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A Method to Screen Anticholesterol Substances produced by Microbes and a New Cholesterol Oxidase produced by *Streptomyces violascens*[1]

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A new method to screen anticholesterol substances produced by microbes was devised and the method was applied for cultured broth of *Streptomyces*. Only one strain, resembled to be *Streptomyces violascens*, out of more than 200 *Streptomyces* was determined to produce an anticholesterol substance by this screening method. Furthermore, a new method to measure the anticholesterol activity was established for fermentation and purification of the active component. The partially purified active component was proved to be a new enzyme which oxidized cholesterol to cholest-4-en-3-one. Nicotinamide adenine dinucleotide or nicotinamide adenine dinucleotide phosphate was not necessary for this enzymatic oxidation. It was found that the enzyme was active against the 3β-hydroxyl group on the steroid skeleton but not against the 3α-hydroxyl group by investigating the substrate specificity of the enzyme.

A new method to screen anticholesterol substances produced by microbes was devised on a basis of the antagonism between polyene antifungal antibiotics and cholesterol against yeasts.[3] A paper strip immersed in a broth filtrate and a paper strip containing trichomycin and cholesterol were placed crosswisely on an assay plate inoculated *Candida yu* 1200 as a test organism. The assay plate was incubated at 37° for 17 hr to examine the antimicrobial zone. Polyene antibiotics, non-polyene antibiotics, synergistic substances for polyene antibiotics and antagonistic substances for cholesterol showed different patterns of antimicrobial zones (pattern A, B, C and D as shown in Fig. 1) by this screening method.

Broth filtrates of more than 200 strains of *Streptomyces* were tested for the anticholesterol activity and only the broth filtrate of *Streptomyces* H 82 N-SY 7, our tentative collection number of *Streptomyces*, was shown to exhibit the antimicrobial zone of pattern D. *Streptomyces* H 82 N-SY 7 was isolated from a soil sample collected at Shiga-kogen, Nagano Prefecture and classified to belong to *Streptomyces violascens*.[4]

Streptomyces H 82 N-SY 7 did not produce an antiyeast antibiotic and the anticholesterol component dit not show antiyeast activity. Therefore, the cylinder agar plate method was modified to measure the anticholesterol activity during fermentation and purification of the

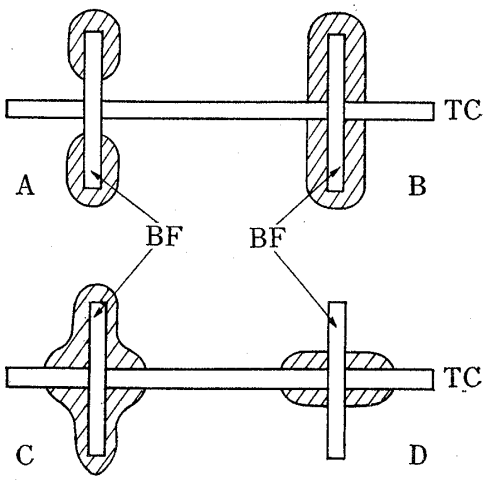

Fig. 1. Screening Method for Anticholesterol Substance

test organism: *Candida yu* 1200
antimicrobial zone: ▨
A: polyene antibiotic
B: non-polyene antibiotic
C: synergist for polyene antibiotic
D: antagonist for cholesterol
TC: trichomycin-cholesterol paper strip
BF: broth filtrate paper strip

1) This is part I of Anticholesterol Substances produced by Streptomyces by S. Nakamura.
2) Location: *1-2-3, Kasumi, Hiroshima.*
3) D. Gottlieb, H.E. Carter, J.H. Sloneker, and A. Ammann, *Science*, **128**, 361 (1958).
4) E.B. Shirling and D. Gottlieb, *Intern. J. Systemat. Bacteriol.*, **18**, 380 (1968).

active component. An assay cup filled with a test sample was placed on the assay plate of *Candida yu* 1200 containing trichomycin and cholesterol in the seed layer and incubated at 37° for 17 hr. The antimicrobial zone due to liberated trichomycin by destruction of cholesterol was measured to determine the anticholesterol activity.

Streptomyces H 82 N-SY 7 was cultured in shaking flasks containing a medium composed of potato starch, glucose, meat extract, peptone, and various inorganic salts at 27—28° for 96 hr on a reciprocal shaker to produce the anticholesterol substance. The active component was not extracted with organic solvents and the activity was lost at acidic pH less than 3 at room temperature or 5 minutes heating at 80° at pH 7.0. The active component was collected from the broth filtrate by cold centrifugation after precipitated by saturation with ammonium sulfate. The crude active powdre was recovered from the precipitate by lyophilization after dialysis in a cellophane tube against water. The crude powder, thus obtained, was fractionated on Sephadex G 75 to obtain the partially purified component. The lyophilized active fractions were tested respectively by electrophoresis on a cellulose acetate film using 0.1 M Tris-HCl buffer (pH 7.0) for 1 hr at 1 mA/cm and Ponceu 3R reagent was used to detect the active component of protein nature. The partially purified component showed the four reddish spots at 5.0, 9.5, 11.0, and 16.5 mm toward the cathode by the electrophoresis.

A mixture of cholesterol and the partially purified component in a phosphate buffer (pH 7.0) was incubated at 27° for 20 hr on a reciprocal shaker and the resulting transformed product was purified as white needles, mp 78°, by thin-layer chromatography on Silica gel G and following recrystalization from methanol. The transformed product was identified to be cholest-4-en-3-one by admixture and comparison of infrared (IR) spectra with an authentic specimen. Thus, the anticholesterol substance produced by *Streptomyces* H 82 N-SY 7 was proved to be an enzyme having an activity of oxidizing cholesterol to cholest-4-en-3-one.

Table I. Substrate Specificity

Substrates	Substituents at 3-	4-	5-	16-	17-	20-	
Cholest-5-en-3β-ol (cholesterol)	β-OH		Δ				+
5α-Androstan-3β-ol-17-one	β-OH		α-H		=O		+
Androst-5-en-3β-ol-17-one	β-OH		Δ		=O		+
5β-Cholestan-3β-ol	β-OH		B-H				+
5α-Cholestane-3β,5α-diol	β-OH		α-H				+
Androst-4-ene-3β,17β-diol	β-OH	Δ			β-OH		+
Androst-5-ene-3β,17β-diol	β-OH		Δ		β-OH		+
Pregn-5-en-3β-ol-20-one	β-OH		Δ			=O	+
5α-Pregnan-3β,16β,20α-triol	β-OH		α-Me	β-OH		α-OH	+
5α-Lanosta-8,24-dien-3β-ol (lanosterol)	β-OH	Me_2	α-OH				−
5β-Pregnane-3α,16β,20α-triol	α-OH		β-Me	β-OH		α-OH	−
5β-Androstan-3α-ol-17-one	α-OH		β-H		=O		−
5α-Androstan-3α-ol-17-one	α-OH		α-H		=O		−
Androst-4-en-17β-ol-3-one	=O	Δ			β-OH		−
5α-Androstan-17β-ol-3-one	=O		α-H		β-OH		−
5β-Androstan-17β-ol-3-one	=O		β-H		β-OH		−
Estr-4-en-17β-ol-3-one	=O	Δ			β-OH		−
Androst-4-en-17α-ol-3-one	=O	Δ			α-OH		−
Estra-1,3,5(10)-triene-3,17β-diol	OH				β-OH		−
Estra-1,3,5(10)-triene-3,16α,17β-triol	OH			α-OH	β-OH		−
Estra-1,3,5(10)-trien-3-ol-17-one	OH				=O		−
3α-Acetoxy-5β-pregnan-16β,20α-diol	AcO		β-Me	β-OH		α-OH	−
Pregn-4-en-16β,20α-diol-3-one	=O	Δ		β-OH		α-OH	−
Pregn-4-en-20α-ol-3-one	=O	Δ				α-OH	−
Pregn-4-en-20β-ol-3-one	=O	Δ				β-OH	−
Cholestan-5α-ol-3-one	=O		α-OH				−

Δ: double bond, +: oxidized, −: not oxidized

Substrate specificity of the enzyme was studied using sterols as substrates and the result was shown in Table I. The enzyme oxidized stereospecifically the hydroxyl group on 3β-position of the steroid skelton to the carbonyl group regardless of junction between A-ring and B-ring. Addition of nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP) was not necessary for this enzymatic oxidation. Nevertheless, the hydroxyl group on 3β-position of 4,4'-disubstituted steroids such as lanosterol was not oxidized by this enzyme. While the hydroxyl groups of 3α-position and of other position on the steroid skeleton were not oxidized by the enzyme.

3β,17β-Hydroxysteroid dehydrogenase (E.C. 1.1.1.51) and 3α-hydroxysteroid dehydrogenase (E.C. 1.1.1.50) were isolated as intracellular enzymes of *Pseudomonas testosteroni* and those enzymes required NAD or NADP for their enzymatic oxidation of hydroxysteroids.[5] 3β,17β-Hydroxysteroid dehydrogenase oxidizes the hydroxyl groups of 3β- and 17β-position of steroids and 3α-hydroxysteroid dehydrogenase is specific for the 3α-hydroxyl group of sterols.[5] The cholesterol oxidase (E.C. 1.1.3.6.) was obtained as an intracellular enzyme produced by *Micobacterium cholesterolicum*[6] and the enzyme oxidized cholesterol to cholest-4-en-3-one without addition of NAD or NADP. Thus, the enzyme produced by *Streptomyces* H 82 N-SY 7 can be resembled to that of *Mycobacterium* origin but the former is unique because of the extracellular nature of *Streptomyces* origin.

Further purification of the enzyme on DEAE-cellulose and thereafter on Sephadex G 200 gives the highly purified enzyme which shows one spot at 5 mm toward the cathode by the electrophoresis. The same results, above mentioned, are obtained by study of substrate specificity using the purified enzyme. Purification procedures and enzymatic studies will be reported in a following paper.

Experimental

Screening Method of Anticholesterol Substances——*Candida yu* 1200 was used as a test microbe on a glucose neutrient agar in an assay plate (7.5×22.5 cm). The trichomycin–cholesterol paper strip and the broth filtrate paper strip were placed crosswisely on the assay plates as shown in Fig. 1. The assay plate was incubated at 37° for 17 hr and the antimicrobial zone was determined. A paper strip (0.4×20 cm) was immersed in the trichomycin–cholesterol solution and dried in air to prepare the trichomycin–cholesterol paper strip. The trichomycin–cholesterol solution was made of 3 volumes of trichomycin solution (1 mg/5 ml in acetone: H_2O=1: 1) and 1 volume of cholesterol solution (1 mg/ml in MeOH: acetone=9: 1). A paper strip (0.4×2 cm) immersed in a broth filtrate was used as the broth filtrate paper strip.

Assay for the Anticholesterol Activity by Cylinder Plate Method——*Candida yu* 1200 was used as a test microbe on a glucose neutrient agar. The seed agar (5 ml) containing 0.075 ml of trichomycin solution (1 mg/ml in EtOH) and 0.025 ml of cholesterol solution (6 mg/ml in EtOH) was placed on the basal agar (10 ml) in an assay plate. The assay plate was kept at 37° for 30 min to remove EtOH. Cylinders were placed on the seed agar and filled with test solutions. After 17 hr incubation at 37°, the diameter of inhibition zone due to activated trichomycin was determined.

Production of the Enzyme——*Streptomyces* H 82 N-SY 7 was cultivated in a shaking flask (500 ml) containing a medium (100 ml) composed of maltose 1% and yeast extract 0.4% (pH 7.0—7.2) for 44 hr at 27—28° on a reciprocal shaker (amplitude 7 cm, 130 rpm) to prepare a inoculation seed. A medium containing potato starch 1.0%, glucose 1.0%, meat extract 3.0%, peptone 0.75%, NaCl 0.3%, $MgSO_4 \cdot 7H_2O$ 0.1%, $MnCl_2 \cdot 4H_2O$ 0.0008%, $CuSO_4 \cdot 5H_2O$ 0.0007%, $ZnSO_4 \cdot 7H_2O$ 0.0002%, and $FeSO_4 \cdot 5H_2O$ 0.0001% (pH 7.2) was used for production of the enzyme. The inoculation seed (2 ml) was inoculated to the production medium (100 ml) in a shaking flask (500 ml) and shake-cultured at 27—28° for 96 hr. The cultured broth showed an antimicrobial zone (diameter 22—24 cm) by the cylinder agar plate method for anticholesterol activity.

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6) T.C. Standtman, A. Cherkes, and C.B. Anfinsen, *J. Biol. Chem.*, **206**, 511 (1954).

Isolation of the Crude Enzyme——The mycelium cake was removed by filtration and $(NH_4)_2SO_4$ (10.2 kg) was added to the broth filtrate (17.0 liters, pH 7.6) adjusting pH 7.0 at 0° by addition of 1N NH_4OH to precipitate the active component. The precipitate was collected by centrifugation after kept at 0° for 5 hr. The crude enzyme mixture (66.09 g) was recovered by lyophilization from the precipitate after dialysis in a cellophane tube against distilled water at 0° for 3 hr.

Purification of the Enzyme——The crude enzyme mixture (5 g) dissolved in H_2O (22 ml) was again dialyzed for 3 hr in a cellophane tube against H_2O at 0° and inactive insoluble materials were removed by centrifugation. The supernatant was purified on a column of Sephadex G 75 (65 cm × 3 cm diameter) eluted with H_2O after decolorized by treatment with active carbon (700 mg). The eluate was collected in 15 ml fractions and the partially purified enzyme was recovered from fract. 14 (69 mg), fract. 15 (87 mg), fract. 16 (187 mg), and fract. 17 (135 mg) by lyophilization.

Transformation of Sterols by the Enzyme——A mixture of 5 ml of the enzyme solution (Fract. 16 partially purified on Sephadex G 75, 1.25 mg/ml) in 1/15M phosphate buffer (pH 7.0) and 0.4 ml of the sterol solution (1—2 mg/0.4 ml) in EtOH was incubated in Erlenmeyer flask (50 ml) at 27° for 20 hr on a reciprocal shaker (amplitude 7 cm, 120 rpm). The resulting mixture was extracted with an equal volume of ether and evaporated to dryness. The residue was chromatographed with the starting sterol on a Silica gel G thin-layer plate with benzene: AcOEt=2: 1 or $CHCl_4$: EtOH=9: 1 and detected by heating after spraying conc. H_2SO_4.

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