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Purification and Characterization of Extra-Cellular Cholesterol Oxidase From *Rhodococcus* sp. PTCC 1633

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Abstract: In this study to isolate cholesterol oxidase (Cho) producing microorganism, a bacterium with high ability to produce Cho was found. It was identified with morphological, biochemical and molecular methods as a new species of *Rhodococcus*. *Rhodococcus* sp. PTCC 1633 has both extra-cellular and intracellular Cho. It was cultured in optimized condition and extra-cellular Cho concentrated by Three Phase Partitioning (TPP) with a recovery of 85%. The concentrated enzyme was purified by one step ion exchange chromatography. On SDS-PAGE the purified enzyme showed a molecular weight of about 55 kDa. The enzyme was active in a wide rang of pH and temperature with an optimum pH and temperature of 7.0-7.5 and 40°C, respectively, for activity. The K_m value for this enzyme was 15 μM. A thermal stability experiment showed high stability at 40°C in 24 h.

Key words: *Rhodococcus* sp., extra-cellular, cholesterol oxidase, purification, three phase partitioning

INTRODUCTION

Cholesterol oxidase (EC 1.1.3.6) catalyses the transformation of cholesterol into 4-cholestene-3-one (Lee *et al*., 1997). This reaction is the first step in the microbial degradation of cholesterol and cholesterol derivative compounds (Sojo *et al*., 1997). Cholesterol oxidases (Cho) are widely used in the quantitative determination of serum and food cholesterol (Richmond, 1973) and have also been used in the production of precursors for the chemical synthesis of pharmaceutical steroids (Sojo *et al*., 1997; Watanabe *et al*., 1989), for degradation and decrease of dietary cholesterol (Kaunitz, 1978) and also as a potent insecticidal protein against boll weevil larvae (Purcell *et al*., 1993).

Due to potential industrial and clinical applications of Cho, screening to find new sources of extra cellular enzyme can be very important (MacLachlan *et al*., 2000). Many microorganisms with Cho production ability have been identified, including *Nocardia* sp. (Richmond, 1973), *Brevibacterium sterolicum* (Fujishiro *et al*., 2002), *Schizophyllum commune* (Sojo *et al*., 1997; Fukuya and Miyake, 1979) and *Streptoverticillum cholesterolicum* (Inouye *et al*., 1982). Recently, several species of *Rhodococcus* have also been reported for ability to produce Cho (Lee *et al*., 1997; Watanabe *et al*., 1989; Chou *et al*., 1999; Kreit *et al*., 1992; Tabatabaei *et al*., 2001a). Cho production in *Rhodococcus* strains has been shown to be primarily of the membrane-bound type, but some strains produce both extra-cellular and membrane-bound types (Sojo *et al*., 1997; Watanabe *et al*., 1989; Kreit *et al*., 1994).

Rhodococcus sp. PTCC 1633 was isolated in a screening program in our laboratory. This strain showed a high ability for the production of an extra-cellular and membrane-bound type of Cho in a relatively short period of time (Tabatabaei *et al*., 2001a). This study describes the results of concentration by TPP, one step purification and characterization of the purified extra-cellular Cho produced by this strain.

MATERIALS AND METHODS

Microorganism and cultivation: *Rhodococcus* sp. PTCC 1633 was identified in our laboratory in a screening program for isolating a high producer of extra-cellular Cho (Tabatabaei *et al*., 2001a). Enzyme production was carried out in an optimized medium consisting of yeast extract

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0.3 g, $(NH_4)_2HPO_4$ 0.1 g, cholesterol 0.15 g, Tween 80 0.05 mL, distilled water 100 mL, with a pH adjusted to 7.0. The media was cultured at 30°C for 24 h (Tabatabaei *et al*., 2001a). After 24 h of incubation the culture broth was centrifuged at 10000 rpm for 10 min at 4°C. The supernatant was removed and used as the source of the extra-cellular enzyme.

Phylogenetic analyses of isolated strain: Identification of the microorganism was performed by microbiological and biochemical properties (Tabatabaei *et al*., 2001a) and phylogenetic analysis. A partial sequence of the 16S rRNA gene was amplified by PCR using primers (5'-GCA CAA GCG GTG GAG CAT GT-3' and 5'-GCC CGG GAA CGT ATT CAC C-3'), which were designed based on universally conserved sequences (Ji *et al*., 2004).

Measurement of enzyme activity: To determine cholesterol oxidase activity, a modified method of the Sasaki *et al*. (1982) was used. In this method, the absorbance of quinoneimine dye formed by coupling with 4-aminoantipyrine, phenol and peroxidase was measured at 500 nm by spectrophotometry. One unit of activity was defined as the formation of 1 μmol of hydrogen peroxide (0.5 μmol of quinoneimine dye) per minute at 37°C and with a pH of 7.0. In some experiments 4-cholesten-3-one, which is the product of isomerization from 5-cholesten-3-one, was measured at 240 nm by spectrophotometry (Gadda *et al*., 1997). One unit of activity was defined as the formation of 1.0 μmol of 4-cholesten-3-one per minute at 37°C and a pH of 7.0. In purification procedure, activity was also estimated by directly reading the absorbance of fractions at 380 and 460 nm (Isobe *et al*., 2003).

Protein measurement: Protein concentration was measured by the method of Bradford (1979) using bovine serum albumin as a standard. Protein estimation during purification steps was carried out by reading the absorption at 280 nm.

Concentration and purification methods: Concentration of the Cho was accomplished by TPP (Roy *et al*., 2005). The supernatant (20 mL) was mixed with various amounts of ammonium sulfate (W/V) and t-butanol (V/V). The mixture was vortexed and incubated at 2-6°C for 5 min, then centrifuged at 10000 g for 5 min. The midlayer, concentrated proteins was collected and dissolved in a minimal volume of 0.1 M (pH 7.0) of phosphate buffer, followed by extensive dialysis against four changes of 1 L of the same buffer. The dialysate was subjected to chromatography on a DEAE-sepharose column (1×20 cm) previously equilibrated with a 0.1 M phosphate buffer of pH 7.0. For eluting the protein, two buffers were used. Buffer A (phosphate buffer, pH 7.0, 0.1 M) and Buffer B (buffer A + 0.5M NaCl). In the first step a liner gradient of 0-100% Buffer B was used in 10 cv. Then column was washed with Buffer A and protein eluting was continued by another gradient from 0-100% Buffer B in 10 cv with the same Buffers, both containing 0.025% Triton X-100. The absorbance at 380 and 460 nm was monitored as an indicator of the presence of a flavoprotein during the purification steps.

Estimation of purity and molecular weight: To analyze the purity and also to estimate the molecular weight of the purified enzyme gel electrophoresis was used in a 10% denaturing polyacrylamide gel (SDS-PAGE) and stained with silver nitrate according to the method of Hames and Richwood (1998). A low molecular weight size marker (Pharmacia) was used to determine the molecular weight of proteins.

RESULTS

Identification of isolated strain: On the basis of microbiological and biochemical characteristics, this strain belonged to the genous *Rhodococcus* (Tabatabaei *et al*., 2001a). In this study a partial 16S rRNA gene sequence was found. It showed 100 % homology to the 16S rDNA sequence of *Rhodococcus* sp. OUCZ16, *Rhodoccocus* sp. equi and *Rhodococcus* sp. MI-11a. The nucleotide sequence of 16S rRNA gene of *Rhodococcus* sp. PTCC 1633 was deposited in the GeneBank under accession number EF117892.

Optimization of TPP for the concentration of Cho: The concentration of Cho was carried out in the presence of different concentrations of ammonium sulfate and in various amounts of t-butanol. The maximum recovery of 85% Cho was obtained with 40% (W/V) ammonium sulfate and the ratio of 1:1 of t-butanol: supernatant.

Purification of Cho: The concentrated proteins were dissolved in a 0.1 M phosphate buffer, pH 7.0 and dialyze against the same buffer and then loaded on DEAE-sepharose. To remove unbound protein, the column was washed with 2 CV of a starting buffer and then the first step of the gradient was used. Fourteen fractions (10 mL) were collected in the first step, but only 20% of enzymatic activity was found in the fractions (5-10) (Fig. 1a). So the elution was continued by the 2nd step of the gradient. Another 14 fractions (15-28) were collected. Five fractions (17-21) were found to have about 70% total Cho activity (Fig. 1b). To evaluate the purity of active fractions, they

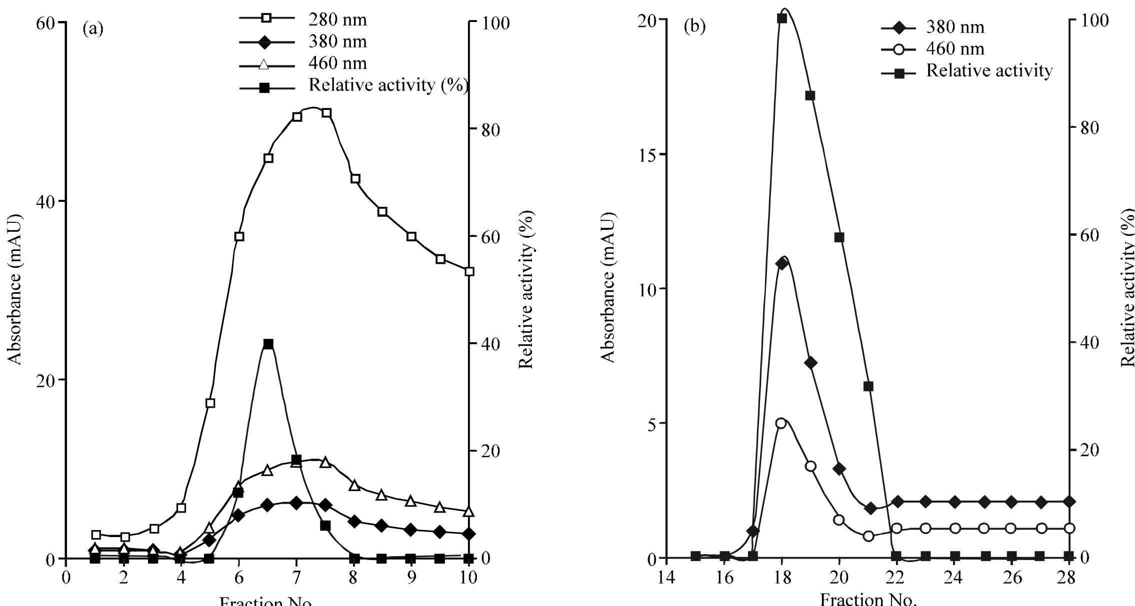

Fig. 1: Purification of Cho by one-step ion-exchange chromatography. (a) First step of gradient and (b) Second step of gradient, *Absorbance at 280 nm is not shown, because triton X-100 has strong absorbance at this region and obscure the 280 nm absorbance

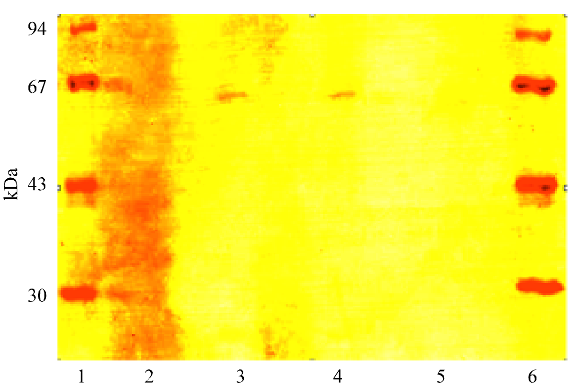

Fig. 2: SDS-PAGE of the purified Cho. Lane 1 and 6, standard low molecular weight size markers. Lane 2-5, fractions 17-21

were subjected to SDS-PAGE on a 10% gel slab. A single protein band with a molecular weight of about 55 kDa was observed in fractions 18 and 19 (Fig. 2). They were pooled and used as a pure enzyme for characterization.

Enzyme characterization: The activity of the purified Cho upon cholesterol, β-sitosterol, stigmasterol, Cholic acid is shown in Table 1. Among these steroid substrates, the enzyme had the highest activity on cholesterol. β-sitosterol and stigmasterol were also oxidized, but their reactions were slower than that of cholesterol, 63, 18%, respectively compared to cholesterol. Cholic acid was not

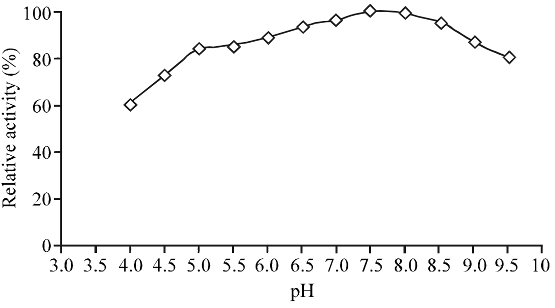

Fig. 3: Effect of pH on Cho. Enzyme activity was assayed under standard conditions except for changing pH of the buffer. Acetate buffer was used for pH 4.5 to 5.5, Phosphate buffer for pH 6.0-7.5 and Tris- HCl buffer for pH 8.0-9.0

Table 1: Substrate specificity of cholesterol oxidase

Substrate	Relative activity (%)
Cholesterol	100
β-sitosterol	63
Stigmasterol	18
Cholic acid	0
Progesterone[A]	0
4-Androsten-3,17-dione(AD)[A]	0
Cholesterol+Progesterone	100
Cholesterol+AD	100

[A]This compound is not the substrate of cholesterol oxidase

oxidized at all. The effect of pH and temperature on enzyme activity showed that Cho has an optimum pH of 7.5 and relatively high activity in a broad pH range of 5.0-9.5 (Fig. 3). The enzyme had relatively good activity

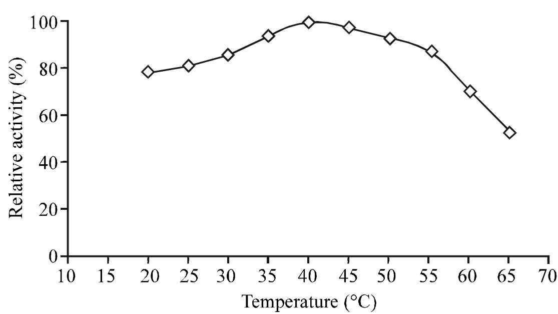

Fig. 4: Effect of temperature on Cho activity. Enzyme activity was assayed under standard assay condition except for the reaction temperature

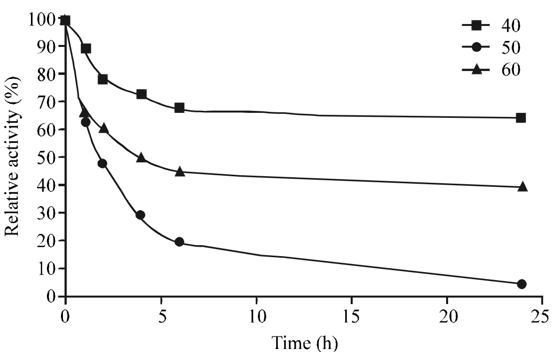

Fig. 5: Effect of temperature on stability of Cho. The enzyme solution was incubated at 40, 50 and 60°C for 24 h. The activity was assayed under standard condition

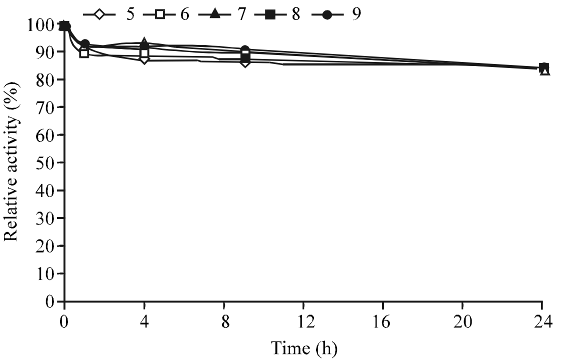

Fig. 6: Effect of pH on stability of Cho. The enzyme solution was incubated at pH 5.0, 6.0, 7.0, 8.0 and 9.0 for 24 h. The activity was assayed under standard condition. Acetate buffer was used for pH 5.0, Phosphate buffer for pH 6.0 and 7.0 and Tris-HCl buffer for pH 8.0 and 9.0

between 25 and 55°C with its optimums at 40°C. At higher temperatures, activity decreased sharply (Fig. 4). Thermal stability of the enzyme was evaluated at pH 7.0 for 24 h.

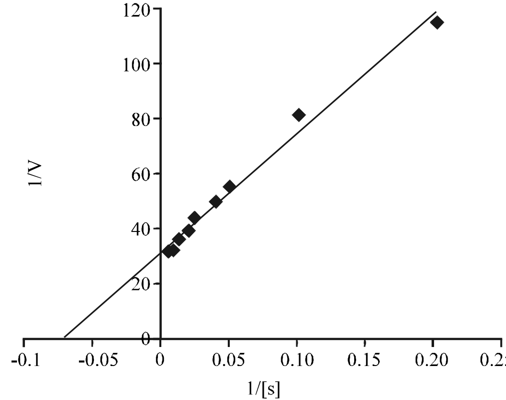

Fig. 7: Lineweaver-Bruk plot for Cho

Results showed that Cho retains its full activity for 1 h at 40°C. After 24 h 65 and 40% of activity remained at 40 and 50°C, respectively. Stability dropped sharply at higher temperatures and at 60°C only about 5% activity remained after 24 h (Fig. 5). The pH stability of purified enzyme was carried out at 2-6°C (Fig. 6). Cholesterol oxidase retained its full activity at pHs between 5.0-9.0 for 24 h. In fact, pH 5-9 had no effect on the stability of Cho at 4°C. To find the K_m of the purified enzyme, V was measured against various concentrations of cholesterol [s], until V reached to max V. To calculate more accurate K_m, 1/V was plotted against 1/[s]. From these data the K_m value was found to be 15 μM (Fig. 7).

DISCUSSION

Rhodococcus sp. PTCC 1633 was introduced as a high Cho producer strain in the earlier study (Tabatabaei *et al*., 2001a). This strain produces both extra- and intracellular forms of Cho. The extra-cellular form of the enzyme was concentrated by a TPP method. In some other reports for concentrating Cho, methods such as precipitation by an organic solvent and by ammonium sulfate have been introduced as efficient for enzyme concentration (Richmond, 1973; Inouye *et al*., 1982). However, these precipitation methods did not yield acceptable results for this enzyme. This may be due to some hydrophobic patches on the Cho molecule, which increase the solubility of the enzyme in organic solvents. This can also be presumed to cause the low efficacy of the ammonium salt precipitation method. However, the salt method has shown some good results in Cho concentration for some sources (Lee *et al*., 1997; Fujishiro *et al*., 2002). By TPP in an optimized condition, Cho can be concentrated by a recovery of 85% which is a higher recovery rate and can be done easily and does not need any specific equipment.

Cho was purified by ion-exchange chromatography on DEAE-sepharose. On DEAE only 20% of the enzyme was eluted by an NaCl-buffer solution and 80% of the enzyme was recovered by NaCl plus Triton X-100. The efficacy of Triton X-100 was four times more than the amount recovered by NaCl alone for eluting the enzyme. This might be due to the ability of Triton X-100 to form micelles with Cho molecules, so the enzyme desorbed easily from the DEAE cellulose. This property has been reported in several other investigations (Inouye *et al*., 1982; Kamei *et al*., 1978). The effect of micelle formation can also be seen in enzyme activity. In the presence of Triton X-100, the enzyme and cholesterol are brought very close together and the enzyme activity increases. On the other hand, the hydrophobic region of each enzyme molecule may be covered by detergent molecules, which would prevent enzyme-enzyme interactions. This is the main reason for increased enzyme aggregation and decreased enzyme activity. They used 0.1% Triton X-100 to elute the enzyme (Kreit *et al*., 1992, 1994). It has also been shown that Triton X-100 can solubilize a cell membrane bound enzyme (Tabatabaei *et al*., 2001a).

The purity of the enzyme was confirmed as a single band on an SDS-PAGE. The molecular weight of the purified Cho was estimated at 55 kDa. The molecular weight of Cho produced by different sources has a range of 30-61 kDa. It has been estimated at 55 and 56 kDa from *Rhodococcus erythropolis* (Sojo *et al*., 1997), 56 kDa from *Rhodococcus equi* No. 23 (Watanabe *et al*., 1989) and 60 kDa from *Streptomyces fradiae* (Tabatabaei *et al*., 2001b).

The pH optimum was found to be 7.5, which is similar to the optimal pH found for *Nocardia rodochrous* (Cheetham *et al*., 1982) and for *Rhodococcus equi* (Watanabe *et al*., 1989). Although Cho of *Rhodococcus* sp. PTCC 1633 has a broad range of pH stability (pH 5.0 to 9.5), but its range is narrower than that of *Streptomyces fradiae* which has a pH stability in the range of 4-10 (Tabatabaei *et al*., 2001b). This enzyme showed complete stability at 4°C in 96 h and relatively good thermal stability at 40 and 50°C; it was less stable than the Cho of *Streptomyces fradiae* which showed full activity at 50°C for 90 min (Tabatabaei *et al*., 2001b).

Progesterone and 4-androsten-3, 17-dione were not substrates for cholesterol oxidase. These substances could not also compete with cholesterol to bind to the active site of Cho. The cholesterol oxidase rapidly oxidized 3β-hydroxysteroids such as cholesterol and β-sitosterol, but had less effect on stigmasterol. The enzyme was inert toward 3β-hydroxysteroids such as cholic acid. These results confirm that the enzyme was specific for steroid substrates with one OH group in position β-C3 and that it showed a marked preference for steroids with a chain at position C17.

The results indicate, extra cellular Cho produced by *Rhodococcus* sp. PTCC 1633 can be purified with high recovery by TPP and one-step chromatography and due to appropriate properties can be considered for further investigation.

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