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ORIGINAL PAPER

Preparation and some properties of cholesterol oxidase from *Rhodococcus* sp. R_{14-2}

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Abstract *Rhodococcus* sp. R_{14-2}, isolated from Chinese Jinhua ham, produces a novel extracellular cholesterol oxidase (COX). The enzyme was extracted from fermentation broth and purified 53.1-fold based on specific activity. The purified enzyme shows a single polypeptide band on SDS-PAGE with an estimated molecular weight of about 60 kDa, and has a *pI* of 8.5. The first 10 amino acid residues of the NH_2-terminal sequence of the enzyme are A-P-P-V-A-S-C-R-Y-C, which differs from other known COXs. The enzyme is stable over a rather wide pH range of 4.0–10.0. The optimum pH and temperature of the COX are pH 7.0 and 50°C, respectively. The COX rapidly oxidizes 3β-hydroxysteroids such as cholesterol and phytosterols, but is inert toward 3α-hydroxysteroids. Thus, the presence of a 3β-hydroxyl group appears to be essential for substrate activity. The Michaelis constant (Km) for cholesterol is estimated at 55 μM; the COX activity was markedly inhibited by metal ions such as Hg^{2+} and Fe^{3+} and inhibitors such as *p*-chloromercuric benzoate, mercaptoethanol and fenpropimorph. Inhibition caused by *p*-chloromercuric benzoate, mercuric chloride, or silver nitrate was almost completely prevented by the addition of glutathione. These suggests that -SH groups may be involved in the catalytic activity of the present COX.

Keywords Cholesterol oxidase · *Rhodococcus* sp. · Purification · Specificity · NH_2-terminal sequence · Inhibitor

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Introduction

Cholesterol oxidase (EC1.1.3.6; COX) catalyses the first step of cholesterol degradation to 4-cholestene-3-one with the reduction of oxygen at C-3 to hydrogen peroxide. COX is one of the key enzymes in microbial sterol metabolism. The interest in this enzyme is due to its industrial importance and its wide utilization in the determination of cholesterol in blood serum and food (Flegg 1973; Parra et al. 2007), and to its assumed potential in the manufacture of diets with reduced cholesterol (Chenfeng et al. 2002). Indeed, dietary cholesterol degradation is considered as a means of protection against atherosclerosis. Other possible applications of COX are in the production of precursors for the chemical synthesis of pharmaceutical steroid hormones (Alexander et al. 1995), and for its insecticidal activity that is vital for pest control strategies employing transgenic crops (Shen et al. 1997). Moreover, an antiobesity effect was observed for 4-cholestene-3-one, the COX reaction product (Suzuki 1993). Due to these multiple and various applications as well as basic aspects, COXs merit thorough scientific investigation.

COX has been isolated from several microbial sources, including species of *Arthrobacter* (Wenhsiung et al. 1988),

Bacillus (Kim et al. 2002), *Brevibacterium* (Pornpen et al. 2006), *Nocardia* (Sojo et al. 1997), *Pseudomonas* (Isobe et al. 2003), *Rhodococcus* (Elalami et al. 1999), *Streptomyces* (Ishizaki et al. 1989; Pornpen et al. 2006) and *Schizophyllum* (Fukuyama et al. 1979). Genes encoding COX have been cloned from *Streptomyces* sp. (Ishizaki et al. 1989), *B. sterolicum* (Ohta et al. 2002) and *A. simplex* (Yenrong et al. 2006). A notable example involved the expression of *B. sterolicum* into *S. lividans*. The choB gene when expressed into *S. lividans* was shown by Ohta et al. (1991) to produce 185 times more active enzyme than the wild-type source.

Previously, we discovered that many fermented foods contain cholesterol-degrading bacteria (Chengtao et al. 2005), and we recently isolated strain *Rhodococcus* sp. $R_{14\text{-}2}$ from Jin-hua ham with a high cholesterol oxidase production. Jin-hua ham is a typical and popular flavour-rich fermented food of China and have a history dating back more than 500 years. We optimized the medium and culture conditions for the production of extracellular COX by strain $R_{14\text{-}2}$, and found that the addition of cholesterol induced a marked increase in the synthesis of the $R_{14\text{-}2}$ COX; significant levels of induction were only achieved when cholesterol was emulsified in the medium, using Tween 80 (Chengtao et al. 2007). The present paper describes the characterization of the secreted COX: production, and purification and analysis of some of its properties.

Materials and methods

Materials

DEAE-Sepharose fast flow, Sephadex G-150, protein standard markers and IEF markers were purchased from Pharmacia Co. (Amersham Biosciences, Sweden). Cholesterol (5-cholestene-3β-ol), 4-cholestene-3-one, β-sitosterol (Sitost-5-en-3β-ol), 7-dehydrocholesterol, cholic acid, deoxycholic acid, 5α-androstan-3α,17β-diol, 3-keto-4-en steroid, fenpropimorph and silica gel plates (Sigma, T6154) were procured from Sigma-Aldrich Chemical Co. (St. Louis, MO). Stigmasterol (Stigmast-5-en-3β-ol), β-Cholestanol (5α-cholestan-5-en-3β-ol), 5-androsten-3β-ol-17-one and kieselguhr were from Merck Co. (Beijing, China). Glutathione, *p*-chloromercuric benzoate, β-mercaptoethanol, EDTA, Tween80 and Triton X-100 were obtained from Fluka. The other chemicals used were of analytical grade.

Isolation and selection of cholesterol oxidase-producing strains

Selective medium I (Arima et al. 1969), containing (g/l): NH_4NO_3, 15; K_2HPO_4, 0.25; $MgSO_4{\cdot}H_2O$, 0.25; $FeSO_4{\cdot}H_2O$, 0.001; NaCl, 0.005; Tween 80, 1 ml and cholesterol, 2; pH 7.0.

We collected 70 samples including fermented foods, soil, waste waters and agricultural compost, from supermarkets, zoo, meat processing factories and tanning industries. The samples (around 1 g) were added to test tubes (20 ml) of selective medium I (5 ml), and were incubated at 30°C at 150 rpm for 3–4 days. To purify the enriched strains, 100 µl of the incubated suspensions were added to new test tubes of the same medium, and incubated under the same conditions for a subsequent 3–4 times. The final subcultures were spread and embedded in the medium PYG (1.0% peptone, 0.4% yeast extract, 0.1% glucose, 0.5% NaCl, 1.8% agar, pH 7.2). The plates were then incubated at 30°C for about 24 h, and bacterial colonies appeared were replica-plated by toothpick onto the isolation medium plates. The replica plates were then incubated at 30°C for 24 h.

In order to select the cholesterol oxidase-producing strains, a colony staining method was used on the agar plates (Yazdi et al. 2000). Strains able to produce the red colour were selected and cultivated at 30°C in 5 ml of liquid isolation medium with constant shaking. The bacterial cultures were centrifuged (7,000 × *g*) for 10 min. The supernatant fluids were assayed for extracellular activity of cholesterol oxidase. To find the cell-bound COX, the cellular pellets were washed twice with 0.1 M phosphate buffer (pH 7.2). When no activity was found in the buffer, the pellet was resuspended in the same buffer containing 0.1% of Triton X-100 and fallen to pieces by sonication (400 W, 8 min) in ice-bath condition. The suspension was recentrifuged (10,000 × *g*, 10 min) and the supernatants were used for the assay of released cell-bound COX.

Fermentation conditions for crude COX

For production of extracellular COX, strain $R_{14\text{-}2}$ was grown in the optimized medium defined by orthogonal array designs (Chengtao et al. 2008), containing (g/l): cholesterol, 2.0; yeast extract, 8.0; peptone, 4.0; NH_4Cl, 1.0; NaCl, 1.0; KH_2PO_4, 0.5; Na_2HPO_4, 0.25; $MgSO_4$, 0.15; $ZnSO_4$, 0.01; $CaCl_2$, 0.02; $FeSO_4$, 0.01 and Tween 80, 2.0 ml/l. To avoid cholesterol coagulation, it was dispersed by sonication of the medium for 10 min. pH was adjusted to 7.4 before sterilization. Optimum culture conditions for strain $R_{14\text{-}2}$ were 33°C on a rotary shaker (150 rpm) (Chengtao et al. 2007). After 60 h of incubation, the culture broth was centrifuged at 7,000 × *g* for 10 min at 4°C. The supernatant obtained was used as the crude enzyme source. Extracellular COX production after 60 h was about 1.5 units/ml.

Extraction and purification of a cholesterol oxidase

For the enzyme extraction, 145 g of ammonium sulfate (60% saturation, 0°C) was slowly added to the supernatant

fluid (400 ml) with stir (200 rpm, on magnetic stirrer) and allowed to stand for 4 h at 4°C. The precipitate thus formed was obtained by centrifugation (10,000 × *g*, 10 min) and dissolved with 10 mM buffer. The insoluble materials were discarded by centrifugation. Then, the supernatant fluid was directly applied to a DEAE-Sepharose FF column (1.5 × 20 cm^2) equilibrated with 10 mM KPB (potassium phosphate buffer, pH 8.5), and the column was washed extensively with 500 ml of the same buffer. The cholesterol oxidase was not absorbed on the column under the conditions employed. The active fractions were pooled, and then concentrated by ultrafiltration on a Pellicon Membrane PTGC OLC M2 (Millipore, Bedford, Mass, USA). Commercial cholesterol was recrystallised in 50% ethanol, and the recrystallised cholesterol was used as the adsorbent for the enzyme. The concentrate (500 mg protein per 100 ml) was loaded onto a kieselguhr-cholesterol affinity column (1.0 × 30 cm^2) equilibrated with 10 mM KPB (Kamei et al. 1978). After washing the column with 100 ml of 10 mM KPB, cholesterol oxidase was eluted with 0.1% Triton X-100, also in 10 mM KPB. To remove cholesterol and Triton X-100 from the enzyme solution, eluates were passed through a Sephadex G-150 column (1.5 × 90 cm^2) equilibrated with 10 mM KPB. The active fractions were combined, concentrated as above and used for the characterization of cholesterol oxidase.

Determination of activity and concentration of the enzyme

The assay of COX activity was based on the conversion of cholesterol to 4-cholestene-3-one (or H_2O_2) according to the method described by Richmond (1973). One unit (U) of COX is defined as the amount of enzyme required to produce 1μmol of 4-cholestene-3-one (or H_2O_2) per minute under the assay conditions. Protein (enzyme) concentration was determined by the method of Bradford (1976) using BSA as a standard for plotting a standard curve, measuring the absorbance at 595 nm. Cell density was determined by measuring the culture absorbance at 600 nm using a spectrophotometer. Cholesterol was measured by the enzymatic method of Allain et al. (1974).

Purification of cholesterol metabolites

To purify cholesterol metabolites, the culture medium was extracted twice and purified by preparative thin layer chromatography (TLC). Cholesterol oxide, present in the ethylacetate extracts, was analyzed by TLC according to the method of Aihara et al. (1986).

The purified substances were identified using gas chromatography (GC)–mass spectroscopy (MS). GC–MS was performed on an Autospec Q high-resolution mass spectrometer (Waters Corp.) housing a 30-mm by 0.32-mm DB-1 (0.25 μm film thickness) fused silica column using temperature-programmed operation from 185 to 295°C at increments of 2°C per minute with initial and final isothermal periods of 2 and 30 min, respectively. Electron ionization (70 electron volts) mass spectra were obtained by repetitive scanning of the mass range 50–1,000 amu throughout the elution of compounds from the GC column.

SDS-PAGE, IEF electrophoresis and NH_2-terminal amino acid sequence

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970) was carried out on 120 g/l resolving and 50 g/l stacking gels in a mini-electrophoresis equipment (ATTO Co., Japan) along with the standard proteins (Pharmacia Co., Sweden).

Isoelectric focusing electrophoresis (IEF) was performed in the Phast System using PhastGel IEF 3-9 (homogenous polyacrylamide gels containing Pharmalyte carrier ampholytes). Broad *pI* calibration kits (proteins *pI* 3.50–9.30) were used as markers in the *pI* value determinations.

NH_2-terminal amino acid sequence of the enzyme was determined by automated Edman method using a gas-phase protein sequencer (Applied Biosystems, Model 476A, USA) at Peking University (Beijing, China).

Substrate specificity of the enzyme

Steroids dissolved in propan-2-ol (50 μl) were mixed with 3 ml of 50 mM phosphate buffer (pH 7.0) containing 0.1 m1 of Triton X-100, 0.1 ml of 4-aminoantipyrine (2.4 mM) and 0.1 ml of phenol (0.4 M) at 30°C. In most instances, the steroids (0.1 mM) were oxidized with 0.02 U of cholesterol oxidase from $R_{14\text{-}2}$. The hydrogen peroxide produced was monitored using horseradish peroxidase (0.18 U) which oxidatively coupled the 4-aminoantipyrine and phenol to give a quinoneimine absorbing at 500 nm. The kinetic constants were determined using Hanes-Woolf plots based on the initial reaction rates (Eisenthal et al. 1974).

Effect of temperature and pH on enzyme activity and stability

The effect of pH and temperature on COX activity were determined according to the method of Kim and Seong (2002). The stability of COX (about 5 U/ml) at buffers of pH 2.0–12.0 was carried out at 4°C for 2 h, and the temperature stability of the enzyme in 0.1 M phosphate buffer of pH 7.0 was carried out between 0 and 80°C for 0.5–1 h. Then, their remained activity of the enzyme liquids was measured with 0.1 mmol/l cholesterol as substrate.

Effect of metal ions and reagents

Various metal salts were examined for their effects on COX activity, each at a final concentration of 0.1 mM. Enzyme (1.8–2.0 μg) was incubated with each metal salt at 30°C for 10 min in the medium of the 4-cholesten-3-one assay (3 ml) devoid of cholesterol. Then, the enzyme reaction was started by adding 1.0 μmol cholesterol (0.2 ml cholesterol solution in isopropanol), and the increase in absorbance at 240 nm was followed for 10 min.

Results and discussion

Isolation and selection of the cholesterol oxidase-producing strains

Many cholesterol oxidase-producing strains were isolated from quite different environments (Chengchun et al. 1999; Yazdi et al. 2001; Chengtao et al. 2005). Fermented foods, especially cholesterol-rich sausage, ham, fish, milk, preserved meat, smoked meat and shrimp paste, were also important sources of cholesterol-degrading strains.

In the primary screening, 322 bacterial colonies with different cholesterol-degrading activities had been isolated from 70 samples. We discovered that many fermented foods contain cholesterol-degrading bacteria. The ability to degrade cholesterol among the isolates was as follows: 8 strains degraded more than 80%, 32 strains degraded 50–79%, 102 strains 20–49% and 180 strains less than 20%. Among them, strain R_{14-2} and R_{22-4}, isolated from Jin-hua ham and La-Rou summer meat, two China traditional fermented meat products, degraded 97.5% and 94.7% of cholesterol in 7 days, respectively. In comparison, in a study carried out by Arima et al. (1969) to determine of cholesterol-degrading potency, among 256 species of bacteria, only 18 species were able to degrade more than 50% under the same conditions, in media containing 0.1% cholesterol.

In order to select the cholesterol oxidase-producing strains, forty of the strains with the highest degrading ability (more than 50%) were selected for further studies. A colony staining method was used on agar plates. Twenty-five of the strains able to produce the red colour were selected and cultivated in liquid medium with constant shaking at 30°C. The culture broths were assayed for intracellular and extracellular activity of cholesterol oxidase and known cholesterol metabolites in the product. Among the twenty-five strains tested, the highest extracellular activities of cholesterol oxidase and cholesterol-degrading efficiency was observed in strain R_{14-2}, and the highest membrane-bound activity in strain R_{22-4}. After 60 h of incubation at the optimized medium and culture conditions, extracellular COX production of R_{14-2} was about 1.5 U/ml (Chengtao et al. 2005). In contrast, the maximum COX production from *Rhodococcus* sp. GKI (Elalami et al. 1999) and *Breibacterium* sp. DGCDC-82 (Lv et al. 2002) were 0.38 U/ml after 90 h cultivation and 1.285 U/ml after 36 h culture, respectively.

From the data from TLC (Fig. 1) and GS–MS (data not shown), we concluded that 4-cholestene-3-one is the first and most probably major accumulating metabolite of cholesterol degradation by strains R_{14-2} and R_{22-4} (Fig. 2).

Identification of selected bacterium

Based on the phenotypical criteria according to Bergey's Manual of Systematic Bacteriology (Krieg et al. 1984) and using API 50 CH (BioMerieux, Marcy l'Etoile, France) and the Vitek BACIL card and corresponding database, the selected strains R_{14-2} and R_{22-4} were preliminarily identified (Chengtao et al. 2005) as *Rhodococcus* sp. and *Brevibacterium* sp. (data not shown), respectively. The *Rhodococcus* sp. strain R_{14-2} having the highest cholesterol oxidase activity was selected for further investigation.

Purification and confirmation of cholesterol oxidase of *Rhodococcus* sp. R_{14-2}

Extracellular COX from the culture supernatants was purified using different purification protocols (summarized in Table 1). Sixty percent ammonium sulfate precipitation followed by ion-exchange chromatography on DEAE-Sepharose FF increased the specific activity 2.4- and 28.2-fold, respectively. The specific activity of COX after gel filtration chromatography on Sephadex G-150 increased more than 53.1-fold. The purified COX had a final specific

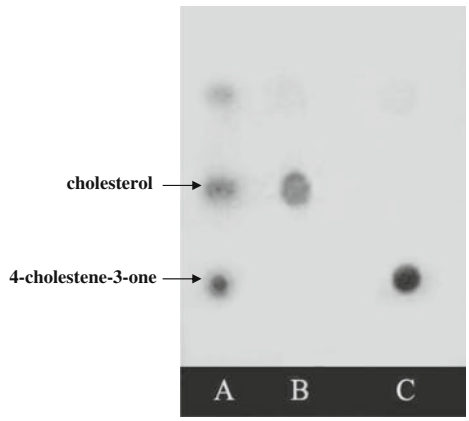

Fig. 1 The determination of metabolites of cholesterol degradation by R_{14-2} on thin layer chromatography (TLC). A: metabolites of cholesterol degradation by $R_{14-2;}$ B: standard of (Sigma); C: standard of 4-cholestene-3-one (Sigma)

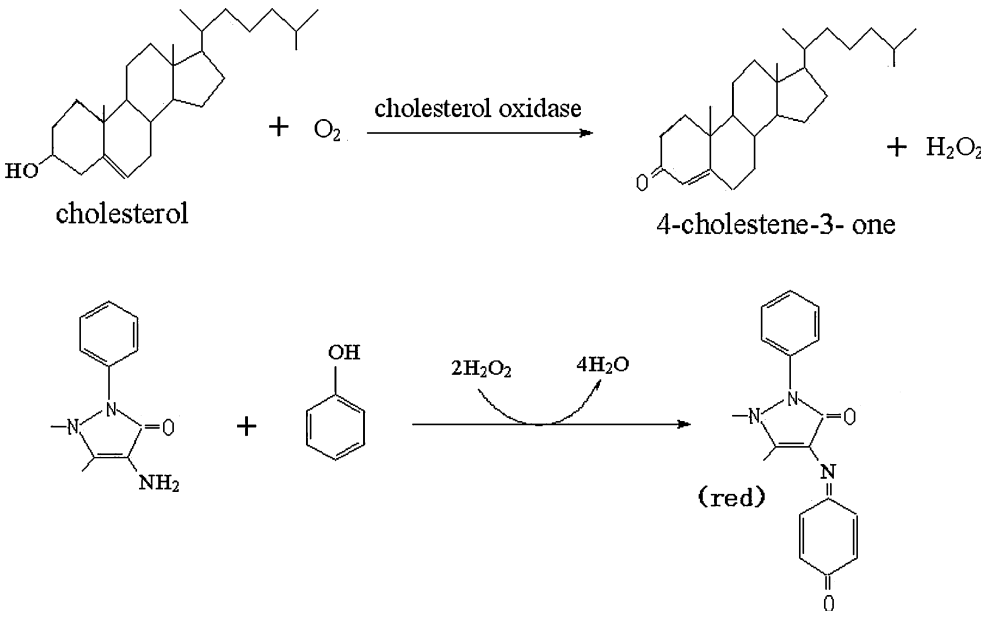

Fig. 2 Mechanism of cholesterol oxidase action and the assay of hydrogen peroxide by the oxidative coupling of phenol 4-aminoantipyrine catalysed by a peroxidase

Table 1 Purification stages of cholesterol oxidase of *Rhodococcus* sp. $R_{14\text{-}2}$

Phases	Total protein (mg)	Total activity (U)	Specific activity[a] (U mg^{-1})	Fold purification	Recovery (%)
Cell free supernatant	1,480	1,560	1.05	1.0	100
$(NH_4)_2SO_4$ precipitation	986	1,490	1.51	1.4	95.5
DEAE-Sepharose FF	35.8	1,060	29.61	28.2	67.9
Kieselguhr-cholesterol affinity chromatography	18.5	740	40.00	38.1	47.4
Sephadex G-150	7.8	435	55.77	53.1	27.9

[a] Specific activity of COX was expressed in U/mg of protein

activity of 55.8 U/mg with a 27.9% recovery based on the initial culture supernatant.

Molecular properties of the enzyme

After the combination of various chromatographic steps, the finally eluted proteins were subjected to SDS-PAGE and IEF. Purified COX migrated as a single polypeptide chain with an apparent molecular weight of 60 kDa on SDS-PAGE under reducing (Fig. 3, lane 1) or non-reducing conditions. And *pI* of the enzyme was approximately 8.7 on isoelectric focusing electrophoresis (IEF). The other COXs (Table 2), which were isolated from, *N. erythropolism*, *B. sterolicum*, *C. cholesterolicum*, *Pseudomonas*, *Proteobacteria* Y-134 (CHO-U), *Streptomyces* and *S. commune*, show similarity to the present COX as they are single polypeptide chains of 67, 54.9, 57, 56, 58, 30 and 53 kDa molecular mass and have *pI* of 8.9, 8.9, 8.7, 5.5, 4.3, 5.1, and 5.1, respectively. In contrast, CHO-A from *Proteobacteria* Y-134 is quite different as it consists of two identical protein subunits of 115 kDa of molecular mass with a *pI* of 7.0 (Isobe et al. 2003).

NH_2-terminal sequence of the first 10 amino acid residues of the enzyme was APPVASCRYC, which was clearly different from other COXs (as shown in Table 2).

Substrate specificity and action mode of the enzyme

The extracellular COX from *Rhodococcus* sp. $R_{14\text{-}2}$ rapidly oxidized 3β-hydroxysteroids such as cholesterol and phytosterols (such as β-sitosterol, stigmasterol), but had less effect on 7-dehydrocholesterol (see Table 3). The enzyme was inert toward 3α-hydroxysteroids (sterols with 3a-OH), such as cholic acid, deoxycholic acid and 5α-androstan-3α,17β-diol. Thus, the C-5 double bond and the alkyl chain in sterol molecules appeared to be necessary for an adequate oxidation of the sterol 3β-ol by the present enzyme. We also studied the effect of the C-5 double bond and the lateral chain in the substrate molecule on COX activity, using either 5α-cholestan-5-en-3β-ol or

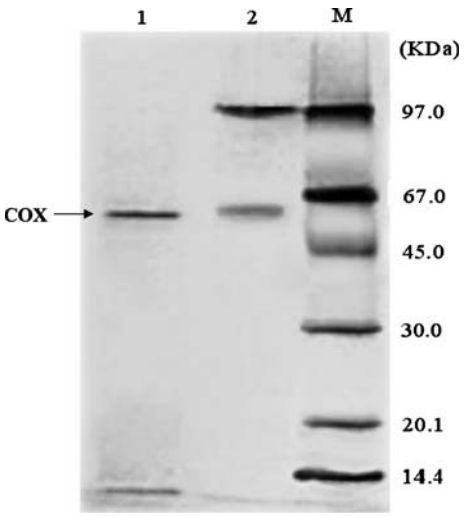

Fig. 3 SDS-PAGE patterns of the purified enzyme. SDS-PAGE was done on 12% gel under reducing conditions. Lane 1: pattern after Sephadex G-150; Lane 2: pattern after DEAE-Sepharose FF; Lane M: protein molecular mass markers: Phophorylase b (97.0 kDa), Albumin (67.0 kDa), Ovalbumin (45.0 kDa), Carbonic anhydrase (30.0 kDa), Soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa)

5-androsten-3β-ol-17-one as substrates. We observed that the lateral chain in the substrate molecule affected on activity of the COX with C-5 double bond. Moreover, the enzyme was inactive with A-ring- or B-ring-modified sterols (e.g. 3-keto-4-en steroid).

The COXs are simply referred to as oxidases, since their mode of action is dependent only on the presence of molecular oxygen (Inouye et al. 1982), whereas others are considered to be true NAD-dependent dehydrogenases, such as the coupled enzyme 3β-hydroxysteroid: NAD(P) oxidoreductase-3-ketosteroid Δ^4, Δ^5-isomerase (3β-hydroxy steroid dehydrogenase: Δ^5-isomerase; Δ: the steroid ring) from the mitochondrial fraction of human placenta (Stadtman et al. 1954). Cholesterol oxidase is a monomeric flavoenzyme (FAD-containing enzyme) that catalyzes the oxidation and isomerization of cholesterol to cholest-4-en-3-one (Stadtman et al. 1954). Two forms of the enzyme are known, one containing the cofactor non-covalently bound to the protein and one in which the cofactor is covalently linked to a histidine residue. The X-ray structure of the enzyme of *B. sterolicum* containing covalently bound FAD has been determined (Vrielink et al. 1993; Kinya et al. 2001). Uwajima et al. (1973) found that this enzyme contains a FAD prosthetic group as the oxidase apoprotein. A model for the steroid substrate, cholesterol, can be positioned in the pocket revealing the structural factors that result in different substrate binding affinities between the two known forms of the enzyme. The enzyme from *B. sterolicum* contains the FAD cofactor covalently bound to His^{121} (Kinya et al. 2001). The

Table 2 Molecular properties of cholesterol oxidase of *Rhodococcus* sp. $R_{14\text{-}2}$, compared with other enzymes in literature data

Enzymes from strains	Molecular weight (kDa)	*pI*	N-terminal amino acid sequence	Km for cholesterol (μM)	References
Rhodococcus sp. $R_{14\text{-}2}$	60	8.5	APPVASCRYC	55	This work
Rhodococcus sp. GKI	59	8.9	APPVASXRYXF[a]	300	Elalami et al. (1999)
Arthrobacter simplex USA18	54			28	Wenhsiung et al. (1988)
Brevibacterium sterolicum	55	4.4–5.1	ADAAPSRTLADGDRV	140	Ohta et al. (1991) and Fujishiro et al. (1990)
B. sterolicum ATCC21387					
Cholesterol oxidase I	54.9	8.9	AOSRTLADGDRVPA	1,100	Ohta et al. (2002)
Cholesterol oxidase II	46.5	4.7	VPAGSAGSLGS	300	Ohta et al. (2002)
Corynebacterium cholesterolicum	57	8.7			Shirokane et al. (2003)
Nocardia erythropolis	55; 67			5,140	Sojo et al. (1997)
Pseudomonas sp.	56	5.0–5.5		200	Noriyuki et al. (1998)
Pseudomonas cepacia					
CHO-A	115	4.3	SXTTPPGFPAGISLYQ	65	Isobe et al. (2003)
CHO-U	58	7.0	SXTTPPGFPAGISLYQ	26	Isobe et al. (2003)
Schizophyllum commune	53	5.1		330	Fukuyama et al. (1979)
Streptomyces hygroscopicus	55	4.4–5.1		240	Giovanni et al. (1997)
Streptomyces sp.	30	5.1	ADAPPSRTLDNGGYV	217	Fujishiro et al. (1990) and Pornpen et al. (2005)

[a] X of N-terminal amino acid sequence indicates an unknown residue

Table 3 Substrate specificities of cholesterol oxidase from *Rhodococcus* sp. R_{14-2}

Substrate	Relative activity (%)	Substrate	Relative activity (%)
Cholesterol (5-cholestene-3β-ol)	100	5-Androsten-3β-ol-17-one	15
β-Sitosterol (Sitost-5-en-3β-ol)	80	3-Keto-4-en steroid	0
Stigmasterol (Stigmast-5-en-3β-ol)	78	Cholic acid	0
7-Dehydrocholesterol	37	Deoxycholic acid	0
β-Cholestanol (5α-cholestan-5-en-3β-ol)	24	5α-Androstan-3α,17β-diol	5

similarities in catalytic properties of the R_{14-2} COX paired with the differences in their primary structures, and a detailed study of the kinetic mechanism of the enzyme are currently underway.

Kinetic analysis of the enzyme

Cholesterol is poorly soluble in water. Thus, enzymatic H_2O_2 generation reactions were carried out using cholesterol emulsified with a surfactant, 0.03% Triton X-100. Reaction kinetics of the COX were examined under standard assay conditions with the substrate concentration as only variable. The saturation curve of COX was typical of a Michaelis-Menten relation (Fig. 4). The Km (Michaelis constant) value and Vmax for cholesterol were determined to be 55 μM and 72.5 μmol min^{-1} mg^{-1} of protein by the method of Lineweaver-Burk (Eisenthal et al. 1974), respectively. The catalytic studies revealed a distinctive feature of its high affinity toward extremely low content, suggesting its potential application to rapid microdetermination of cholesterol in serum and food.

Effect of temperature and pH on enzyme activity and stability

The effect of temperature and pH on enzyme activity was studied with cholesterol as substrate. The heat stability of COX is useful when supplying it in a process involving cholesterol degradation, e.g. food-cholesterol degradation. The R_{14-2} COX is very stable, possessing a broad activity range around pH 7.0. Maximum activity can be retained over 30 min at 50°C in 0.1 M phosphate buffer of pH 7.0, but at temperatures higher than 60°C, COX rapidly lost its activity and at 70°C was completely inactivated within 1 h. In comparison, the optimum temperatures of the COXs from *S. polychromogenes* (Kim and Seong 2002), *A. simplex* (Wenhsiung et al. 1988) and *B. sterolicum* (Kinya et al. 2001) were 37, 50, and 55°C, respectively. The pH stability of purified enzyme was carried out at 4°C. COX retained its full activity at pHs between 4 and 10 for 2 h, with a maximum activity at pH 7.0, but at higher or lower pHs, enzyme activity was decreased significantly. After freezing and thawing for four times, 92% of the activity remained.

Effect of chemical reagents on enzyme activity

The effect of metal ions such as Ag^+, Li^+, Ca^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Pb^{2+}, Hg^{2+}, $Co^{2+}$and Fe^{3+} on the enzyme activity was also investigated (Table 4). None of the metal salts, $CaCl_2$, $Co(NO_3)_2$, $Pb(CH_3COO)_2$, $FeSO_4$, $MnSO_4$, $MgSO_4$,Li_2SO_4 showed, at 0.1 mM, a significant inhibitory effect on the enzyme activity. The inhibition was only around 15% of the control activity. Under the same conditions, however, Zn^{2+}, Cu^{2+} and Ag^+ inhibited the enzyme activity by 70%, 65% and 72% respectively. Hg^{2+} at final concentrations of 50, 10, and 1 μM inhibited COX activity by 95%, 80%, and 15%, respectively. The activity of the enzyme was seriously inhibited by the addition of Fe^{3+} ($FeCl_3$). Thus, the present COX is extremely sensitive

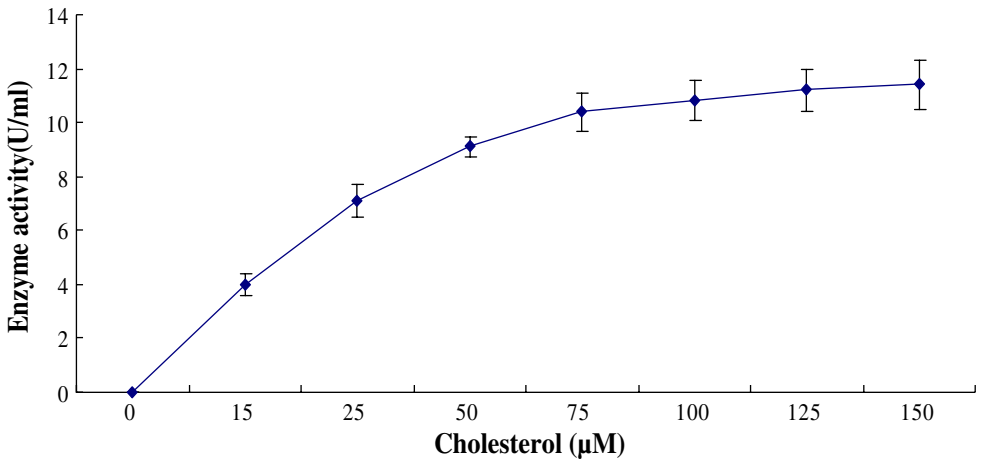

Fig. 4 Substrate saturation curves of cholesterol oxidase (n = 3)

Table 4 Effect of defined inhibitors on activity of purified cholesterol oxidase of *Rhodococcus* sp. R_{14-2}

Inhibitors	Concentration (mM)	Relative activity (%)	Inhibitors	Concentration (mM)	Relative activity (%)
None (control)		100	Hg^{2+} ($HgCl_2$)	50 μM	5
NaN_3	0.1	102		10 μM	20
p-Chloromercuric benzoate	0.1	15		5 μM	85
Fenpropimorph	50 mg/l	20	Co^{2+} [$Co(NO_3)_2$]	0.1	85
EDTA	0.1	95	Mg^{2+} ($MgSO_4$)	0.1	103
Ag^{+} ($AgNO_3$)	0.1	28	Ca^{2+} ($CaCl_2$)	0.1	97
Zn^{2+} ($ZnSO_4$)	0.1	30	Pb^{2+} [$Pb(CH_3COO)_2$]	0.1	88
Cu^{2+} ($CuCl_2$)	0.1	35	Mn^{2+} ($MnSO_4$)	0.1	95
Fe^{3+} ($FeCl_3$)	0.1	10	Li^{+} (Li_2SO_4)	0.1	93
Fe^{2+} ($FeSO_4$)	0.1	92			

to Hg^{2+} and Fe^{3+}, but unaffected by metal chelating reagents (Such as EDTA), indicating that the enzyme is not a metalloprotease.

To improve our understanding of the properties of these COXs, we continue investigating their response to diverse chemical reagents. Inhibition caused by *p*-chloromercuric benzoate, mercuric chloride, or silver nitrate was almost completely prevented by the addition of glutathione. This finding suggests that -SH groups may be involved in the catalytic activity of the present COX. In addition, fenpropimorph, a morpholine derivative, had been shown to block selectively the enzyme activity, 50 mg/l inhibiting about 80% of the enzyme activity. Furthermore, the inhibition is instantaneous and followed a reversible competitive mechanism for the COX from *Rhodococcus* sp. R_{14-2}. However, a reversible noncompetitive mechanism for COXs from *N. erythropolis* and *S. commune* (Smith et al. 1975), and an irreversible type of inhibition is observed for *P. testeroni* cholesterol oxidase (Hesselink et al. 1990).

Although cholesterol oxidases from some bacteria have some similarities with the present COX, such as rapid oxidation of 3β-hydroxysteroids and inactivity towards 3α-hydroxysteroids, The electrophoretic pattern on SDS-PAGE, together with NH_2-terminal sequence, kinetic data, stability, *pI* value, temperature and pH for optimal activity and the estimated molecular mass of 60 kDa, strongly indicate that the extracellular cholesterol oxidase from strain R_{14-2} is a different form of the enzyme cholesterol oxidase. Our results also show that *Rhodococcus* sp. R_{14-2} can be considered as a potentially interesting source of COX for clinical and industrial uses, because of its good thermal stability, higher affinity for cholesterol and broad pH activity range.

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