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Purification of Cholesterol Esterase from *Aeromoans* sp.

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Aeromoans sp.가 생산하는 콜레스테롤 에스테라아제의 정제

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ABSTRACT

A cholesterol esterase-producing microorganism, strain CES 506, isolated from soil was identified as *Aeromonas* sp. This strain produce about 0.023 units of cholesterol esterase per ml of culture broth. The cholesterol esterase produced by this strain was purified 370 fold to homogeneity in an overall yield of 24% from culture broth. The apparent molecular weight was 64,000, as estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The enzyme showed a high substrate specificity for cholesterylpalmitate and the Km value for the hydrolysis of cholesterylpalmitate by this enzyme was 0.15mM.

INTRODUCTION

Cholesterol esterase(EC 3.1.1.13) catalyses the synthesis or hydrolysis of cholesterol ester of fatty acids. Since the first report on cholesterol esterification in mammals(1), the same reaction has been reported in a number of microorganisms such as *Pseudomonas fluorescens*(2), *Streptomyces lavendulae*(3). Morover, this enzyme has been purified and partially characterized from several microorganisms(4, 5), and mainly used for determination of cholesterol by coupling with cholesterol oxidase and peroxidase in clinical specimens (6). In the course of a study on the decomposition of cholesterol by bacterial cells, this laboratory found a new bacterial strain that produce extracellular cholesterol oxidase(7). As the second step of bacterial decomposition of cholesterol, we initiated the screening for cholesterol esterase-producing microorganisms from soil. Here we present the taxonomic characteristics of a strain isolated from soil that exhibited highest activity in culture broth among the bacterial strains tested and the purification of extracellular cholesterol esterase from the organism.

MATERIALS AND METHODS

Microorganism and culture conditions

Cholesterol esterase producing microorganisms were isolated by the following procedure. Soil suspension was spread onto agar plates containing L-broth(1.0% peptone, 0.4% yeast extract, 0.1% glucose, 0.5% NaCl, pH 7.2) and was solidified with 1.5% agar. The plates were incubated at 30℃ for about 24 h and bacterial colonies that appeared were replica plated by toothpick onto the L-broth plates. The replica plates were inc-

ubated at 30℃ for 24 h. To selected the cholesterol esterase-producing strains, we used a colony straining method on agar plates. Filter papers were dipped into 0.5% cholesterylpalmitate, 1.76% 4-aminoantipyrine, 6% phenol, 300 units/liter cholesterol oxidase and 3,000 units/liter horseradish peroxidase, 1.0% Triton X-100 in 100 mM potassium phosphate buffer, pH 7.0 (KPB), were placed on colonies grown on agar medium and incubated at 37℃. The cholesterol esterase activity of the test colonies was indicated by a red color, due to the formation of quinoneimine dye. Strains able to produce the red color were selected and cultivated at 30℃ in 5 ml liquid L-broth with constant shaking. The cells were collected by centrifugation, and the culture broth was assayed for extracellular activity of cholesterol esterase. A bacterial strain, named strain CES 506, was selected. The medium used for the purification of the enzyme was a L-broth and the cells were grown at 30℃ for 36 h with reciprocal shaking in 500 ml Sakaguchi flasks containing 100 ml medium.

Characterization and identification of the strain

Taxonomic characteristics of strain CES 506 were investigated by the procedures described in Bergey's Manual of Systematic Bacteriology(8).

Determination of cholesterol esterase activity

Cholesterol esterase activity was assayed by the method of Allain et al.(6). One unit of the enzyme activity was defined as the amount of enzyme which catalysed the decomposition of 1 μmole of cholesterylpalmitate per min. at 37℃.

Protein determination

Protein was determined by the method of Lowry et al.(9) using bovine serum albumin as a standard.

Determination of molecular weight

Molecular weight of the enzyme was estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis(SDS-PAGE) (10).

RESULTS AND DISCUSSION

Characterization of the strain

Isolation of soil microorganisms that produce extracellular cholesterol esterase was carried out using a colony staining method on agar plates. From 600 samples of soil, two strains of microorganism produce extracellular cholesterol esterase. One of them, named CES 506, was selected. This strain was a Gram-negative, rod shaped with rounded ends to coccoid(1.0×1.8μm), facultatively anaerobic, motile bacterium with polar flagella. This strain reacted positively to the following tests: catalase; oxidase; hydrolysis of gelatine, starch and casein; oxidation and fermentation; indole and pyoverdin formation. It reacted negatively urease. The pH range for growth was 5.5–9.0 and optimum temperature for growth was 30℃. The above morphological and physiological characteristics of the strain seems to fit the genus *Aeromonas*(8) and thus the strain was tentatively named *Aeromonas* strain CES 506. A electron micrograph of this strain is shown in Fig. 1.

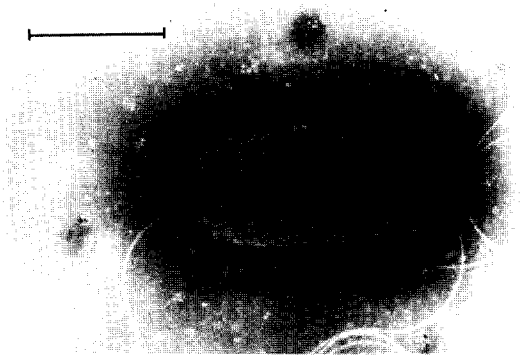

Fig. 1. Electron micrograph of *Aeromonas* sp. strain CES 506. Bar, 1.0μm.

Purification of cholesterol esterase

All purification steps were conducted at 0–5℃, unless otherwise specified.

Step 1. The culture broth(7,000 ml) was centrifuged at 10,000 rpm for 10 min and the clear superatants obtainted were used for purification of the enzyme. The clear supernatants(5,670 mg protein, 65,500 ml) was concentrated by ultrafiltration on a Pellicon Membrane PTGC OLC M2 (Millipore) to 100ml.

Step 2. To the concentrated culture broth from Step 1, 24.3 g of solid ammonium sulfate was added and then applied to a Butyl Toyopearl 650M column(3cm × 15cm) equilibrated with 10 mM KPB(pH 7.0) con-

taining 40% ammonium sulfate. After eluting the column with decreasing linear concentration gradient of ammonium sulfate(40% – 0%, volume 400ml) in 10 mM KPB. The enzyme was eluted with linear gradient of Triton X–100(0% – 3%, 400 ml) in 10mM KPB. The active fractions were pooled and dialyzed against 10 mM KPB.

Step 3. The dialyzates from Step 2(60 ml) were loaded onto a palmitic acid affinity column(2 cm × 30 cm) equilibrated with 10mM KPB and the enzyme was eluted with linear gradient of Triton X–100(0% – 3%, 200 ml) in 10 mM KPB. Active fractions were combined and used for characterization. The procedures for purification of the enzyme are summarized in Table 1 and in Fig. 2. The purified enzyme is apparently homogeneous as judged by SDS–PAGE(Fig. 2. lane 4).

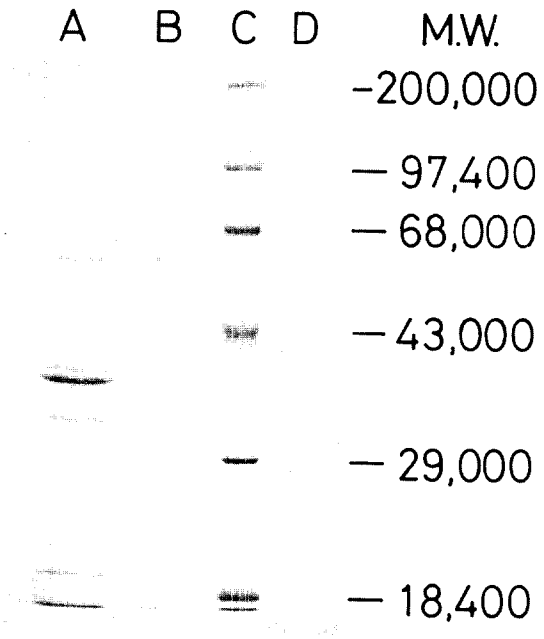

Fig. 2. SDS–Polyacrylamide gel electrophoresis of cholesterol esterase at the various purification steps. Lane A, culture broth: lane B, after Butyl Toyopearl 650M: lane C, marker proteins and lane D, after palmitic acid column chromatography: The marker proteins and their molecular weights were as follows: myosin(H-chain) (200,000): phosphorylase b(97,400): bovine serum albumin(68, 000): ovalbumin (43,000): carbonic anhydrase(29,000) and β-lactoglobulin(18,400). The gel was stained for protein with Coomassie Brillant Blue R-250.

Some properties of cholesterol esterase

The molecular weight of the enzyme was estimated to be 64,000 by SDS–PAGE(Fig. 2). The Km value for the hydrolysis of cholesterylpalmitate was calculated to be 0.15 mM from Lineweaver-Burk plot. The enzyme was most active at pH 7.0 when activity was assayed in 100 mM KPB at 37℃. To determine the pH stability of the enzyme, 2.5μg enzyme in 0.5 ml of 10 mM Tris-malate buffer was incubated at various

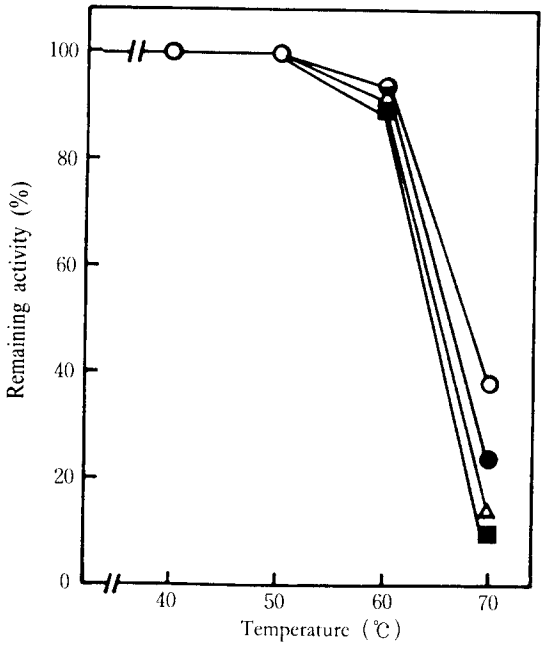

Fig. 3. Effect of temperature on the stability of cholesterol esterase activity. The enzyme(10 μg) was incubated in 2.0 ml of 100 mM potassium phosphate buffer (pH 7.0) at various temperatures for 5(○), 10(●), 30(△) and 60(■) min, and remaining activity was measured. The activity at 40℃ for 5 min was taken as 100%.

Table 1. Summary of the purification of the cholesterol esterase from *Aeromonas* strain CES 506

Step	Protein	Total activity (unit*)	Specific activity (unit/mg)	Yield (%)	Fold
Culture borth	5,670	152	0.026	100	1
Butyl toyopearl	58	87	1.5	57	58
Palmitic acid	3.8	37	9.7	24	373

* unit; μmole/min.

pH values for 1 min at 60℃ and the remaining activity was assayed under the standard conditions. No appreciable loss in activity was found between pH 5.0 and 9.0(data not shown). Thirty percent activity loss was observed at pH 10.0. To determine the heat stability of the enzyme, 10 μg enzyme was treated in 2.0 ml of 100 mM KPB for several minutes at various temperatures. After treatment, the enzyme solutions were immediately chilled to 20℃ and remaining activities were assayed. The enzyme retained its full activity on heating at 60℃ for 30 min, and about 90% of the original activity after heating at 60℃ for 30 min. On the other hand, the activity was rapidly lost by heating at 70℃ (Fig. 3). The heat and pH stability of this enzyme gives a great advantage in the clinical uses.

Although the productivity of cholesterol esterase in this strain is low, available source of cholesterol esterase gene is now provided, and consequently next step is cloning of the gene. Gene library construction and screening of transformants are in progress.

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요 약

콜레스테롤 에스테라아제를 생산하는 미생물 strain CES506 균주를 토양으로부터 분리하여 *Aeromonas* 속의 한 세균으로 동정하였다. 이 균주는 배양액 1ml 당 0.023 단위의 콜레스테롤 에스테라아제를 생산하였다. 이 균주가 생산하는 본 효소를 균주 배양액으로부터 약 370배, 24%의 수율로 균질한 상태의 단백질로 정제하였다. 본 효소의 분자량은 SDS-PAGE로 산출한 결과 64,000으로 계산되었다. 본 효소는 cholesteryl-palmitate에 대해 높은 기질 특이성을 나타내었으며 cholesterylpalmitate의 가수분해에 대한 Km값은 0.15 mM이었다.

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