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Purification and Characterization of Extracellular 3β-Hydroxysteroid Oxidase produced by *Streptoverticillium cholesterolicum*

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3β-Hydroxysteroid oxidase activity was detected in the broth filtrate of strain H 1109 MY 12. Based on taxonomic studies, this strain was shown to be a new species of genus *Streptoverticillium* and the name *Stv. cholesterolicum* is proposed for this strain. The enzyme was purified by ammonium sulfate precipitation and affinity column chromatography on crystalline cholesterol, and the product showed a single band on SDS-polyacrylamide gel electrophoresis. The enzyme showed optimum activity at pH 7.0—7.5 and was stable over a rather wide pH range of 4.0 to 12.5. Cholesterol and dihydrocholesterol were oxidized rapidly. The K_m value for the oxidation of cholesterol by this enzyme was about 0.4 mM. The enzyme activity was greately inhibited by $HgCl_2$, and $AgNO_3$. $FeCl_3$ and $CuSO_4$ were also inhibitory though to lesser extents. Iodine and *N*-bromosuccinimide completely inhibited the enzyme activity at concentrations of less than 0.01 mM. Neither metal-binding agents nor *p*-chloromercuribenzoic acid had any inhibitory effect on the oxidation of cholesterol by this enzyme. The molecular weight of the enzyme was estimated to be 56000 by SDS-polyacrylamide gel electrophoresis. The enzyme was proved to be a flavoprotein containing flavin adenine dinucleotide as a prosthetic group.

Keywords——3β-hydroxysteroid oxidase; *Streptoverticillium*; affinity column chromatography; flavoprotein; extracellular enzyme; flavin adenine dinucleotide

Due to the increasing importance of the specific estimation of steroids in various clinical samples, 3β-hydroxysteroid oxidases have been studied in many microorganisms.[1-6] Although these enzymes share some enzymatic and physicochemical properties, there is considerable variation in their substrate specificities. Recently Okada *et al.* reported the partial purification of 3β-hydroxysteroid oxidase from the sonicated mycelia of a strain of genus *Streptoverticillium.*[7] This enzyme seems to be unique in being exclusively specific for cholesterol with the lowest K_m value so far reported. However, the details of the properties of this enzyme remain to be elucidated.

In our screening program, *Streptoverticillium* strain H 1109 MY 12, a monospore isolate from a soil sample collected in Wakayama Prefecture, was found to release a high titer of 3β-hydroxysteroid oxidase into the culture fluid (1—3 units/ml). The enzyme was purified by using cholesterol as a specific adsorbent and characterized comparatively with the enzyme from other microorganisms.

Materials and Methods

Materials——Horseradish peroxidase was purchased from Sigma Chemical Co. Steroids were products of Merck Darmstadt and Sigma Chemical Co., and were kindly donated by Dr. Kamei of Banyu Pharmaceutical Co., Ltd., Tokyo. Flavin adenine dinucleotide, flavin mononucleotide and flavin were purchased from Nakarai Chemical Co. All other materials were commercial products of analytical grade.

Taxonomic Studies——Taxonomic studies were conducted in accordance with the methods described by Shirling and Gottlieb,[8] and Waksman.[9] Color names were determined according to the "Guide to Color Standard."[10] The procedures of Becker *et al.*[11] were used for the preparation of whole cell hydrolysate and the chromatographic detection of the isomers of diaminopimelic acid.

Cultivation——Strain H 1109 MY 12 was cultured in a medium that consisted of 1.0% potato starch, 1.0% glucose, 3.0% peptone, 3.0% meat extract, 0.75% NaCl, 0.1% $MgSO_4$ and a trace amount of divalent metal salts at 27°C for 4 d. Culture conditions were described in detail in the previous paper.[4a]

Assay of the Enzyme Activity——Assay Method I: Hydrogen peroxide generated by the oxidation of 3β-hydroxysteroid was measured by the oxidative coupling of 4-aminoantipyrine with phenol catalyzed by horseradish peroxidase to give a quinone-imine dye absorbing at 500 nm.[12] A 0.5 ml aliquot of the enzyme solution was mixed with 2.0 ml of 0.1 M phosphate buffer (pH 6.0) containing 2 μmol of 4-aminoantipyrine, 35 μmol of phenol and Triton X-100 at the final concentration of 0.1%. The mixture was allowed to stand at 37°C for 3 min, then 50 μl of isopropanol solution of steroid (13 mM) was added and incubation was continued at the same temperature for a further 30 min. The reaction was terminated by boiling the reaction mixture for 3 min.

Assay Method II: The oxidation of cholesterol was determined according to the method of Stadtman.[13] Briefly, 0.4 ml of 0.125 M Tris-HCl buffer (pH 7.5) was mixed with 0.1 ml of enzyme solution in an ice-bath. After 3 min of preincubation at 37°C, 25 μl of an isopropanol solution of cholesterol (12 mM) was added in order to start the reaction, which was conducted for 30 min. At the end of the incubation, 2.0 ml of ethanol was added to the reaction mixture and the amount of 4-cholesten-3-one was determined by measurement of the absorbancy at 240 nm ($\varepsilon = 1.6 \times 10^4$). One unit of cholesterol oxidase activity was defined as the amount of the enzyme forming 1 μmol of 4-cholesten-3-one per min at 37°C.

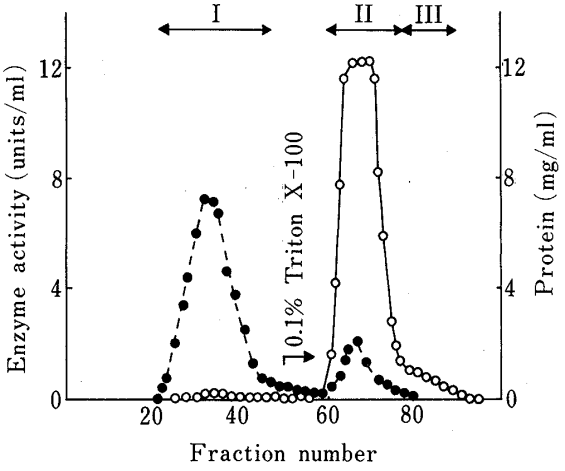

Fig. 1. Affinity Column Chromatography of 3β-Hydroxysteroid Oxidase on Crystalline Cholesterol

An ammonium sulfate-precipitated and dialyzed sample (1967 units, 10.61 g as protein) was subjected to affinity column chromatography on cholesterol (ϕ5.0×26.0 cm). The column was washed with a sufficient amount of water, then the adsorbed enzyme was eluted with 0.1% Triton X-100; 10 ml fractions were collected. ○, cholesterol oxidation activity; ●, protein concentration.

Determination of Protein——Protein concentration of the enzyme preparation was measured by the method of Lowry[14] using bovine serum albumin as a standard.

Electrophoresis——Electrophoresis was performed in 7.0% polyacrylamide gel in the presence of sodium dodecyl sulfate according to the method of Weber and Osborn.[15] For estimation of the molecular weight of the enzyme protein, the following reference proteins were used: bovine serum albumin (68000), ovalbumin (45000), chymotrypsinogen (26000) and lysozyme (14300).

Purification of 3β-Hydroxysteroid Oxidase——After 4 days of cultivation, mycelia were removed from the culture broth by centrifugation and filtration. Solid ammonium sulfate was added to the filtrate to give 40% saturation, and the solution was left to stand overnight. The resulting precipitate was collected by centrifugation and dissolved in 1/10 volume of distilled water. The dialyzed sample was subjected to affinity chromatography on a column of crystalline cholesterol (ϕ 5.0×26.0 cm) which was prepared as reported previously.[4c] The enzyme bound to solid cholesterol was washed with a sufficient amount of distilled water and then eluted with 0.1% Triton X-100 (Fig. 1). The enzyme solution thus obtained was further concentrated by ammonium sulfate precipitation, dialysis and lyophilization for the analysis of its physicochemical properties.

Results

Characterization of Strain H 1109 MY 12

Strain H 1109 MY 12 exhibited poor sporulation on all media tested. Spores developed on oatmeal agar (ISP medium No. 3) in cylindrical form, measuring 0.3—0.4×1.1—1.2 μm in size. Sporophores were straight and predominated by secondary umbels with some primary ones. Each spore chain consisted of less than 10 spores with smooth surface. LL-Diaminopimelic acid was detected in the whole cell hydrolysate. Thus, strain H 1109 MY 12 was classified as genus *Streptoverticillium*.

By consulting "Bergey's Manual of Determinative Bacteriology"[16] and ISP reports by Shirling and Gottlieb,[17] *Streptoverticillium orinoci* and *Streptoverticillium parvisporogenes* were

Table I. Physicochemical Properties of Strain H 1109 MY 12

	Characteristics
Spore wall ornamentation	Smooth
Spore chain morphology	Verticillate
No. of spores per chain	5—10
Color of substrate mycelium	Yellowish white to pale yellowish brown
Color of mature sporulated aerial mycelium	White or yellowish white
Soluble pigments	None
Melanoid pigments	None
Starch hydrolysis	Positive
Gelatin liquefaction	Negative
Action on skimmed milk	
Coagulation	Positive
Peptonization	Positive
Production of H_2S	Negative
Utilization of sugars	
L-Arabinose	Negative
D-Xylose	Negative
D-Glucose	Positive
D-Fructose	Negative
Sucrose	Negative
Inositol	Positive
L-Rhamnose	Negative
Raffinose	Negative
D-Mannitol	Negative
D-Galactose	Positive
Salicin	Negative
Cell wall type	I (LL-DAP)

Table II. Purification of 3β-Hydroxysteroid Oxidase from the Broth Filtrate of *Streptoverticillium cholesterolicum* H 1109 MY 12

Step	Total protein (mg)	Total units (units)	Specific activity (units/mg)	Yield (%)
Broth filtrate (1310 ml)	10610	1967	0.19	100.0
$(NH_4)_2SO_4$ ppt.	606	1036	1.71	52.7
Cholesterol column				
Peak-I	558	5	0.01	0.3
Peak-II	47	992	21.1	50.4
Peak-III	1	19		1.0

selected as the species most resembling strain H 1109 MY 12. *Stv. orinoci* and strain H 1109 MY 12 are not chromogenic, hydrolyze casein and do not liquefy gelatin. However, the former can be differentiated from the latter in the following respects: production of hydrogen sulfide, no hydrolysis of starch, no growth on inositol, and formation of white to yellowish gray aerial mycelia and yellow to dull yellow substrate mycelia. *Stv. parvisporogenes* is chromogenic, liquefies gelatin, and lacks the ability to hydrolyze casein. In addition to D-glucose and inositol, sucrose is also used by this species. As a result of the above comparisons, strain H 1109 MY 12 was concluded to be a novel species of genus *Streptoverticillium*. The name *Stv. cholesterolicum* is proposed for this strain, referring to the product of this strain.

Purification of 3β-Hydroxysteroid Oxidase and Estimation of Molecular Weight

The results of the purification procedure are summarized in Table II. The enzyme was purified about 100 times in terms of specific activity with a recovery of 50%, giving a specific activity of 21.1 units per mg protein. The enzyme could be stored in 0.1% Triton X-100

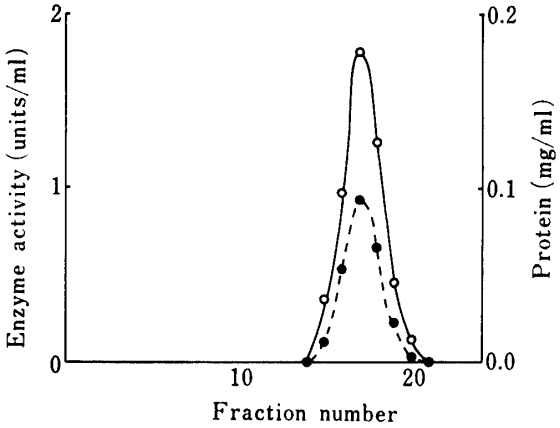

Fig. 2. Sephadex G-75 Column Chromatography of 3β-Hydroxysteroid Oxidase

The concentrated enzyme solution obtained from affinity column chromatography (21.1 units, 1 mg as protein) was charged on a column (φ 1.5×60.0 cm) of Sephadex G-75 equilibrated with 50 mM phosphate buffer (pH 7.0) and eluted with the same buffer. Four ml fraction were collected. ○, cholesterol oxidation activity; ●, protein concentration.

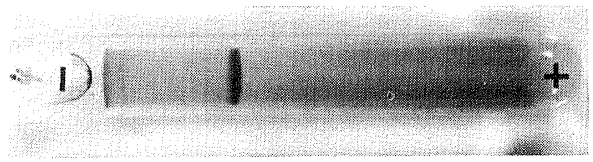

Fig. 3. SDS-Polyacrylamide Gel Electrophorogram of the Purified Enzyme

for an extended period without losing activity. The concentrated enzyme solution was subjected to Sephadex G-75 gel filtration (Fig. 2). The enzyme was eluted after the void volume as a single symmetrical peak with a constant specific activity. The purified enzyme gave a single band on SDS-polyacrylamide gel electrophoresis (Fig. 3). The molecular weight of the enzyme was estimated to be 56000 from the relative mobilities with respect to reference proteins on SDS-polyacrylamide gel electrophoresis and from the elution profile on Sephadex G-75 gel filtration.

Optimum pH for the Enzyme Activity

The effect of pH on 3β-hydroxysteroid oxidase activity was tested using cholesterol as a substrate. The results (shown in Fig. 4) indicate that maximum activity is seen at pH 7.0—7.5.

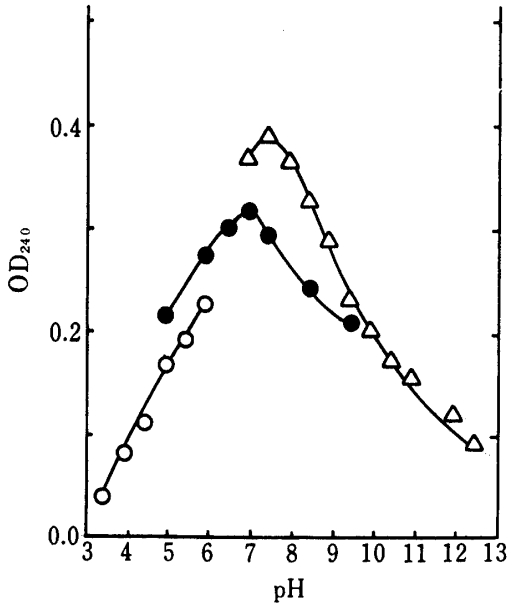

Fig. 4. Effect of pH on Cholesterol Oxidation Activity

Enzyme activity was measured by assay method II in 0.1 M buffer solution using 2.06 μg/ml of enzyme solution. ○, acetate buffer (pH 3.5—6.0); ●, Tris-HCl buffer (pH 5.0—10.0); △, glycine-NaOH buffer (pH 7.0—12.5).

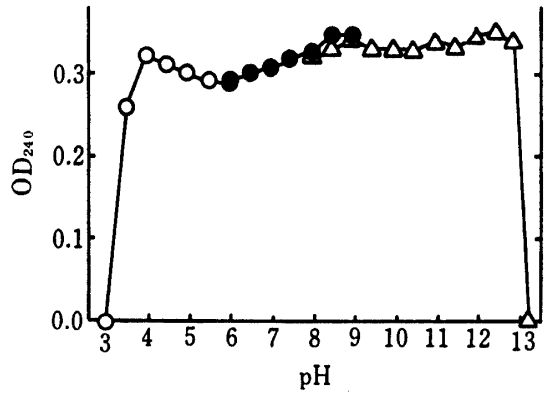

Fig. 5. pH Stability of 3β-Hydroxysteroid Oxidase

A mixture of 0.1 ml of enzyme solution (20.6 μg/ml) and 0.1 ml of 0.1 M buffer solution was kept at room temperature for 1 h. The pH was changed to 7.5 and the total volume was made up to 1 ml, then enzyme activity was measured by assay method II. ○, acetate buffer (pH 3.0—6.0); ●, Tris-HCl buffer (pH 6.0—9.0); △, glycine-NaOH buffer (pH 8.0—13.3).

Stability of Enzyme Activity at Different pH Values

As can be seen in Fig. 5, the enzyme retained full activity after being treated at room temperature for 1 hour at pH between 4.0 and 12.5.

Effects of Metal Salts

The effects of metal salts on the enzyme activity were tested by assay method II using cholesterol as a substrate, and the results are shown in Table III. The marked inhibitory effects of $HgCl_2$ and $AgNO_3$ were prevented by the addition of either cysteine or glutathione. $CuSO_4$ and $FeCl_3$ were also inhibitory to the enzyme activity.

TABLE III. Effects of Metal Salts on Cholesterol Oxidation Activity

Metal salt	Concentration (mM)	Inhibition (%)
$CaCl_2$	10	0
$MgCl_2$	10	0
$CoCl_2$	10	0
$NiCl_2$	10	0
$MnCl_2$	1	6
Na_2MoO_4	1	2
$FeSO_4$	1	13
	0.1	8
$FeCl_3$	1	32
	0.1	12
$CuSO_4$	1	17
	0.1	11
$AgNO_3$	1	73
	0.1	23
	1	
	+10 mM Glutathione	14
	+10 mM Cysteine	15
$HgCl_2$	1	51
	0.1	10
	1	
	+10 mM Glutathione	0
	+10 mM Cysteine	0

The effects of metal salts were measured by assay method II with 0.21 μg of the enzyme in 0.5 ml of 0.1 M Tris–HCl buffer (pH 7.5).

Effects of Metal-binding Agents and Other Chemicals

In order to test the possible involvement of metal ions in the catalytic site of the enzyme, the effects of various metal-binding agents on the enzyme activity were investigated. None of them showed inhibition of the oxidation of cholesterol by the enzyme (Table IV, above the broken line).

The enzyme was highly sensitive to inhibition by either iodine or *N*-bromosuccinimide and was slightly inhibited by 1-fluoro-2,4-dinitrobenzene (Table IV, below the broken line). The other chemicals tested in this study failed to affect the enzyme activity.

Substrate Specificity

The relative oxidation rates of various hydroxysteroids by the enzyme were measured by assay method I in terms of the formation of hydrogen peroxide. The enzyme showed specificity for 3β-hydroxysteroids, and cholesterol and dihydrocholesterol were the preferred substrates. 4-Methyl groups (lanosterol), conjugated double bonds in the steroid B-ring (7-dehydrocholesterol and ergosterol) and a 17β-hydroxy group (androstendiol) adversely affected the oxidation of the 3β-hydroxy group by the enzyme.

Michaelis Constant (K_m) Value for Cholesterol Oxidation

In order to obtain the K_m value for the oxidation of cholesterol, the enzyme activity was measured by assay method II. The K_m value was estimated to be approximately 0.4 mM.

Other Physicochemical Properties

The enzyme showed the characteristic absorption spectrum of a flavoprotein with maxima at 390 and 470 nm (Fig. 6). The flavin moiety was released from the enzyme protein by heating

TABLE IV. Effects of Various Chemicals on Cholesterol Oxidation Activity

Chemical	Concentration (mM)	Inhibition (%)
EDTA	10	0
8-Hydroxyquinoline	0.1	0
α,α'-Dipyridyl	0.1	12
NaN_3	20	9
	1	0
p-Nitrophenol	1	29
	0.1	0
N-Bromosuccinimide	0.1	100
	0.01	89
1-Fluoro-2,4-dinitrobenzene	0.1	40
	0.01	6
Iodine	0.1	100
	0.01	95
	0.001	10
Hydrogen peroxide	10	14
	1	9
p-Chloromercuribenzoic acid	0.1	0
Monoiodoacetic acid	1	5

The enzyme activity was measured by assay method II. Each inhibitor was incubated with 0.21 μg of the enzyme in 0.5 ml of 0.1 M Tris-HCl buffer (pH 7.5) at 37°C for 30 min prior to the addition of cholesterol.

TABLE V. Relative Oxidation Rates of Various Steroids by 3β-Hydroxysteroid Oxidase

Substrate	Relative activity (%)
3β-Hydroxysteroids	
Cholest-5-en-3β-ol (Cholesterol)	100
5α-Cholestan-3β-ol (Dihydrocholesterol)	96
Cholesta-5,7-dien-3β-ol (7-Dehydrocholesterol)	4
Androst-5-en-3β-ol-17-one (Dehydroepiandrosterone)	48
Androst-5-ene-3β,17β-diol (Androstenediol)	9
Pregn-5-en-3β-ol-20-one (Pregnenolone)	37
Ergosta-5,7,22-trien-3β-ol (Ergosterol)	5
Stigmast-5-en-3β-ol (β-Sitosterol)	39
Stigmasta-5,22-dien-3β-ol (Stigmasterol)	17
Lanosta-8,24-dien-3β-ol (Lanosterol)	0
Other steroids	
Estra-1,3,5(10)-triene-3,17β-diol (β-Estradiol)	0
5α-Androstan-3α-ol-17-one (Androsterone)	0
5α-Androstane-3α,17β-diol	0
Androst-4-en-17β-ol-3-one (Testosterone)	0
Cholesterol linolate	0

A 0.5 ml of aliquot of the enzyme solution (1.05 μg/ml) was mixed with 2.0 ml of standard reaction mixture (see "Assay method I" in the text). After 3 min of preincubation at 37°C, 50 μl of an isopropanol solution of steroid (13 mM) was added to the reaction mixture.

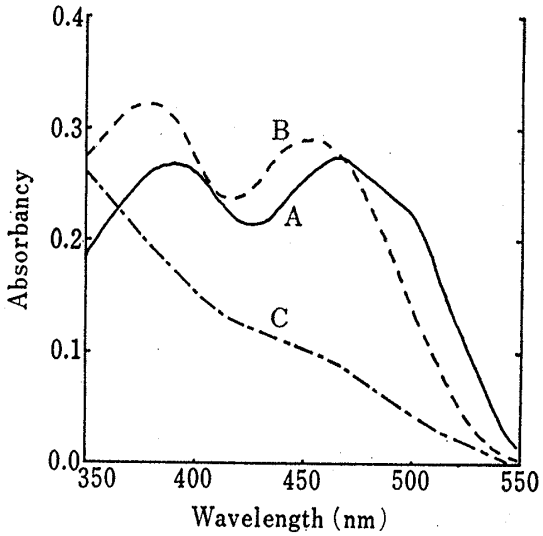

Fig. 6. Absorption Spectra of 3β-Hydroxysteroid Oxidase

Curve A: native enzyme, 1.4 mg/ml in 0.1 M citrate buffer (pH 6.0). Curve B: flavin extracted from the enzyme solution of the same concentration. Curve C: reduced enzyme after the addition of cholesterol under anaerobic conditions, 1.4 mg/ml in 0.1 M citrate buffer (pH 6.0).

Table VI. Identification of the Flavin Moiety of 3β-Hydroxysteroid Oxidase by Paper Chromatography

Compound	*Rf*	
	Solvent I	Solvent II
Riboflavin	0.62	0.36
Flavin mononucleotide	0.54	0.24
Flavin adenine dinucleotide	0.38	0.04
Flavin extract	0.39	0.04

Solvent I: *n*-butanol–acetic acid–water (2: 1: 2).
Solvent II: *tert*-amyl alcohol–formic acid–water (3: 1: 1).

at 100°C for 3 min and was identified as flavin adenine dinucleotide by paper chromatography (Table VI). The amount of flavin adenine dinucleotide released from 1.4 mg of protein (2.5×10^{-8} mol on the basis of the molecular weight of 56000) was estimated to be 2.6×10^{-8} mol from the absorption at 450 nm ($\varepsilon=1.13\times10^4$). Addition of cholesterol under anaerobic conditions caused the disappearance of the absorption maxima at 390 and 470 nm. The peaks were restored after bubbling air through the solution.

Discussion

Extracellular 3β-hydroxysteroid oxidase produced by *Streptoverticillium cholesterolicum* H 1109 MY 12 was conveniently purified by affinity column chromatography on cholesterol as previously reported by Kamei *et al.*[4c] In a preliminary experiment, 1 cm³ of packed cholesterol was found to be the capacity to retain 2 units of 3β-hydroxysteroid oxidase of this strain.

Physicochemical and enzymological properties of the enzyme were compared with those of the enzymes produced extracellularly by *Streptomyces*, *Corynebacterium* and *Brevibacterium*. A molecular weight of 32500 was reported for the enzyme from *Brevibacterium sterolicum*,[5a] and values of 56000, 57000 and 61000 were reported for those from *Stv. cholesterolicum*, *Corynebacterium cholesterolicum*[6] and *Streptomyces violascens*,[4c] respectively. The 3β-hydroxy configuration of the steroid is essential for substrates of these enzymes. However, the enzymes showed some differences in their substrate specificities as follows. 3β-Hydroxysteroid oxidase from *B. sterolicum*[5a] cannot oxidize dihydrocholesterol, which is the preferred substrate of the enzymes from *Str. violascens*[4b,c] and *Stv. cholesterolicum*. The presence of a side chain containing more than two carbon atoms at the C_{17} position of the steroid is required for efficient oxidation by the enzyme from *C. cholesterolicum*.[6] This substrate specificity resembles that of the intracellular enzyme from *Nocardia erythropolis*.[18] In contrast to the enzyme from *Str. violascens*,[4b,c] 3β-hydroxysteroid oxidase from *Stv. cholesterolicum* cannot oxidize 5-androstene-3β,17β-diol effectively. The K_m values for the oxidation of cholesterol by the enzymes from *Stv. cholesterolicum* and *Str. violascens* are similar. Thus, the enzyme produced by *Stv. cholesterolicum* is closely related to the enzyme from *Str. violascens* with a rather wide substrate specificity, in contrast to the presumption in the previous report.[7]

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