

Isolation of Cholesterol Oxidases from *Rhodococcus equi* ATCC 33706

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Rhodococcus equi ATCC 33706 was shown to possess a membrane-bound and a secreted form of cholesterol oxidase. Several detergents (*n*-octyl glucoside, Tween-20, Tween-60, Tween-80, and Brij-35) were tested in 50 mM K₂HPO₄-KH₂PO₄ (pH 7.4) for efficacy to release protein from membrane fragments and prevent aggregation of the secreted enzyme. Brij-35 was most efficient at 0.5%, releasing 35% of membrane-bound protein. Under experimental conditions, 50% of the total cholesterol oxidase activity detected in pelleted fragments was solubilized. *n*-Octyl glucoside decreased the activity of the membrane-bound enzyme and failed to release protein from the fragments. However, octyl glucoside at a 2.0% concentration prevented aggregation of the secreted enzyme. The membrane-bound enzyme was purified 38-fold by Bio-Gel A-1.5m chromatography, whereas isolation from a Sephacryl S-200 column resulted in a 58-fold purification of the extracellular enzyme. Cholesterol oxidase activity for both enzyme preparations was optimal at approximately 40°C; however, the pH optimum (8.0) for the membrane-bound enzyme was much broader for the secreted enzyme (pH 6.0-8.0).

Microbial cholesterol oxidases (EC 1.1.3.6) catalyze the transformation of cholesterol to 4-cholesten-3-one. Interest in these enzymes is in part due to their utility in the determination of serum cholesterol. Since the report by Stadtman *et al.* in 1954 (1) on crude enzyme preparations from *Mycobacterium* sp., cholesterol oxidases have been described in a number of organisms, including *Arthrobacter simplex* (2), *Nocardia erythropolis* (3, 4), *Nocardia rhodochrous* (5, 6, 7) *Brevibacterium sterolicum* (8, 9), *Streptomyces griseocarneus* (10), *Streptomyces violascens* (11), *Corynebacterium sterolicum* (12), *Actinomyces lavendulae* (13), *Streptovercillium cholesterolicum* (14), *Schizophyllum commune* (15), and *Rhodococcus* strains (16). The *Nocardia* enzyme was reported to be intrinsically membrane bound, extractable from whole cells by treatment with Triton X-100 or trypsin but not by mechanical cell disruption nor changes in pH or ionic strength (5). However, the cholesterol oxidases of *Brevibacterium*, *Streptomyces*, and *Streptovercillium* are extracellular enzymes and have been purified to homogeneity from culture filtrates (8, 11, 14, 15). The

Cholesterol Oxidase Assay

Cholesterol oxidase activity was assayed by estimating the decrease in cholesterol concentration, using the colorimetric ferric chloride test (21). The 2-ml reaction mixture contained 540 μM cholesterol (supplied in 200 μl of a 2 mg/ml stock solution of cholesterol in *n*-propanol), 200 μl enzyme extract, and 1.6 ml of PO buffer. The reaction was carried out at 37°C for 1 h.

One unit of activity was defined as the amount of enzyme required to oxidize 1 μmol of cholesterol per minute at 37°C. The specific activity was expressed as units of activity per milligram of protein (U/mg). The protein concentration was determined with the BCA (bicinchoninic acid) protein assay reagent (Pierce Chemical Co., Rockford, IL), according to the manufacturer's recommendations.

Determination of Cholesterol

Test samples were extracted with 4 ml of ethyl acetate and 1.0-ml aliquots of the ethyl acetate layer were evaporated to dryness. Residues were dissolved in 3 ml of the ferric chloride reagent (ferric chloride dissolved at 70 mg/100 ml in glacial acetic acid) and 2 ml of concentrated sulfuric acid was added with vigorous vortexing to obtain thorough mixing. Reaction mixtures were allowed to cool to room temperature and absorbance was read at 560 nm (21).

Samples containing detergent required saponification before determination of residual cholesterol concentration. Saponification was carried out by mixing 1.0 ml of the reaction mixture with 2.0 ml of 95% ethanol and 2.0 ml of 50% KOH. This mixture was incubated at 60°C for 10 min and after cooling it was extracted with 4.0 ml of ethyl acetate. Cholesterol was then determined as described above.

Solubilization of Cholesterol Oxidase

Each of the detergents (*n*-octyl glucoside, Tween-20, Tween-60, Tween-80, and Brij-35) was mixed with CX in PO buffer. Mixtures were held at 4°C for 1 h, inverting the tubes every 15 min to facilitate solubilization of membrane-bound protein. The mixtures were centrifuged at 105,000g and 4°C for 1 h. The supernatant was then removed and the pellet resuspended in the same volume of buffer and detergent concentration. Both the pellet suspension and the supernatant were tested for cholesterol oxidase activity.

BX samples were also treated with each of the detergents in PO buffer at 4°C. After repeatedly inverting the tubes as described above, the tubes were checked for clarity.

Gel Filtration Chromatography

Detergent-extracted CX was applied to Bio-Gel A-1.5m resin (BioRad Laboratories, Richmond, CA) in a 1.0 \times 27 cm column at 4°C. Fractions were eluted with PO buffer containing detergent at the concentration used to extract the membrane-bound enzyme.

Chromatography of detergent-treated BX was with Sephacryl S-200 resin (Pharmacia). The sample was applied to a 1.0 \times 30 cm column, and fractions were eluted with PO buffer at 4°C containing detergent at the concentration used to prevent aggregation of the secreted enzyme.

TABLE I
Solubilization of Free Cholesterol by Various Detergents

Detergent	cholesterol extracted (%) at detergent concentration		
	1.0%	0.1%	0.01%
Tween 80	36	74	95
Tween 20	40	86	97
Triton X-100	98	100	86
Brij-35	0	8	85
Octyl glucoside	96	99	100

Note. Detergents in 50 mM potassium phosphate buffer (pH 7.4) were mixed (1800 μ l) with 200 μ l of cholesterol (2 mg/ml in *n*-propanol). The mixture was extracted with 4 ml of ethyl acetate and the solvent (1-ml aliquots) evaporated. The residue was tested for cholesterol by the ferric chloride method (21).

terol and possibly to its interaction with the membrane fragments that may prevent substrate-enzyme interaction.

Certain detergents formed complexes with cholesterol, resulting in the inextractability of the latter by ethyl acetate without prior saponification of the mixtures. The data in Table I show that Brij-35 solubilized cholesterol more efficiently than other detergents. This characteristic of the polyoxyethylene-fatty alcohol ether class of detergents was studied in detail by Mueller-Goymann and Usselman (23). These authors showed that polyoxyethylene ethers form micellar solutions of aggregates with a leaflet shape which help solubilize cholesterol. Saponification of such mixtures breaks down the complexes and cholesterol may then be extracted by ethyl acetate.

To extract cholesterol oxidase from the membrane fragments, several detergents were brought to 1.5% concentration in the presence of the CX. The effect of the detergents on the activity of cholesterol oxidase was studied by observing enzyme activity in the mixture before centrifugation. With respect to the total activity of the enzyme in the absence of detergent, it increased in the presence of Brij-35 (Table II). To determine which of the detergents was effective in solubilizing membrane-bound

TABLE II
Effect of Detergents on Cholesterol Oxidase Activity

Detergent	Relative Activity (%)
None	100.0
Tween 20	100.7
Tween 60	100.7
Tween 80	106.6
Triton X-100	100.9
Brij-35	111.8
Octyl glucoside	19.5

Note. Enzyme preparations in 50 mM potassium phosphate buffer were treated with each detergent at 1.5% final concentration for 1 h.

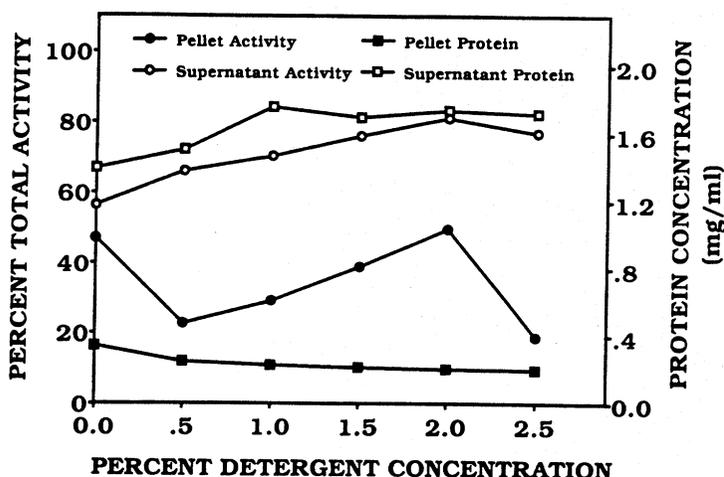


FIG. 2. Solubilization of membrane-bound cholesterol oxidase by Brij-35. Enzyme extracts were treated for 1 h with Brij-35 and centrifuged at 105,000g and 4°C for 1 h. The supernatant was collected and the pellet resuspended in the same volume buffer with detergent. Solubilized cholesterol oxidase was calculated as percentage total activity of the Brij-35-treated extract before centrifugation.

creased with increasing detergent concentration up to 1.0% Brij-35, while the protein content of pellets decreased by approximately 200 $\mu\text{g}/\text{ml}$ with the increase of the detergent to 1.5% concentration.

According to centrifugation studies, 2.0% Brij-35 was optimal for extracting membrane-bound cholesterol oxidase (Fig. 2). During gel filtration chromatography (Bio-Gel A-1.5m) at this detergent concentration, all the activity was eluted from the column in the void volume (peak A in Fig. 3). This apparent contradiction with the results of centrifugation studies was caused by the formation of large micellar aggregates.

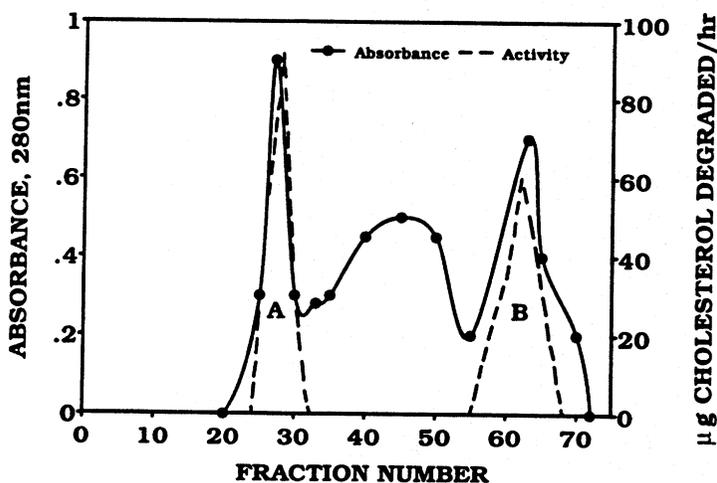


FIG. 3. Gel filtration of CX extracted with 0.5% Brij-35. Enzyme extract (2 mg protein) extracted with 0.5% Brij-35 was fractionated on a 27.4-ml BioGel A-1.5m column. Elution was with 50 mM potassium phosphate buffer (pH 7.4) containing 0.5% Brij-35.

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