[Chem. Pharm. Bull. 25(12)3190—3197(1977)]

UDC 577.153.07 : 576.852.098

Cholesterol Esterase produced by *Streptomyces lavendulae*

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(Received April 13, 1977)

Cholesterol esterase was purified from the culture filtrate of *Streptomyces lavendulae* by a procedure involving precipitation with ammonium sulfate and acetone, gel filtration on Sepharose CL-4B, and rechromatography on Sepharose CL-4B after treatment of Triton X-100. The purified enzyme was detected as a single band by polyacrylamide disc electrophoresis, while one main band and three minor bands were observed by SDS gel electrophoresis. The molecular weight of the main band was 29000. The enzyme was inhibited by Hg^{2+}, Ag^{+} and DFP. Long-chain fatty acid esters of cholesterol were hydrolyzed preferentially and pH optimum was 6.0. This enzyme could be used to the determination of total serum cholesterol with cholesterol oxidase from *Streptomyces violascens*.

Keywords——cholesterol esterase; *Streptomyces lavendulae*; cholesterol oxidase; *Streptomyces violascens*; total serum cholesterol determination

Enzymes which hydrolyze long-chain fatty acid esters of cholesterol are found in many mammalian tissues[2] and have been given the name of cholesterol esterase (EC 3.1.1.13). Cholesterol esterase is known to be important in the absorption and metabolism of cholesterol,[3] and is well studied in a variety of mammalian tissues.[2] However, as for microbial cholesterol esterase except *Pseudomonas fluorescens*,[4,5] little has been known about its properties. We have investigated the enzymatic determination of total serum cholesterol using bovine pancreatic cholesterol esterase and *Streptomyces* cholesterol oxidase.[6]

But, mammalian enzyme is less available than microbial one. So, cholesterol esterase has been sought from microorganisms by screening many strains of *Streptomyces*. *Streptomyces lavendulae* was found to produce a remarkable amount of cholesterol esterase.

The present paper is described on the cultural conditions, purification, some properties and utilization of cholesterol esterase from *Streptomyces lavendulae*.

Materials and Methods

Materials——Cholesterol oxidase (EC 1.1.3.6) was prepared from the culture filtrate of *Streptomyces violascens* as previously described.[7] Peroxidase was obtained from Sigma Chemical Co. and had an activity of 100 units/mg. Other materials were obtained as follows: sodium cholate (Merck Darmstadt), Emulgen 109P (Kao-Atlas Co.), Triton X-100 (Wako PureChemicalCo.), Serachol (Ono Pharmaceutical Co.), Sepharose CL-4B (Pharmacia Fine Chemicals). All other reagents were obtained from commercial sources and were of analytical grade.

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Medium and Cultivation——A screening and seed medium was composed of 1.0% cholesterol palmitate, 0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.05% KCl. A cultivation medium was composed of 1% palmitic acid, 0.2% corn gluten meal, 0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.05% KCl. Initial pH of the medium was adjusted to 7.0 with 1 N NaOH. Cultivation was carried out in a 500-ml Erlenmyer flask containing 80 ml of the medium on rotary shaker (amplitude, 70 mm, 180 rpm) at 28° for 4 days.

Determination of Enzyme Activity——Assay Method I: In the screening of microbes and enzyme purification, enzyme activity was measured by the method of Allain, *et al.*[8] In this method, chloesterol oxidase was used from *Streptomyces violascens* and incubation was performed for 30 min at 37°. One unit of cholesterol esterase activity was defined as the amount of enzyme hydrolyzing 1 μmol of cholesterol ester per min at 37°.

Assay Method II: In the investigation of the enzyme properties, another method was employed to avoid the effect of other enzymes. This method was based on the determination of free fatty acid which was another product of cholesterol ester hydrolysate. The reaction mixture was composed of 0.1 ml of the enzyme solution and 0.5 ml of 0.1 M citrate phosphate buffer (pH 6.0) containing 0.5 μmol of cholesterol linolate and 5 mg of Triton X-100. The reaction was carried out at 37° for 15 min and terminated by the addition of chloroform and *n*-heptane (1 : 1). The produced free fatty acid was measured by the method of Maehata.[9]

Determination of Fatty Acid Specificity for Hydrolysis of Cholesterol Esters——Hydrolyzing rate of some fatty acid esters of cholesterol was measured by the modified method of Allain, *et al.*[8] 1 ml of micellar cholesterol ester (300 nmol) in 1% Triton X-100 was added to 2 ml of 0.1 M citrate phosphate buffer (pH 6.0) containing 0.82 mM 4-aminoantipyrine, 14 mM phenol, 3.8 units of peroxidase, 0.074 unit of *Streptomyces* cholesterol oxidase and 0.001 unit of cholesterol esterase. The reaction was carried out at 37° for 30 min and terminated by heating at 100° for 3 min. The produced colour was measured at 500 nm.

Protein Determination——Protein concentration of an enzyme preparation was measured by the method of Lowry, *et al.*[10] Protein concentration in eluate of Sepharose CL-4B column was measured by the fluorometric method using fluorescamine.[11]

Sugar Determination——Qualitative analysis of sugar was performed by the methods of Molish[12] and anthrone-H_2SO_4 reaction.[13] Quantitative analysis of sugar was performed by the method of Dubos, *et al.*[14]

Electrophoresis——Polyacrylamide disc gel electrophoresis was performed by the method of Davis.[15] SDS polyacrylamide gel electrophoresis was also performed by the method of Weber, *et al.*[16]

Determination of Molecular Weight——The molecular weight of the purified enzyme was estimated by SDS polyacrylamide gel electrophoresis. The marker proteins used were cytochrome C (mol. wt. 11700), trypsin (23300), alcohol dehydrogenase (41000) and bovine serum albumin (68000).

Results

Selection of Microorganisms

The primary screening was performed by examining thin layer chromatograms of the incubation product of the culture filtrate and cholesterol linolate. Thirteen strains out of 205 *Streptomyces* strains were shown to produce cholesterol esterase. The strains to produce cholesterol esterase were recultivated in the screening medium which contained cholesterol palmitate as a sole carbon source. One species of *Streptomyces* was found to produce a remarkable amount of cholesterol esterase. This strain was resemble to *Streptomyces lavendulae*[17] in the properties and identified as *Streptomyces lavendulae* H-646-SY2.

Cultural Condition for Production of Cholesterol Esterase by *Streptomyces lavendulae* H-646-SY2

Effect of various lipids on the extracellular production of cholesterol esterase was investigated. As shown in Table I, the highest enzyme activity was obtained when cholesterol

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palmitate or palmitic acid was used as the carbon source. Using palmitic acid as a sole carbon source, the most suitable nitrogen source was corn gluten meal (Table II). In the cultivation medium, cholesterol esterase activity was detected at 2 days, and reached maximum at 4—5 days after cultivation.

Table I. Effect of Various Lipids on the Formation of Cholesterol Esterase

Lipids	Cultivation time, days	Cholesterol esterase activity, units/ml
Cholesterol palmitate	17	0.280
Cholesterol acetate	—	0
Cholesterol laurate	26	0.060
Cholesterol stearate	26	0.083
Cholesterol oleate	26	0.029
Cholesterol linolate	—	0
Lauric acid	—	0
Palmitic acid	13	0.289
Stearic acid	—	0
Oleic acid	—	0
Linolenic acid	—	0
Linoleic acid	—	0
Olieve oil	—	0
Soybean oil	—	0

Lipid was added to the medium composed of 0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.05% KCl at a concentration of 1% and pH of the medium was adjusted to 7. The cultivation was carried out at 28°. The enzyme activity was measured by "Assay Method I."

Table II. Effect of Nitrogen Sources on the Formation of Cholesterol Eeterase

Nitrogen source	Cultivation time, days	Cholesterol esterase activity, units/ml
Soya meal	7	0.118
Pharma media	7	0.590
Corn gluten meal	4	0.962
Meat extract	6	0.017
Polypepton S	—	0
Glycine	—	0
Yeast extract	—	0

Nitrogen source was added to the medium composed of 1% palmitic acid, 0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.05% KCl at a concentration of 0.2% and pH of the medium was adjusted to 7. The cultivation was carried out at 28°. The enzyme activity was measured by "Assay Method I."

Purification of Cholesterol Esterase from the Culture Filtrate

All procedures were carried out at 4° unless otherwise stated. After the four days cultivation, mycelia was removed by centrifugation. Solid ammonium sulfate was added to the supernatant up to the concentration of 65% saturation. After standing overnight, precipitate was collected by centrifugation and was dissolved in a minimum volume of distilled water. The solution was then dialyzed against distilled water for 24 hr. Cold acetone was added to the dialyzed solution up to the concentration of 75%, and the resulting precipitate was collected by centrifugation and was dissolved in a minimum volume of 0.1 M phosphate buffer (pH 7.0). It was confirmed by the Molish[12] and anthrone–H_2SO_4[13] reactions that this enzyme preparation contained sugar moiety.

Gel Filtration on Sepharose CL-4B

15 ml of acetone precipitate fraction (23.4 units, 5.2 mg as protein, 9.8 mg as glucose) was subjected to gel filtration on a Sepharose CL-4B column (5×70 cm), which was previously equilibrated with 0.1 M phosphate buffer (pH 7.0). The elution pattern of the enzyme is shown in Fig. 1a. The enzyme activity was eluted in relatively large molecular weight fractions and these fractions contained sugar moiety. The active fractions (22.3 units, 2.8 mg as protein, 8.1 mg as glucose) were combined and concentrated by ultrafiltration.

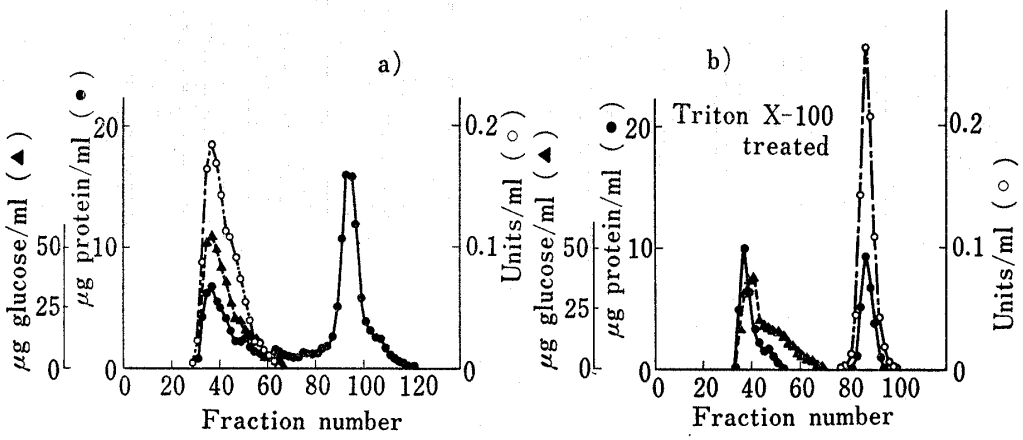

Fig. 1. Gel Filtration Patterns of Cholesterol Esterase on Sepharose CL-4B

a) Acetone ppt. fraction (23.4 units) was dissolved in 15 ml of 0.1 M phosphate buffer (pH 7.0), and applied to a column (5×70 cm) of Sepharose CL-4B. Column was eluted with 0.1 M phosphate buffer (pH 7.0) at a flow rate, 60 ml/hr.

b) Active fractions from a) were collected and concentrated to 15 ml. After treatment by Triton X-100, enzyme preparation (16.8 units) was rechromatographed on Sepharose CL-4B column (5×70 cm) equilibrated with 0.1 M phosphate buffer (pH 7.0) containing 1% Triton X-100. Column was eluted with the same buffer at a flow rate, 60 ml/hr. 10 ml fractions were collected.

Gel Filtration of the Enzyme treated with Triton X-100 on Sepharose CL-4B

Concentrated enzyme was treated with Triton X-100 (final concentration 1%) at 4° overnight. 15 ml of treated enzyme solution (16.8 nuits, 2.6 mg as protein, 7.2 mg as glucose) was subjected again to gel filtration on Sepharose CL-4B column (5×70 cm), which was previously equilibrated with 0.1 M phosphate buffer (pH 7.0) containing 1% Triton X-100. The elution pattern of the enzyme is shown in Fig. 1b. The enzyme activity was separated from the fractions containing sugar moiety, and eluted in relatively low molecular weight fractions. The active fractions (15.8 units, 1.6 mg as protein, sugar was not detected) were combined and concentrated by ultrafiltration. This fraction was used for the characterization of the enzyme. The purification procedures are summarized in Table III. The enzyme preparation was purified to 14.5 folds in specific activity with a recovery of 43% of the original activity.

Table III. Summary of Purification of Cholesterol Esterase

Fraction	Total protein (mg)	Total units	Specific activity (units/mg)	Recovery (%)
Broth sup.	2059	874	0.43	100
$(NH_4)_2SO_4$ ppt	210	726	3.5	83
Acetone ppt	125	560	4.5	64
Sepharose CL-4B (a)	57	465	8.0	53
Sepharose CL-4B (b)	38	377	9.9	43

Electrophoresis of the Purified Preparations

As shown in Fig. 2, the purified preparation showed one band by the disc electrophoresis. But, one main band and three minor bands were detected by the SDS polyacrylamide gel electrophoresis.

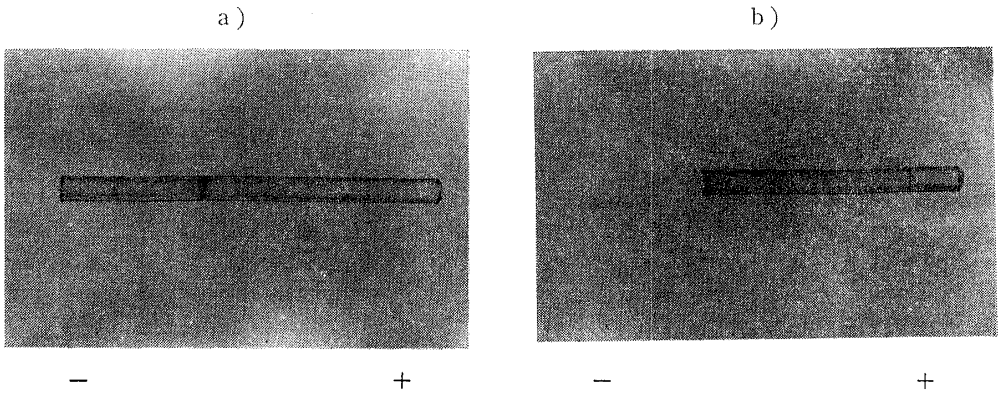

Fig. 2. Electrophoretic Patterns of Purified Cholesterol Esterase

a) SDS-Polyacrylamide gel electrophoresis.
b) Polyacrylamide gel disc electrophoresis.

Molecular Weight

The molecular weights of main band and three minor bands were estimated to be about 29000, 10000, 12000 and 92000, respectively by SDS polyacrylamide gel electrophoresis.

Effect of pH

As shown in Fig. 3, cholesterol esterase was the most active at pH 6, and its activity decreased rapidly above pH 6. Cholesterol esterase was stable in the range from pH 5.5 to 8.0 at 37° C for 1 hr.

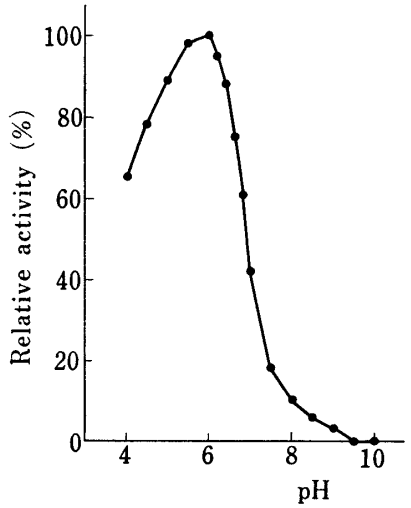

Fig. 3. Optimum pH for the Activity of Cholesterol Esterase

The enzyme activity was measured by "Assay Method II" with 0.1 M buffer as indicated. The buffers used were citrate-phosphate buffer pH 4—6, phosphate buffer for pH 6—8, Tris-HCl buffer for pH 8—9 and carbonate-bicarbonate buffer for pH 9—10.5.

Table IV. Effect of Inhibitors on Cholesterol Esterase Activity

Inhibitor (1 mM)	Inhibition (%)
$AgNO_3$	41
$HgCl_2$	100
$CoCl_2$	14
$NiCl_2$	15
EDTA	0
8-Hydroxyquinoline	0
N-Ethylmaleimide	0
PCMB	0
Diisopropyl fluorophosphate	96

The residual activity was measured by "Assay Method II" after treatment with the inhibitor for 30 min at 37°.

Effects of Metal Ions and Some Reagents

As shown in Table IV, cholesterol esterase activity was inhibited by heavy metal ions such as Ag^{+} and Hg^{2+}, and also by DFP. No inhibition was observed with metal chelating agents and –SH reagents.

Substrate Specificity of Cholesterol Esterase

Table V shows that cholesterol esterase hydrolyzed preferentially long-chain fatty acid esters of cholesterol. Cholesterol linolate was the most suitable substrate for the present cholesterol esterase.

Table V. Fatty Acid Specificity of Cholesterol Esterase

Cholesterol ester	Relative activity (%)
Linoleate (18:2)	100
Oleate (18:1)	42
Stearate (18:0)	40
Palmitate (16:0)	20
Acetate (2:1)	4

The assay procedures are described under Method.

Requirement of Detergents for Cholesterol Esterase Activity

Table VI shows the requirement of detergents for cholesterol esterase activity. The enzyme activity was very low against cholesterol esters in Serachol without a detergent. Non-ionic detergents such as Triton X-100 and Emulgen 109 P activated the enzyme effectively at a concentration between 0.03 and 0.125%. Sodium cholate did not stimulate the enzyme activity as much as non-ionic detergents and did not enhance the activity stimulated with non-ionic detergents.

Table VI. Effect of Detergents on Cholesterol Esterase Activity

Detergent	Optimum Concn.	Relative activity (%)
None	—	100
Triton X-100	0.125—0.031%	508
Emulgen 109 P	0.125—0.061%	518
Sodium cholate	5—10 mM	380
Triton X-100 Sodium cholate	0.0625% 10 mM	483
Emulgen 109 P Sodium cholate	0.0625% 10 mM	500

Cholesterol esterase activity was measured with various detergents against cholesterol esters in Serachol by "Assay Method I".

Application of Cholesterol Esterase for the Determination of Total Serum Cholesterol

Optimum condition for the determination of total serum cholesterol was summarized in Table VII. Under the condition in Table VII, cholesterol esters in Serachol were completely hydrolyzed to free cholesterol by the present cholesterol esterase, and free cholesterol was completely oxidized to cholest-4-en-3-one by cholesterol oxidase isolated from *Streptomyces violascens*. These enzymatic reaction products were confirmed by thin layer chroma-

tography. As shown in Table VIII, human total serum cholesterol could be determined, and obtained values were well correlated with those obtained by the method of Zak-Henly.[18]

Table VII. Assay Condition for the Determination of Total Human Serum Cholesterol

Ingredient	Concn.
4-Aminoantipyrine	0.82 mM
Phenol	14 mM
Triton X-100	0.0625%
Cholesterol esterase	180 U/l
Cholesterol oxidase	37 U/l
Peroxidase	1900 U/l
in 0.05 M sodium phosphate buffer (pH 6.0)	

25 μl of human serum was added to 2.5 ml of above reagent, incubated for 30 min at 37° and the absorbance of produced quinoneimine dye was measured at 500 nm.

Table VIII. Comparison of the Total Serum Cholesterol Value by the Present Method and the Method of Zak-Henly

Sample	Present method (mg/dl)	Method of Zak-Henly (mg/dl)
A	230	217
B	219	209
C	208	207
D	188	189
E	152	164

Discussion

Streptomyces lavendulae H-646-SY2 produced a remarkable amount of cholesterol esterase extracellulary when cultured in a medium containing palmitic acid as a sole carbon source. This cholesterol esterase was purified by ammonium sulfate fractionation, acetone precipitation, Sepharose CL-4B gel filtration and rechromatography on Sepharose CL-4B after treatment with Triton X-100. Differences of gel filtration patterns of the enzyme suggest that cholesterol esterase might exist with macromolecule, which was separated by the treatment of Triton X-100. From the result of disc electrophoresis, the purified enzyme preparation was almost homogeneous. Hyun, *et al.*[19] has purified cholesterol esterase from rat pancreatic juice and characterized the enzyme properties. Also, Uwajima, *et al.*[4,5] has isolated and purified cholesterol esterase from *Pseudomonas fluorescens*, and reported on the enzymatic properties. Several properties were different among these cholesterol esterases. Cholesterol esterase described here was inhibited by heavy metal ions such as Hg^{2+} and Ag^{+}, but not inhibited by Cu^{2+}, which was described to inhibit the enzyme of *Pseudomonas* origin.[5] PCMB was reported to inhibit rat pancreatic cholesterol esterase,[19] but not to inhibit the present and *pseudomonas* enzyme.[5] The present purified enzyme was inhibited by DFP, which was

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described to inhibit the mammalian enzyme[2] and not to inhibit the *Pseudomonas* emzyne.[5] Inhibition by DFP suggested that the present cholesterol esterase contained serine in the active site. It was reported[19] that rat pancreatic enzyme hydrolyzed cholesterol oleate (18: 1), cholesterol stearate (18: 0) and cholesterol palmitate (16: 0) more rapidly than cholesterol linolate (18: 2). Also, Sakamoto, *et al.*[20] reported that rat liver cholesterol esterase hydrolyzed rapidly cholesterol acetate (2: 0) more than long-chain fatty acid esters. The present enzyme hydrolyzed cholesterol linolate effectively and its property was similar to the enzyme from *Pseudomonas*.[5] Our previous paper[6] described that bovine pancreatic cholesterol esterase was effectively activated by sodium cholate. However, present purified enzyme was effectively activated by non-ionic detergents such as Triton X-100 and Emulgen 109 P. Activation of cholesterol esterase by non-ionic detergents are thought that the structure of substrate is changed into the micellar form. The present cholesterol esterase could be used to the specific and sensitive determination of total serum cholesterol, together with the use of cholesterol oxidase from *Streptomyces violascens*.[7]

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