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Purification of 3β-Hydroxysteroid Oxidase of *Streptomyces violascens* Origin by Affinity Chromatography on Cholesterol

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Cholesterol could be used to adsorb the 3β-hydroxysteroid oxidase of *Streptomyces violascens* origin from a crude enzyme solution, and the adsorbed enzyme could be eluted with a suitable detergent such as Triton X-100. The enzyme was purified by ammonium sulfate precipitation and affinity chromatography on cholesterol with a recovery of 79% from the culture filtrate. The purified enzyme was detected as a single band on SDS-polyacrylamide gel electrophoresis. The molecular weight of the enzyme was 61000 by SDS-polyacrylamide gel electrophoresis. The purified enzyme exhibited a characteristic spectrum of flavoenzyme. The flavin moiety of the enzyme was isolated and identified as flavin adenine dinucleotide.

Keywords——3β-hydroxysteroid oxidase; *Streptomyces violascens*; affinity chromatography; flavoenzyme; flavin adenine dinucleotide

We have devised a new method of screening anti-cholesterol substances produced by microbes on the basis of the phenomenon that polyene antifungal antibiotics lost antiyeast activity by cholesterol.[2] In the course of screening, an active substance was found in the culture filtrate of *Streptomyces violascens*. This active substance was identified as a new 3β-hydroxysteroid oxidase.[2] In our previous report,[3] purification procedures involving DEAE-cellulose column chromatography and gel filtration on Sephadex G-75 were described. Also, enzymatic characterization of the 3β-hydroxysteroid oxidase was reported. The present study was undertaken to develop a new method for the purification of the enzyme from the culture filtrate, based on the affinity of the 3β-hydroxysteroid oxidase for cholesterol.

Materials and Methods

Materials—Horseradish peroxidase was purchased from Sigma Chemical Co. and had an activity of 100 units/mg. Steroids were purchased from Merck Darmstadt and Sigma Chemical Co. Other materials were purchased as follows: Flavin adenine dinucleotide and flavin mononucleotide (Nakarai Chemical Co.), Triton X-100 (Wako Pure Chemical Co.), Cutscum (Fisher Scientific Co.), Brij 35 and Emulgen 109P (Kao-Atlas Co.). All other reagents were purchased from commercial sources and were of analytical grade.

Cultivation of the Microbe——Cultivation of *Streptomyces violascens* H82-N-SY7 was performed as previously described.[3]

Determination of Enzyme Activity——The assay of the enzyme is based on the formation of the quinoneimine dye. The rate of hydrogen peroxide formation is determined by measuring the color development at 500 nm. To 2 ml of phosphate citrate buffer (0.1 M, pH 6.0) containing 2 μmol of 4-aminoantipyrine, 35 μmol of phenol and 2375 units/l of horseradish peroxidase, 0.5 ml of the enzyme preparations were added and mixed well. After standing for 3 min at 37°, 0.1 ml of 13 μmol/ml cholesterol in 10% Triton X-100 solution was added to this incubation mixture, and incubated for 30 min at 37°. The enzyme reaction was terminated by heating in boiling water bath for 3 min. After cooling to room temperature, the absorbance was read against the blank at 500 nm. One unit of 3β-hydroxysteroid oxidase was defined as the amount of enzyme forming 1 μmol of hydrogen peroxide per min at 37°.

1) Location: *a) 2-9-3, Shimomeguro, Meguro-ku, Tokyo, 153, Japan; b) 1-2-3, Kasumi, Hiroshima, 734, Japan.*
2) H. Fukuda, Y. Kawakami, and S. Nakamura, *Chem. Pharm. Bull.* (Tokyo), **21**, 2057 (1973).
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Determination of Protein——Protein concentration of an enzyme preparation was measured by the method of Pollack.[4]

Electrophoresis——Polyacrylamide gel disc electrophoresis was performed by the method of Davis.[5] SDS-polyacrylamide gel electrophoresis was also performed by the method of Weber and Osborn.[6]

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

Assay of 3β-Hydroxysteroid Oxidase

3β-Hydroxysteroid oxidase assay of Stadtman[7,8] based on the absorption of cholest-4-en-3-one at 240 nm could not be used, because *Streptomyces violascens* produced melanin pigments in the culture broth. So, we used the assay method based on the formation of the quinoneimine dye. Under the assay conditions, the enzyme activity was a linear function for both incubation time and enzyme concentration at least up to the absorbance of 0.5 at 500 nm (Fig. 1).

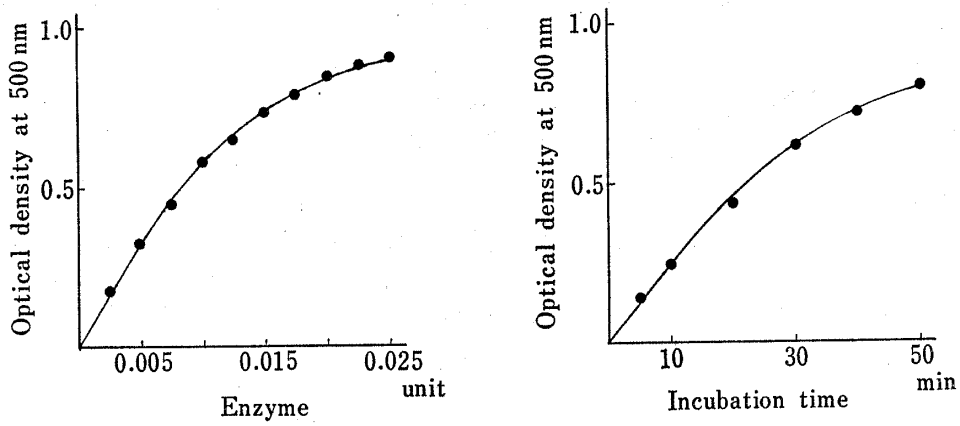

Fig. 1. Linearity of the Assay of 3β-Hydroxysteroid Oxidase Activity with Enzyme Concentration (Left) and Incubation Time (Right)

In the left, incubation was carried out for 30 min. In the right, 0.01 unit of 3β-hydroxysteroid oxidase was used.

Affinity Chromatography of 3β-Hydroxysteroid Oxidase on Cholesterol as the Adsorbent

Cholesterol is not usually used as the adsorbent of the enzyme because it floats on water. But, cholesterol heated in boiling water precipitates in water. Water precipitable cholesterol after boiling was the good adsorbent of 3β-hydroxysteroid oxidase. About 15 units of enzyme obtained from the broth filtrate by 40% saturation with ammonium sulfate precipitation was adsorbed on 1 ml of cholesterol suspension. The adsorbed enzyme on cholesterol could be eluted by various detergents (Table I). Triton X-100 and Cutscum could elute the enzyme effectively among the detergents tested. All the retained activity was eluted with 0.1% (w/v) Triton X-100 solution.

Purification of 3β-Hydroxysteroid Oxidase

All procedures were carried out at 4° unless otherwise stated. After the 6 days cultivation, mycelia were removed by filtration with the aid of 1% Celite 545. Solid ammonium

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Table I. Desorption of 3β-Hydroxysteroid Oxidase from Cholesterol with Various Detergents

Detergent	Concentration (%, w/v)	Elution (%)
Triton X-100	0.01	0
	0.1	102
	0.5	100
	1	103
Tween 20	0.01	21
	0.1	89
	1	—
Emulgen 109P	0.01	0
	0.1	64
	1	—
Sodium Cholate	0.01	0
	0.1	0
	1	15
Brij 35	0.01	62
	0.1	78
	1	—
Cutscum	0.01	0
	0.1	99
	1	104

15 units of enzyme was applied on micro cholesterol column (0.46×6.00 cm). After washing with 10 ml of water, adsorbed enzyme was eluted by various detergents. Elution % was expressed in percent relative to adsorbed enzyme.
—; Column was stopped by the detergent and recovery was not calculated.

sulfate was added to the filtrate up to the concentration of 40% saturation. After standing overnight, precipitate was collected by centrifugation and was dissolved in distilled water. Then, this solution was dialyzed against distilled water for 24 hr. One hundred eighty ml of the dialyzed solution (2314 units, 1.8 g as protein) was subjected to affinity chromatography on cholesterol (5×30 cm). The charged column was washed with a sufficient volume of water and then eluted with 0.1% Triton X-100. The elution profile was shown in Fig. 2. The active fractions were combined and 7 volumes of EtOH was added. After standing overnight at −20°, the precipitate was collected by centrifugation and was dissolved in distilled water. This fraction was lyophilized. The lyophilized residue was recovered as yellow powder. This powder was used for the characterization of the enzyme. The purification procedures are summarized in Table II. The enzyme was purified to 62 folds in specific activity recovering 79% of the original activity.

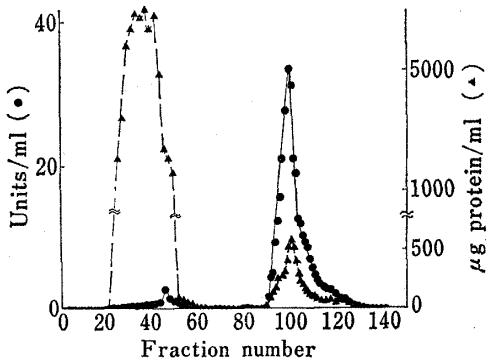

Fig. 2. Chromatography of 3β-Hydroxysteroid Oxidase on Cholesterol Column

Ammonium sulfate precipitated and dialyzed fraction (2314 units, 1.8 g as protein) was applied to affinity chromatography on cholesterol (5×30 cm). After washing with sufficient water, column was eluted with 0.1% Triton X-100 at a flow rate, 60 ml/hr and 10 ml of fractions were collected.

Electrophoresis of the Purified Preparations

As shown in Fig. 3, the purified preparation showed two bands on the polyacrylamide gel disc electrophoresis. But, only one band was detected on SDS-polyacrylamide gel electrophoresis.

Table II. Summary of Purification of 3β-Hydroxysteroid Oxidase

Fraction	Total protein (mg)	Total units	Specific activity (units/mg)	Recovery (%)
Broth filtrate	5856	2723	0.465	100
$(NH_4)_2SO_4$ ppt.	1986	2315	1.17	85
Dialysate	1821	2321	1.27	85
Cholesterol column	73.8	2141	29.0	79

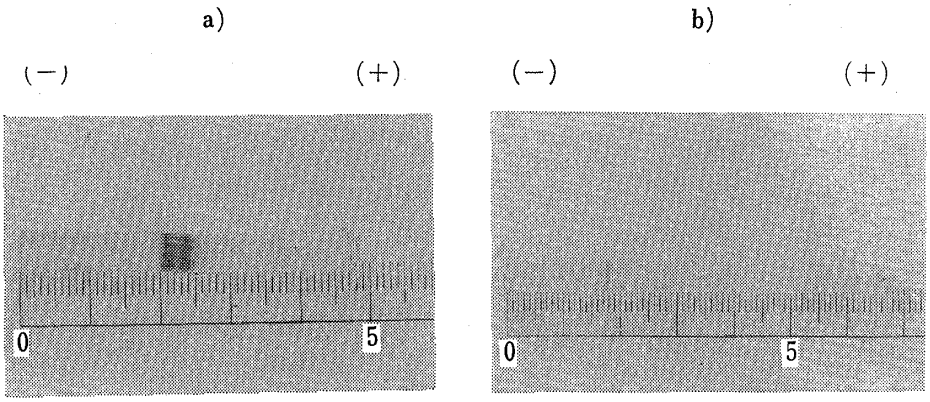

Fig. 3. Electrophoretic Pattern of Purified Cholesterol Oxidase

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

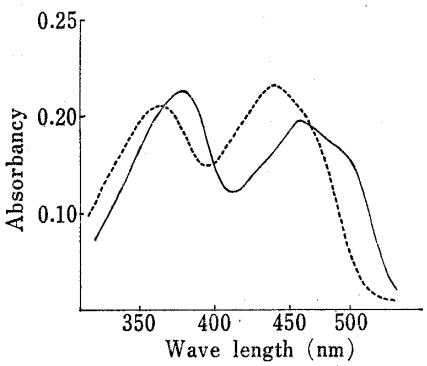

Fig. 4. Absorption Spectrum of 3β-Hydroxysteroid Oxidase and Extracted Flavin Moiety

The solid line; native enzyme, 1.18 mg/ml in 0.1 M, pH 6.0 phosphate-citrate buffer.
The broken line; extracted flavin, flavin released from the enzyme solution of the same molar concentration by treatment at 100° for 3 min.

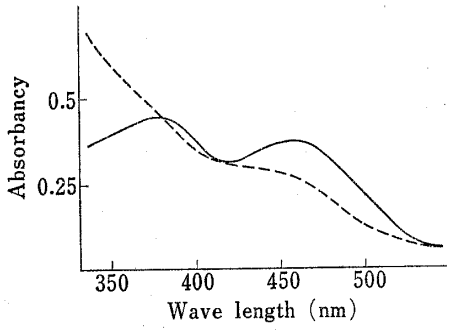

Fig. 5. Reduction Spectrum of 3β-Hydroxysteroid Oxidase by Cholesterol under Anaerobic Conditions

Solid line represents the native enzyme in 0.1 M, pH 6.0 phosphate-citrate buffer. Broken line represents the reduced enzyme after the addition of 1.3 μmol of cholesterol under anaerobic condition.

Molecular Weight

In our previous report,[3] 3β-hydroxysteroid oxidase was eluted after the void volume on Sephadex G-75 gel filtration. It was suggested that the molecular weight of this enzyme was below 70000. In SDS-polyacrylamide gel electrophoresis, the molecular weight of this enzyme was estimated to be about 61000. It was considered that the molecular weight of this enzyme was about 61000.

Prosthetic Groups of 3β-Hydroxysteroid Oxidase

The purified enzyme showed the characteristic absorption spectrum of a flavoprotein with absorption maximum at 280, 390 and 470 nm with a shoulder at 490 nm (Fig. 4). When

cholesterol was added to the enzyme solution under anaerobic conditions, disappearance of peaks at 390 and 470 nm was observed. The peaks were subsequently restored by bubbling oxygen into the solution (Fig. 5). The ratio of E_{280}/E_{470} was 12.8. Flavin was dissociated from the enzyme by treatment at 100° for 3 min and exhibited the absorption maximum at 370 and 450 nm (Fig. 4). The component was identified as flavin adenine dinucleotide by cellulose powder thin-layer chromatography (Table III). Furthermore, pH dependency of the fluorescence intensity of this flavin measured at 520 nm with excitation at 345 nm showed the characteristic change of flavin adenine dinucleotide.

Table III. Identification of the Flavin Moiety of 3β-Hydroxysteroid Oxidase by Cellulose Powder Thin-Layer Chromatography

Compound	*Rf*	
	Solvent I	Solvent II
Riboflavin	0.71	0.44
Flavin adenine dinucleotide	0.43	0.06
Flavin mononucleotide	0.60	0.28
Extracted flavin	0.42	0.06

The flavin spots were detected by their fluorescence under a UV light. All chromatographic experiments were performed in the dark.
Solvent I; *n*-butanol–acetic acid–water (4: 2: 4).
Solvent II; *t*-amyl alcohol–formic acid–water (3: 1: 1).

Flavin Adenine Dinucleotide Content

When 1.18 mg of the enzyme (1.93×18^{-8} mol on the basis of the molecular weight of 61000) was heated at 100° for 3 min, the amount of the recovered flavin adenine dinucleotide was estimated to be 2.06×10^{-8} mol as judged by the absorption at 450 nm ($\varepsilon=1.13\times10^4$). From the above results, it was considered that the enzyme contained 1 mol of flavin adenine dinucleotide per mol of protein.

Substrate Specificity

The relative oxidation rates of steroids by 3β-hydroxysteroid oxidase were measured by the formation of hydrogen peroxide and shown in Table IV. The enzyme oxidized various 3β-hydroxysteroids. Cholesterol and dihydrocholesterol were the most suitable substrates among the steroids tested. Steroids lacking the 3β-hydroxy group were not oxidized.

Table IV. Relative Oxidation Rates of Various Steroids by 3β-Hydroxysteroid Oxidase

Substrate	Relative rate	Substrate	Relative rate
Cholesterol	100	Ergosterol	14
Dihydrocholesterol	101	7-Dehydrocholesterine	9
β-Sitosterol	64	Cholesterol linolate	0
Stigmasterol	50	Cholesterol palmitate	0
5α-Androstan-3β,17β-diol	62	Vitamine D_3	0
5α-Androstan-3α,17β-diol	0	β-Estradiol	0
5-Androstene-3β,17β-diol	37	Testosterone	0
Dehydro-epi-androsterone	37	Androsterone	0
Pregnenolone	42	Cholic acid	0

The reaction mixture consisted of 1.3 μmol of substrate, 10 mg of Triton X-100, 0.26 mmol, pH 6.0 phosphate-citrate buffer, 2.7 μmol of 4-aminoantipyrine, 45.5 μmol of phenol, 6.175 units of peroxidase and 0.01 unit of 3β-hydroxysteroid oxidase in total volume 2.6 ml. The reaction was carried out at 37° for 30 min and terminated by heating at 100° for 3 min. The produced color was measured at 500 nm.

Discussion

W. Richmond[9] reported that 3β-hydroxysteroid oxidase was purified by using substrate-(cholesterol)-affinity chromatography. In his method, Sephadex LH-20 was swollen in a saturated ethanolic solution of cholesterol. A slurry prepared in this manner was packed into a column, which was then washed with distilled water to precipitate cholesterol throughout the gel. This column was used to adsorb the enzyme. However, in his method, binding capacity was low because the amount of cholesterol packed in a column was small. In our method, cholesterol precipitated in a boiling water was packed directly, and binding capacity was high. An adsorbed enzyme was easily eluted by suitable detergents such as Triton X-100. For convenience in handling and inexpensive preparation of the adsorbent, cholesterol column may be more usefull in large scale purification of 3β-hydroxysteroid oxidase than Richmond's method and conventional purification methods. In our previous report,[3] 3β-hydroxysteroid oxidase of *Streptomyces violascens* origin was considered to be a non-flavoenzyme. In the present and previous experiment,[3] specific activities of the most purified enzymes measured by the method of Stadtman[7,8] were 21.0 U/mg and 19.7 U/mg, respectively. In our previous experiment, it was considered that most purified enzyme was almost homogeneous. However, because the recovery of highly purified enzyme was very low (2.46 mg) in our previous experiment,[3] the absorption spectrum of the enzyme was measured at low enzyme concentration. So, it was considered that the absorption of flavin was not detected. In the present experiment, the recovery of the purified enzyme was extremely improved. So, the absorption spectrum could be measured at high concentration. Therefore, the spectrum of the flavin could be detected. Flavin moiety of this enzyme was identified as flavin adenine dinucleotide. 3β-Hydroxysteroid oxidase from *Brevibacterium sterolicum* was also reported to be a flavoenzyme containing flavin adenine dinucleotide.[10,11] The molecular weights of *S. violascens* and *B. sterolicum* enzymes were 61000 and 31000, respectively. In the substrate specificity, *B. sterolicum* origin did not oxidize ergosterol,[10] but *S. violascens* oxidized it. *S. violascens* enzyme was not inhibited by *p*-chloromercuribenzoate.[3] This result does not accord with that reported to *B. sterolicum* enzyme.[10] Though *S. violascens* and *B. sterolicum* enzymes were flavoenzymes containing flavin adenine dinucleotide, many different characters were observed. In SDS-polyacrylamide gel electrophoresis, the purified enzyme was detected as a single band, but in polyacrylamide gel disc electrophoresis, it was detected as major two bands. It may be considered that holoenzyme and apoenzyme are in equilibrium.

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