Properties of the Purified Extracellular Cholesterol Oxidase from *Rhodococcus equi* No. 23

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The cholesterol oxidase produced by *Rhodococcus equi* No. 23 (JCM 6819) was purified from the culture broth by procedures including DEAE-Sephadex A-50 and batchwise treatment utilizing the affinity to cholesterol. The purified enzyme was detected as a single band in SDS-PAGE (molecular weight 56 000) but in an aggregated form in the electrophoretic patterns on a linear gradient polyacrylamide gel in the presence of Triton X-100. Antibodies from the purified preparation reacted with the monomer type of enzyme blotted after SDS-PAGE, so the aggregate could be said to be formed from the enzyme–enzyme interaction without the loss of enzyme activity and the conformational change. The properties of the purified enzyme were also examined in terms of amino acid composition, isoelectric point, K_m value, stability of enzyme activity, metal inhibition, and sensitivity to various modification agents.

Since the first report of microbial degradation of cholesterol by Turfitt (1944), many microorganisms that degrade cholesterol have been isolated. In a previous study (Watanabe et al., 1986), we also isolated cholesterol-degrading organisms from food of animal origin such as butter, bacon, pork fat, and chicken fat, and most of the strains were identified as belonging to the genus *Rhodococcus*. One of those strains, *Rhodococcus equi* No. 23 (JCM 6819; JCM stands for the Japan Collection of Microorganisms, RIKEN, Wako, Saitama 351-01, Japan), was found to degrade cholesterol without accumulating steroid intermediates to non-steroid compounds. So, we have used the strain for the degradation of cholesterol in egg yolk (Aihara et al., 1988a,b).

R. equi No. 23 produced more extracellular cholesterol oxidase, which catalyzes the first step in cholesterol degradation to cholest-4-en-3-one, than the other isolated and references strains.

Cholesterol oxidase has been described in *Nocardia erythropolis* (Smith and Brooks, 1974) and *Nocardia rhodochrous* (Cheetham et al., 1982). These enzymes were shown to be an intrinsic membrane-bound type that could be extracted from cells by treatment with Triton X-100 or trypsin. In contrast to such membrane-bound enzymes, extracellular cholesterol oxidases were also isolated from the broth filtrates of *Streptomyces violascens* (Tomioka et al., 1976), *Brevibacterium sterolicum* (Uwajima et al., 1974), and *Streptoverticillium cholesterolicum* (Inouye et al., 1982). In such reports, many different characteristics of cholesterol oxidase about the molecular weight, substrate specificity, and so on have been described.

The increased need for a rapid, simple method for determining cholesterol in food and blood serum has become apparent with the advent of voluntary food-labeling regulations and the increase in clinical assay for human health. The enzymatic method using cholesterol oxidase, which is an enzyme reagent for the specific estimation of cholesterol, has been developed (Allain et al., 1974; Smith and Brooks, 1974). The properties of cholesterol oxidases produced by newly isolated bacteria should be elucidated for the development of better cholesterol-degrading enzyme reagent. In the present study, the extracellular cholesterol oxidase from *R. equi* No. 23 described above was purified in the form of aggregate, and the properties of this enzyme were clarified.

MATERIAL AND METHODS

Microorganism and Cultivation. *R. equi* No. 23, which was isolated from butter and stocked in JCM, was used. The culture medium contained (g/L) the following: NH_4NO_3, 1.0; KH_2PO_4, 0.25; $MgSO_4 \cdot 7H_2O$, 0.25; $FeSO_4 \cdot 7H_2O$, 0.001; yeast extract, 5; cholesterol, 1; pH 7.0 (Arima et al., 1969). After sterilization followed by homogenization of cholesterol by sonication in the culture medium as described in the previous report (Aihara et al., 1986), the culture [1L] was grown at 37 °C for 40 h on a mechanical shaker. After centrifugation for 30 min at 9500*g* at 4 °C, supernatant was filtered and used for isolation of cholesterol oxidase.

Purification of Cholesterol Oxidase. To the resulting filtrate was added 0.1 N HCl with stirring to lower the pH to 7.0. About 100 g of DEAE-Sephadex A-50 (Pharmacia Co.) equilibrated with 10 mM potassium phosphate buffer (pH 7.0, designated as buffer A) was added to this solution [1L]. After it was stirred for 10 min, the mixture was filtered. The cholesterol oxidase was not adsorbed on the DEAE-Sephadex A-50 under the condition employed. The combined filtrate and washed solution containing cholesterol oxidase were subjected to batchwise treatment on cholesterol after boiling, which was prepared as reported by Kamei et al. (1978). Thus, about 20 g of the cholesterol equilibrated with buffer A was added to the solution (1.3 L). The mixture was gently stirred for 1 h and then centrifuged for 30 min at 9500*g*. The precipitated cholesterol was washed with buffer A (50 mL) and centrifuged again. These washing and centrifugation procedures were repeated five times. The recovery treatments (elution and centrifugation) of enzyme bound to the washed cholesterol were repeated five times with 0.01% Triton X-100 and three times with 0.05% Triton X-100, under the same condition as described above.

Determination and Unit of Enzyme Activity. The assay of cholesterol oxidase is based on the formation of cholest-4-en-3-one from cholesterol as described in the previous paper (Watanabe et al., 1986). The formed cholest-4-en-3-one was extracted from the reaction mixture with ethyl acetate and analyzed by high-performance liquid chromatography. One unit of cholesterol oxidase activity is defined as the amount of enzyme producing 1 μmol of cholest-4-en-3-one/min.

Determination of Protein. The protein concentration was determined by the method of Lowry et al. (1951) using crystalline bovine serum albumin as a standard. To the samples was added 50% trichloroacetic acid to yield a final trichloroacetic acid concentration of 7%, and the mixture was then allowed to stand overnight at 5–6 °C. The samples were centrifuged at 900*g* for 15 min, and the obtained precipitates were dissolved in 2 mL of 0.1 N NaOH–10% Na_2CO_3 and used to determine protein concentrations.

Polyacrylamide Gel Electrophoresis. SDS-PAGE was performed according to the method of Laemmli (1970). Polyacrylamide gradient gel electrophoresis (PGGA) in the present of Triton X-100 (0.2 × 8 × 8 cm, gradient concentration of acrylamide from 4 to 10% containing 0.05% Triton X-100; upper gel; acrylamide 4%) with 10 mM imidazole–acetic acid buffer (pH 6.4) was used for the native enzyme. Proteins were silver-stained with the kit of Daiichi Pure Chemicals, Ag-STAIN DAIICHI. Activity of the enzyme on a Triton X-100 gel was visualized by the formation of the quinone imine dye (Allain et al., 1974) as follows: To 10 mL of reagent A [Tris-HCl buffer (0.15 M, pH 7.0), 100 mL containing 40 mg of 4-aminoantipyrine, 20 mg of

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Table I. Purification of Cholesterol Oxidase from Broth Filtrate of *R. equi* No. 23

fraction	total act., units	total protein, mg	sp act., units/mg	yield, %	purificn, fold
broth filtrate	49.7	134.3	0.37	100.0	1
DEAE-Sephadex A-50 unadsorption	45.1	92.9	0.49	90.7	1.3
affinity chromatography					
unadsorption	4.6	70.8		9.3	
0% Triton X-100	0.3	1.6		0.6	
0.01% Triton X-100	1.75	0.8		3.5	
0.05% Triton X-100	6.75	1.5	4.5	13.6	12.2

p-chlorophenol, and 2 mg of horseradish peroxidase] was added 100 μL of reagent B (cholesterol/isopropyl alcohol, 100 mg/mL). Filter paper (Toyoroshi No. 5C) was soaked in the reagent mixture, put on the gel after electrophoresis, and kept for 2 h at 37 °C. The enzyme band on the gel could be judged by the color development.

Preparation of Antibodies. A part of the enzyme solution, which was eluted with 0.05% Triton X-100 as described above, was divided into two parts. The one was concentrated 10-fold by centrifugal evaporator (Sakuma, EC-57) keeping the native form, and the protein in the other was precipitated with the addition of 8-fold ethanol, collected in denatured form, and suspended in water. The native and denatured enzymes were emulsified with equal volumes of Freund's complete adjuvant, respectively. Five mice (BALB/C, approximately 7 weeks old, male; Shizuoka Agricultural Association for Laboratory Animals) were immunized by intraperitoneal injection of 200 μL of either of those emulsions per mice. The mice received two booster injections of 200 μL of antigen emulsions mixed with Freund's incomplete adjuvant in a similar manner, 28 and 35 days after the first injection. Blood was collected 7 days after the last injection by puncture of the opthalmic retroorbital veneous plexus. The serum was separated from the pooled blood of five mice and stored at −80 °C before use.

Immunological Techniques. Double immunodiffusion in agarose gel was performed according to Ouchterlony (1949). Immunoblotting was performed according to the method of Towbin et al. (1979). The proteins electrophoresed on 0.1% SDS and 10% polyacrylamide slab gels (0.2 × 8 × 8 cm) were transferred electrophoretically to a nitrocellulose paper, and the protein cross-reacted with the anti-mouse serum was visualized with peroxidase-coupled goat anti-mouse IgG (Cappel).

Molecular Weight Determination. In the SDS-PAGE method, enzyme molecular weight was estimated by comparing the mobility of the enzyme protein with those of standard proteins after SDS-PAGE. The mobility of each standard protein was plotted against its molecular weight on a logarithmic scale. In the gel filtration method, the column of Sephadex G-100 (2.0 × 105 cm) was equilibrated with 10 mM phosphate buffer (pH 7.0) containing 1 mM cysteine. A set of protein standards of Combithek (Calibration Proteins II) was used for the molecular weight determination.

Amino Acid Analysis. The enzyme was hydrolyzed with 6 N HCl in evacuated sealed tubes at 110 °C for 20 h. The hydrolysates were analyzed with a JEOL 6AH amino acid analyzer.

Isoelectric Focusing. The isoelectric point was determined by isoelectric focusing on a column (1.9 × 24 cm) with Pharmalyte covering the pH range from 5.0 to 11.5 in a sucrose linear gradient from 0 to 50% containing 1 mM cysteine and 0.2% Triton X-100, with constant voltage (800 V for 24 h followed by 1500 V for 72 h). After electrophoresis, the solution was fractionated and the pH values and the enzyme activity in each fraction were detected.

RESULTS

Isolation, Purity, and Molecular Weights of Prepared Enzyme. As shown in Table I, approximately 12.2-fold purification of specific activity compared with the original broth filtrate was achieved by the above procedure. Triton X-100 at the concentration of 0.05% was needed to effectively elute the bound cholesterol oxidase to cholesterol.

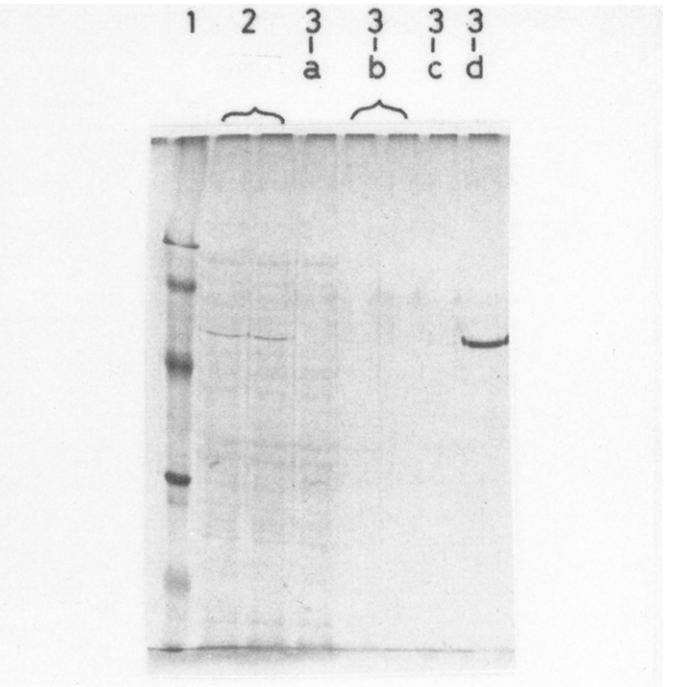

Figure 1. SDS–polyacrylamide gel electrophoresis of samples taken at each stage of purification of cholesterol oxidase. Lanes: 1, broth filtrate (protein less than 5 μg); 2, DEAE-Sephadex A-50 unadsorption Fr (protein less than 5 μg); 3, affinity chromatography, (a) unadsorption Fr (less than 5 μg), (b) 0% Triton X-100 Fr (less than 5 μg), (c) 0.01% Triton X-100 Fr (less than 5 μg), (d) 0.05% Triton X-100 Fr (less than 5 μg). Electrophoresis was carried out on a 10% gel by the method of Laemmli (1970), and protein was stained with silver stain kit. Concentration of each sample was obtained by blowing nitrogen on sample solution.

The patterns of protein band in the purification step were examined by SDS-PAGE (Figure 1). The final preparation gave a single band as seen in 3-d in Figure 1. These results indicated that the preparation obtained in the present study was highly homogeneous. The apparent molecular weight of the reduced and denatured protein was found to be 56 000 as determined in the SDS-PAGE.

Electrophoretic Patterns of PAGE. Electrophoretic patterns on a linear gradient polyacrylamide gel in the presence of Triton X-100 of both the broth filtrate and the purified preparation were shown in Figure 2, in which A and B are patterns for protein detected by silver staining and for enzyme by its assay, respectively.

Two bands (a and b in A-1 of Figure 2) in the broth filtrate and one band (b in A-2 of Figure 2) in the purified preparation could be detected by the silver staining. On the other hand, when the strips were treated for enzyme activity in both samples (B-1 and B-2), patterns similar to those in silver staining were also found.

It has been demonstrated that polyacrylamide gel gradient in the electrophoresis has a high potential value for size evaluation of native protein molecules in the absence and presence of nonionic detergents in the gel; components of lower molecular weight generally migrate on the gel much more than those of higher molecular weight (Andersson et al., 1972; Nakashima et al., 1981). The main band in the broth filtrate was the component that migrated most and had the lowest molecular weight. Its band did not exist in the purified preparation, suggesting that extracellular cholesterol oxidase from *R. equi* No. 23 aggregates without any apparent loss of enzyme activity in the purification procedure.

Figure 3 shows the patterns of SDS-PAGE of the gels of a section in B-1 and b section in B-2 in Figure 2, with

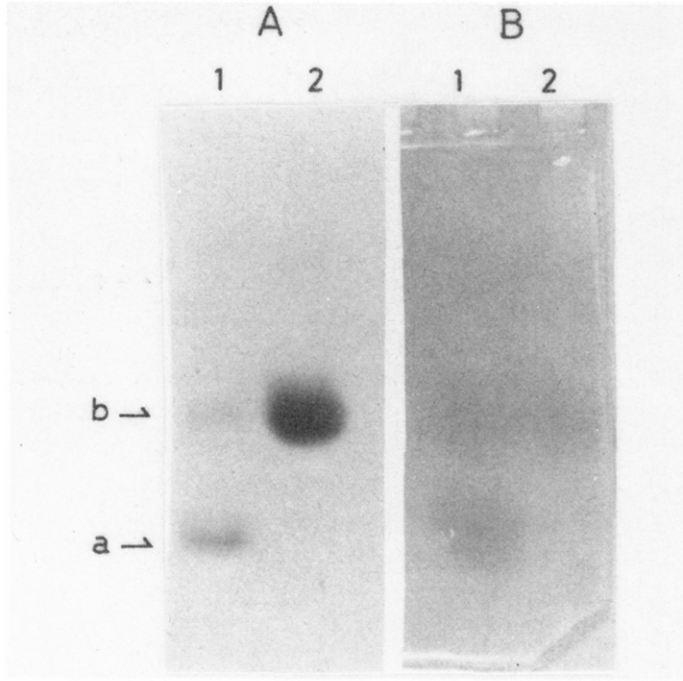

Figure 2. Polyacrylamide gradient gel electrophoresis (4–10%) in the presence of Triton X-100 (0.05%). Key: A, protein staining with silver staining kit; B, activity staining of cholesterol oxidase as described in Materials and Methods; lanes A-1 and B-1, broth filtrate (protein 30 μg); lanes A-2 and B-2, purified preparation (protein 30 μg). The isoelectric point of the purified preparation was found to be around pH 9.1, as shown in Figure 5. So, cathode at the upper side and anode at the lower side were set.

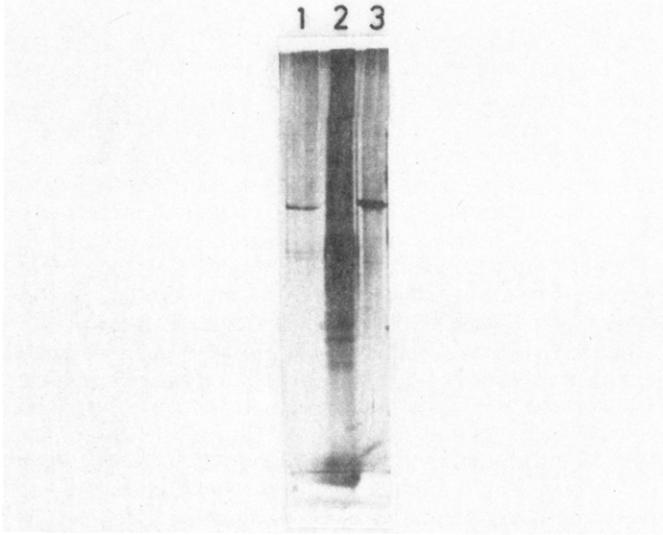

Figure 3. SDS–polyacrylamide gel electrophoresis of the fractions separated by polyacrylamide gradient gel electrophoresis. Key: lane 1, gel of B-1-a in Figure 2; lane 2, activity staining kit; lane 3, gel of B-2-b in Figure 2. SDS electrophoresis was carried out as shown in Figure 1. Protein was stained with silver stain kit.

the activity staining kit. In Figure 3-1,3, one component with identical molecular weights in both samples was observed with some contaminants of proteins in the determining kit. This pattern supported the result obtained in Figures 1 and 2 that the purified preparation was the aggregate of cholesterol oxidase.

The broth filtrate (5 mL) was subjected to Sephadex G-100 gel filtration. One main symmetrical cholesterol oxidase activity peak was eluted (data not shown). From comparison with the molecular weight of standard protein, the molecular weight of cholesterol oxidase in the broth filtrate was estimated to be about 55 000 which corresponded to the value obtained in SDS-PAGE. This means that extracellular cholesterol oxidase mainly existed in the form of monomer.

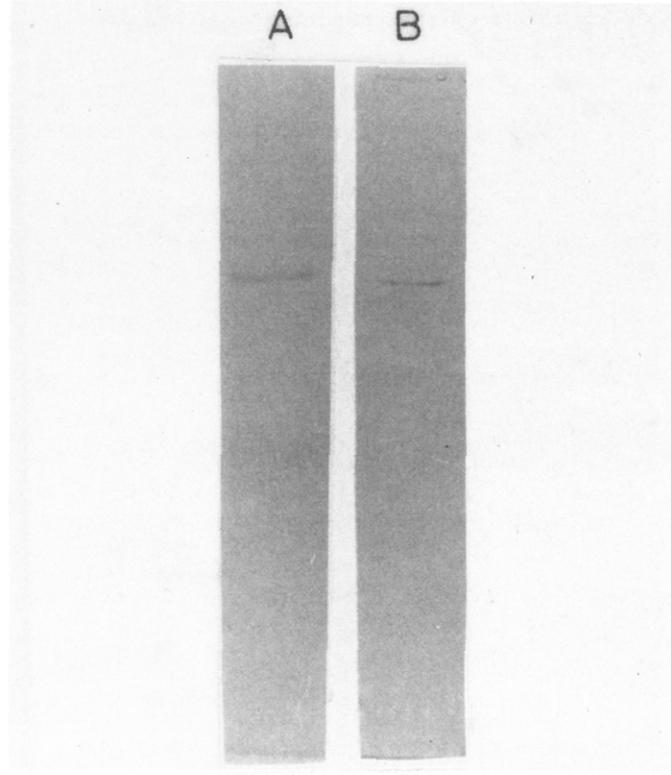

Figure 4. Immunoblotting analysis of the purified cholesterol oxidase. Key: lane A, protein staining with silver stain kit after SDS electrophoresis; lane B, binding to antibodies prepared against the purified preparation to the cholesterol oxidase blotted to nitrocellulose after SDS electrophoresis.

Table II. Amino Acid Composition

amino acid	mole, %	amino acid	mole, %	amino acid	mole, %
Asp	9.1	Ala	11.4	Tyr	3.0
Thr	8.5	$^1/_2$Cys	0.3	Phe	4.6
Ser	6.6	Val	6.6	His	1.1
Glu	10.8	Met	2.0	Lys	6.5
Pro	3.9	Ile	4.3	Arg	4.2
Gly	9.0	Leu	8.3		

Immunologic Properties. Immunodiffusion experiments were carried out with the antisera against native and denatured purified preparations, at different ratios of the purified preparation to antisera. A single precipitation line against antiserum from the native purified preparation was observed, but that from the denatured one was not. The immunoreactivity of the purified preparation was further investigated by the immunoblotting procedure. The purified preparation was submitted to SDS-PAGE. After electrophoretic transfer of protein separated in SDS-PAGE to nitrocellulose, the blots were incubated with specific antibody. The antigen–antibody complex was detected by second antibody conjugated to peroxidase when the antibody was prepared from the native purified enzyme (Figure 4). These results indicate that the antigen determinants depend on the conformation of the purified enzyme and the purified preparation was an aggregate of the cholesterol oxidase that bound to one another without conformational change.

Amino Acid Composition. The amino acid composition of the purified preparation is shown in Table II. The amide content was not determined.

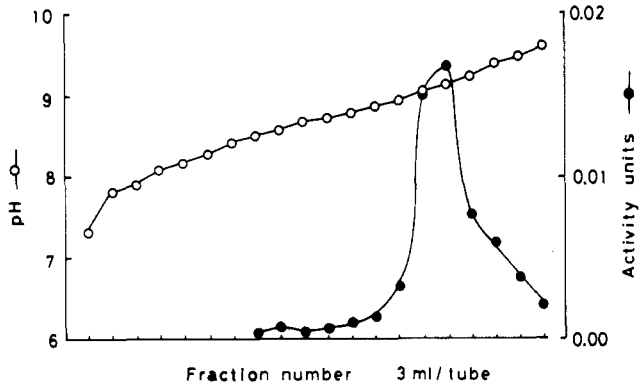

Figure 5. Isoelectric focusing chromatogram of the purified preparation. See the text for experimental details.

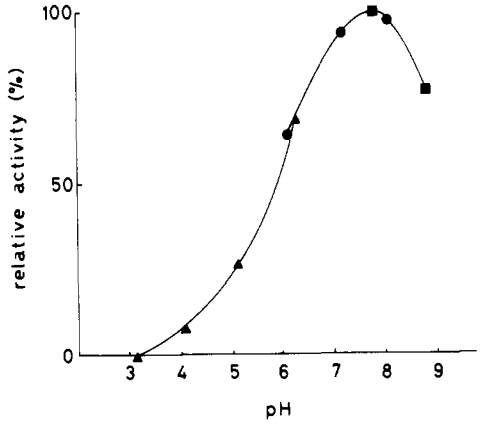

Figure 6. Optimum pH of the purified preparation. The amount of the purified preparation used in this experiment was 0.005 unit/reaction tube. The assay procedure was described in Materials and Methods. Buffer solutions used: 0.1 M acetate (▲—▲, pH 3–6); 0.1 M phosphate (●—●, pH 6–8); 0.1 M Tris-HCl (■—■, pH 8–9).

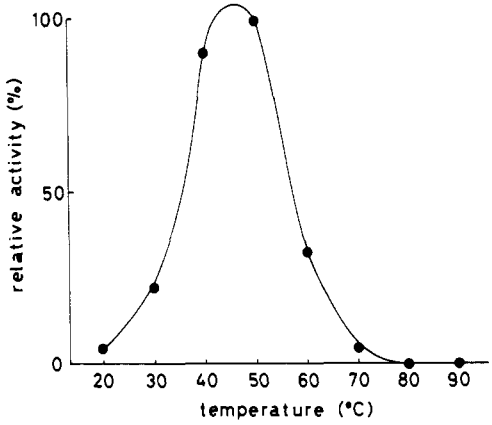

Figure 7. Optimum temperature of the purified preparation. Enzyme reaction in a mixture consisting of 10 mM cholesterol in isopropyl alcohol (50 μL), 0.1 M phosphate buffer (pH 6.3) (100 μL), and the purified preparation (200 μL) (0.004 unit) was carried out at a temperature given in the figure for 10 min, and the resulting cholest-4-en-3-one was analyzed as described in Materials and Methods.

Isoelectric Point. The isoelectric point of the purified preparation was found to be around pH 9.1, as shown in Figure 5.

Enzymatic Properties. The enzymatic properties of the purified preparation were examined.

The effect of pH on enzyme activity was studied with cholesterol as a substrate; the enzyme was most active at about pH 7.8 (Figure 6).

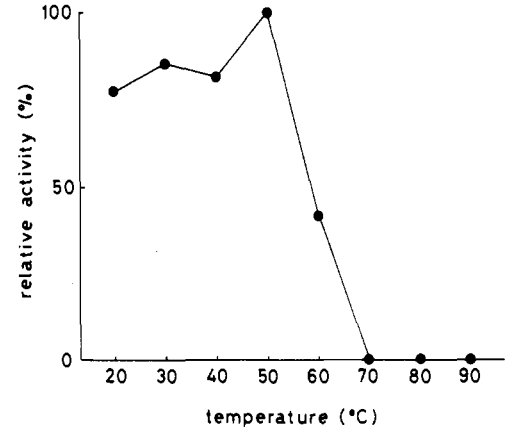

Figure 8. Thermal stability of the purified preparation. The purified preparation (200 μL, 0.004 unit, pH 6.3) was incubated at a temperature in the figure for 10 min and then cooled immediately. The remaining activity was assayed as described in Materials and Methods.

Table III. Effects of Metal Salts and Agents for the Chemical Modification of Protein on Cholesterol Oxidase Activity

addn[a]	concn, mM	rel act., %
$CaCl_2$	10	43.0
$MgCl_2$	10	80.0
$FeSO_4$	1	94.7
$CuSO_4$	1	93.8
$MnCl_2$	1	89.5
$AgNO_3$	1	6.1
	+10 mM glutathione	14.9
	+10 mM cysteine	109.6
	0.1	7.0
$HgCl_2$	1	1.8
	0.1	6.1
	+10 mM glutathione	14.0
	+10 mM cysteine	104.4
EDTA	10	59.6
1-fluoro-2,4-dinitrobenzene	0.1	95.6
iodine	0.1	99.1
hydrogen peroxide	10	93.0
p-chloromercuribenzoic acid	0.1	76.3
monoiodoacetic acid	1	89.5
N-bromosuccinimide	0.1	1.4
	0.01	39.1

[a] The chemical was added to the assay medium, and the mixture was assayed by the assay procedure described in Materials and Methods. Activities are expressed as percent of a control without the addition of chemical.

The effect of temperature on enzyme activity is presented in Figure 7; the optimum temperature was approximately 47 °C, and the enzyme activity was lost at 20 °C and above 70 °C.

The heat stability was examined at pH 6.3. Each sample was heated at the desired temperature for 10 min, and after cooling the residual activity was determined at 37 °C (Figure 8). The enzyme was stable up to 50 °C and rapidly inactivated at a temperature higher than 50 °C.

The initial oxidation rates of cholesterol to cholest-4-en-3-one were determined at various substrate concentrations. Double-reciprocal plots of the data fitted a straight line, and the K_m value was calculated as 9.0×10^{-4} M.

The effects of various metal salts and agents for the chemical modification of protein on enzyme activity were studied with cholesterol as a substrate (Table III). The activity was remarkably inhibited by the addition of $AgNO_3$ or $HgCl_2$. The inhibition caused by their addition was almost completely prevented by the addition of cys-

teine. Other metal salts such as $FeSO_4$, $CuSO_4$, and $MnCl_2$ at 1 mM concentration essentially did not affect the oxidation rate of cholesterol by the enzyme. The enzyme was highly sensitive to *N*-bromosuccinimide, but not much inhibited by sulfhydryl reagents, *p*-chloromercuric benzoate, iodoacetate, or hydrogen peroxide.

DISCUSSION

The extracellular cholesterol oxidase from *R. equi* No. 23 was found to exist mainly in monomer form in culture broth, but it aggregated easily through enzyme–enzyme interactions without apparent loss of enzyme activity or the conformational change during purification of enzyme, from PAGE, gel filtration, SDS-PAGE, and immunoblotting. The fact that aggregates could be detected in the broth filtrate and monomers did not exist in the purified preparation led to the above estimation of the aggregation. It has been demonstrated that there were three forms of cholesterol oxidase extracted from *Nocardia rhodochrous* by treatment with Triton X-100, trypsin, or buffer alone, which differ chiefly in the presence or absence of a hydrophobic anchor region connected by a trypsin-sensitive region; and the enzyme extracted with buffer had a hydrophobic region responsible for binding the whole enzyme to membranes (Cheetham et al., 1982). The extracellular cholesterol oxidase obtained in this report might have a hydrophobic domain in the same situation as the buffer-extracted enzyme described above.

The molecular weight (56 000) of cholesterol oxidase from *R. equi* No. 23 was the same as that found for the extracellular cholesterol oxidase from *S. cholesterolicum* (Inouye et al., 1982) but was larger and smaller than those (32 000 and 61 000) from *B. sterolicum* (Uwajima et al., 1974) and *S. violascens* (Kamei et al., 1978), respectively. Although the particle weights of aggregated enzymes were not closely examined in this study, they could well be dimers judging from the migrated distances in PGGE.

Inhibition studies showed that cholesterol oxidase from *R. equi* No. 23 was more or less inhibited by SH reagents and that this inhibition was almost completely prevented by the addition of cysteine. These findings suggest that SH groups may be involved in the catalytic activity of the enzyme, as shown in the enzyme from *B. sterolicum* (Uwajima et al., 1974). The high sensitivity of the purified enzyme to *N*-bromosuccinimide might depend on the oxidation of cysteine residue, although there are some possibilities that the loss of enzyme activity resulted from oxidation of tryptophan residue (Warwick et al., 1972).

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