIN, J. C. & PARK, B. K. (1989). Purification and characterization of cholesterol oxidase from *Pseudomonas* sp. and taxonomic study of the strain. *Applied Microbiology and Biotechnology* **31**, 542–546.
- RICHMOND, W. (1973). Preparation and properties of bacterial cholesterol oxidase from *Nocardia* sp. and its application to the enzymatic assay of total cholesterol in serum. *Clinical Chemistry* **19**, 1350–1356.
- TOMIOKA, H., KAGAWA, M. & NAKAMURA, S. (1976). Some enzymatic properties of 3 β -hydroxysteroid oxidase produced by *Streptomyces violaceus*. *Journal of Biochemistry* **79**, 903–915.
- UWAJIMA, T., YAGI, H., NAKAMURA, S. & TERADA, O. (1973). Isolation and crystallization of extracellular 3 β -hydroxysteroid oxidase of *Brevibacterium sterolicum* nov. sp. *Agricultural and Biological Chemistry* **37**, 2345–2350.