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Extracellular 3β-hydroxysteroid oxidase of *Mycobacterium vaccae* VKM Ac-1815D

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Abstract

Extracellular 3β-hydroxysteroid oxidase (SO) has been isolated from cell-free cultivation broth at the growth of *Mycobacterium vaccae* VKM Ac-1815D on glycerol-mineral medium in the presence of sitosterol. The enzyme is responsible for the transformation of 3β-hydroxy-5-ene- to 3-keto-4-ene-moiety of steroids including dehydrogenation of 3β-hydroxy function followed by $\Delta^{5\rightarrow}\Delta^{4}$ isomerization. 6-Hydroxy-4-sitosten-3-one and 6-hydroxy-4-androsten-3,17-dione were revealed among the metabolites at the incubation of the enzyme preparations with sitosterol and dehydroepiandrosterone (DHEA), respectively. The enzyme was strongly NADH or NADPH dependent. SO has been purified over 300-fold using cultivation broth concentration on hollow fibers followed by fractionation by ammonium sulphate, column chromatography on DEAE-Toyopearl, hydroxyapatite Bio-Gel HTP and double gel-filtration on Bio-Gel A 0.5 M. SDS-electrophoresis gave a molecular mass estimate of 62 ± 4 kDa. The purified SO obeyed Michaelis–Menten kinetics, double reciprocal plots kinetics revealed K_m value towards DHEA 5×10^{-4} M.

Along with SO activity, 17-hydroxysteroid dehydrogenase (17-OH SDH) and 3-ketosteroid-1(2)-dehydrogenase (1(2)-SDH) activities were detected in cell-free cultivation broth. The extracellular steroid transforming activities of C-17-ketosteroid producing mycobacteria were hitherto unreported.

Keywords: Microbial transformation; Steroid; *Mycobacterium*; Sitosterol; Dehydroepiandrosterone; Androstenedione; Testosterone; 3β-Hydroxysteroid oxidase; 17-Hydroxysteroid dehydrogenase; 3-Ketosteroid 1(2)-dehydrogenase

1. Introduction

Conversion of 3β-hydroxy-5-ene to 3-keto-4-ene-moiety of steroid nucleus was described as an initial step of bacterial catabolism of sterols. The process includes dehydrogenation of 3β-hydroxy function forming 3-keto-5-ene-steroid. The following $\Delta^{5\rightarrow}\Delta^{4}$ isomerization results in the formation of 3-keto-4-ene-structure. This A-ring modification is generally independent on the oxidation of sterol side chain at C-17—it can either forego, or proceed in parallel with side chain oxidation [1].

As reported for many microorganisms, the enzyme responsible for 3β-hydroxy-5-ene to 3-keto-4-ene modification belongs to oxidases (cholesterol oxidase, ChO, EC 1.1.3.6). At present, microbial ChO is widely used for analytical purposes for cholesterol content determination in blood and other biological liquids, as well as for quantitative determination of dehydroepiandrosterone (DHEA) in human breast cyst and duct fluids [2,3].

Numerous microorganisms of different taxonomy position have been demonstrated to possess ChO activity: *Brevibacterium*, *Nocardia*, *Rhodococcus*, *Corynebacterium*, *Schizophyllum*, *Streptomyces*, etc. [4–8]. The strains able to overproduction of ChO were obtained by genetic engineering [9].

The oxidation and isomerization functions of ChO were shown to be separate in *Streptomyces* ChO. Along with 3β-hydroxy dehydrogenation and $\Delta^{5\rightarrow}\Delta^{4}$ isomerization products, 4-cholesten-6β-ol-3-one was formed [10]. This compound was also detected among the metabolites of cholesterol, or 4-cholesten-3-one by extra- or intracellular

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ChO from recombinant strains *Streptomyces lividans* and *Escherichia coli* with cloned DNA-fragment from *Brevibacterium sterolicum*. As established by genetic analysis, the enzyme responsible for the formation of 6-hydroxy derivative is ChO [9]. Similar results were described for commercial ChO (EC 1.1.3.6.) preparations from *Streptomyces* sp., *Brevibacterium sterolicum* and *Pseudomonas* sp. [11].

The evidence of two ChO isoforms—cell-linked and extracellular, with molecular mass 55 kDa was demonstrated for *Rhodococcus erythropolis*. The synthesis of extracellular ChO was induced by cholesterol emulsified with Tween-80. Recently, the effective method for commercial ChO production was developed using Triton X-114 [12].

The data on the enzyme responsible for oxidation of sterol 3-OH-function in fast-growing mycobacteria are scarce [13,14]. The ChO from *M. fortuitum*, *M. vaccae*, *M. phlei*, *M. smegmatis* were localized intracellularly [14]. To our knowledge, the data on extracellular ChO from fast-growing mycobacteria were hitherto unreported.

A strain of *Mycobacterium vaccae* VKM Ac-1815D (syn. *Mycobacterium* sp. VKM Ac-1815D) actively oxidizes sterols forming 4-androsten-3,17-dione as a major product. 4-Sitosten-3-one was detected as an intermediate product [15].

This work purposed the isolation and characterization of extracellular enzyme responsible for the transformation of 3β-hydroxy-5-ene to 3-keto-4-ene moiety of steroids in *M. vaccae* VKM Ac-1815D, study of its correlation with other steroid transforming activities and elucidation of its possible role in cell-substrate interaction.

2. Materials and methods

2.1. Microorganism and cultivation

Mycobacterium vaccae VKM Ac-1815D (syn. *Mycobacterium* sp. VKM Ac-1815D) was obtained from All-Russian Collection of Microorganisms (VKM IBPM RAS). The strain was grown on glycerol mineral medium supplemented with 3 mM of β-sitosterol in a 100-L fermenter BIOR (Russia) [15]. The fermentation was carried out at 30 °C, pH 6.8–7.0, 220–250 rpm, DO 50–60%.

2.2. Reagents

β-Sitosterol (91.4% purity) was purchased from Kaukas Co. (Finland), 4-androstene-3,17-dione (AD), 1,4-androstadiene-3,17-dione (ADD), dehydroepiandrosterone (DHEA) were obtained from Sigma (USA), testosterone (T): from Serva (Sweden), 1(2)-dehydrotestosterone (DHT): from Laboratory MTOC (IBPM RAS). Electrophoresis reagents were purchased from Bio-Rad (Sweden) and DiaM (Russia), yeast extract: from Difco (USA). Other materials were of reagent grade and purchased from domestic companies.

2.3. Isolation and purification of SO

All steps were performed at 0–4 °C. After 20 h growth in BIOR fermenter, cells were harvested by centrifugal separator at 20,000 × *g* for 2 h.

2.3.1. Isolation of enzyme associated with cell wall

A sample of biomass (100 g, wet weight) was treated using 100 ml of 0.1% Triton X-100 suspension in 10 mM potassium phosphate buffer (pH 6.8, buffer A). After 10 min mixing, the cells were separated by centrifuge (4000 × *g* for 1 h). The supernatant (T-I) was examined on steroid transforming activity and subjected to SDS-electrophoresis as described in Section 2.5.

2.3.2. Isolation of extracellular enzyme

The cell-free cultivation broth obtained after biomass separation was subjected to the following procedures.

(a) Concentration

The cell-free cultivation broth (50 L) was concentrated to 2 L using membrane ultrafiltration on the hollow fibers. To the concentrate obtained (K-I) fine-dispersed ammonium sulphate was added to 85% saturation. The residue was collected by centrifugation, dissolved in 100 ml of buffer A and dialysed against 2 L of buffer A with double change, thus obtaining preparation D-I.

(b) DEAE-Toyopearl chromatography

Preparation D-I was applied to DEAE-Toyopearl column (0.9 cm × 40 cm) preliminary developed with buffer A. The proteins were eluted with a linear gradient of 0–1 M NaCl in the same buffer. Active fractions of 20 ml volume were dialysed against 2 L buffer A with double change, thus obtaining preparation D-II.

(c) Hydroxyapatite Bio-Gel HTP chromatography

Preparation D-II was applied to hydroxyapatite Bio-Gel HTP column (1.5 cm × 20 cm) preliminary developed with buffer A. The proteins were eluted with a linear gradient of 0.01–1 M K-phosphate buffer (pH 6.8). Active fractions (peaks 1 and 2) were concentrated to 1.8 ml thus obtaining preparations HA-I and HA-II, respectively.

(d) Gel-filtration on Bio-Gel A 0.5 M

Preparation HA-I was applied to Bio-Gel A 0.5 M column (0.8 cm × 55 cm) preliminary developed with buffer A. Proteins were eluted by buffer A. The fractions eluted in a free volume were concentrated to 1.8 ml and subjected again to the gel-filtration.

The gel-filtration of HA-II preparation was carried out using the same procedure. The active fractions obtained were concentrated against buffer A supplemented with 50% glycerol, and stored at −20 °C.

The preparations obtained were assayed on steroid transforming activities as described below. Steroids were analyzed by TLC, HPLC and MS.

2.4. Enzyme assay

(a) 3β-Hydroxysteroid oxidase (SO)
Assay mixture (2 ml) contained: 20 μm Tris–HCl buffer (pH 6.8); 0.2 μm substrate (sitosterol, or DHEA) in 10 μl dimethylformamide (DMF), 0.2 μm NADPH and 100–500 μl of enzyme preparation. The mixture was air-saturated during 30 min at 37 °C and incubated on a rotary shaker (200 rpm) for 30 min at 37 °C. Steroids were extracted with ethyl acetate (3 ml), evaporated on a rotary evaporator and assayed by TLC/HPLC. One unit of enzyme activity oxidized 1 μm of steroid per min.

(b) 17-Hydroxysteroid dehydrogenase (17-OH SDH)
Assay mixture (2 ml) contained: 20 μm Tris–HCl buffer (pH 8.9); 0.2 μm T in 10 μl DMF; 0.2 μm NAD; 100–500 μl of the enzyme preparation. The mixture was incubated on a rotary shaker (200 rpm) for 12 h at 37 °C. Steroids were extracted with ethyl acetate (3 ml), evaporated on a rotary evaporator and assayed by TLC/HPLC. In some experiments, the intracellular *M. vaccae* enzymes (17-OH SDH 1 and 17-OH SDH 2) obtained as described earlier [16] were used as a control.

(c) 3-Ketosteroid-1(2)-dehydrogenase (1(2)-SDH)
The activity of 1(2)-SDH was assayed in accordance with [17]. Protein was determined by Lowry method [18].

Molecular weight of SO was estimated from the plot of elution volume versus M_r of marker proteins: amylase (M_r = 200 kDa), yeast alcohol dehydrogenase (M_r = 150 kDa), bovine serum albumin (M_r = 66 kDa), carbonyl anhydrase (M_r = 29 kDa).

Kinetic parameters were derived for DHEA. K_m was determined in accordance with [19]. The reaction mixture contained: 20 μm Tris–HCl (pH 6.8); 0.5 μg enzyme; 0.2 μm NADH and DHEA (1.99×10^{-5} to 22×10^{-5} M).

2.5. Electrophoresis

Electrophoresis in a non-denaturing system was performed according to Davis [20]. Demonstration of 17-OH SDH activity by staining was carried out in accordance with [16]. SDS-electrophoresis was carried out according to [21].

2.6. Metabolite isolation

At the end of the incubation period, the steroids were extracted with ethyl acetate. The extract was subjected to preparative TLC plate. Individual substances were eluted from the plates with ethyl alcohol followed by evaporation to dryness. Chromatographic purity of the substances was controlled by TLC.

2.7. Steroid analyses

2.7.1. TLC

Steroids were extracted with ethyl acetate, aliquots were applied to Silufol UV 254, or Kieselgel 254 (Merck) plates, developed in benzene/acetone (3:1, v/v), and visualized under UV light (254 nm). 3-Hydroxy steroids were visualized using spraying by 10% phosphoric–molybdic acid solution in ethanol.

2.7.2. HPLC

3-Ketosteroids were analyzed by reversed-phase HPLC using C_{18}-column 250 mm × 4.6 mm and UV detection at 240 nm. Mobile phase was composed of acetonitrile:water:glacial acetic acid (55:45:0.01, v/v/v). AD, ADD, T and DHT were used as standards.

2.7.3. Mass spectrometry (MS)

MS analysis was carried out using Finnigan MAT-8430 mass spectrometer (Germany) with direct inlet and ionization energy of 70 eV.

3. Results

After 20 h growth of *M. vaccae* VKM Ac-1815D in 100-L fermenter, the cells (650 g, wet weight) were harvested by centrifugation obtaining 50 L supernatant. A sample of cells was used for testing of SO activity associated with cell wall; cell-free supernatant was used for the isolation and purification of extracellular enzymes.

3.1. Isolation and purification of extracellular 3β-hydroxysteroid oxidase

A summary of represented SO purification from the cell-free cultivation broth concentrate is shown in Table 1. At DEAE-Toyopearl chromatography, majority of SO activity was eluted from the column at 425–500 mM NaCl (Fig. 1). At hydroxyapatite Bio-Gel HTP chromatography, two active fractions were revealed: first (HA-I), eluted at 10 mM; and second (HA-II), at 300–345 mM K-phosphate buffer.

At the gel-filtration of HA-I preparation, two SO activities were revealed designated as SO-I, SO-II and eluted in a free volume (20 ml) and 36.5 ml, respectively. At the gel-chromatography of the second hydroxyapatite fraction (HA-II), the enzyme was revealed eluted in a volume of 36.5 ml. Molecular weight of SO-II was determined by gel-filtration as 64 kDa (Fig. 2). The data were confirmed by SDS-electrophoresis showed 62 ± 4 kDa (Fig. 3).

The purification scheme provided almost 300-fold purification of the extracellular SO from *M. vaccae*. Specific activity after gel-filtration was 8.67 μmol/min × mg. The purified SO obeyed Michaelis–Menten kinetics (Fig. 4). Double reciprocal plots kinetics revealed K_m value towards DHEA 7.9×10^{-4} M.

Table 1
Purification of extracellular SO of *M. vaccae* VKM Ac-1815D[a]

Purification step/(preparation name)	Total protein (mg)	Total activity	Specific SO activity (μmol/min × mg)	Recovery (%)
Concentration of cell-free cultivation broth (K-I)	300	6.9	0.023	100
Dialysis of $(NH_4)_2SO_4$ fraction (D-I)	250	5.94	0.024	86
DEAE-Toyopearl chromatography (D-II)	17.6	3.20	1.09	46
Hydroxyapatite chromatography, peak 1 (HA-I)	1.08	3.4	3.17	49
Hydroxyapatite chromatography, peak 2 (HA-II)	1.2	2.88	2.40	42
HA-I gel-filtration on Bio-Gel A 0.5 M (first)	0.70	2.46	3.51	36
HA-I gel-filtration on Bio-Gel A 0.5 M (second)	0.20	1.38	6.90	20
HA-II gel-filtration on Bio-Gel A 0.5 M (SO-II)	0.15	1.3	8.67	19

[a] All enzymatic activities were determined towards DHEA oxidation.

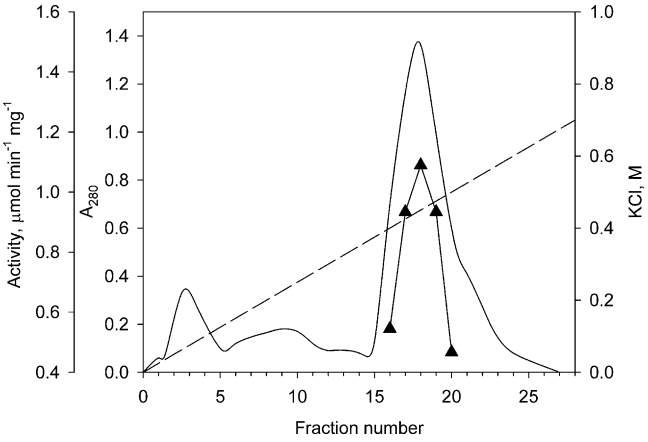

Fig. 1. DEAE-Toyopearl chromatography of SO from *M. vaccae*. D-I preparation was applied to a column (0.9 cm × 40 cm) equilibrated with buffer A. The column after developing with 150 ml of buffer A was eluted with 200 ml linear gradient of 0–1 M NaCl in the same buffer. The flow rate was 10 ml/h and volumes of 5 ml were collected. The enzyme activity (▲), absorbance at 280 nm (—), NaCl concentration (– – –) of eluates are indicated.

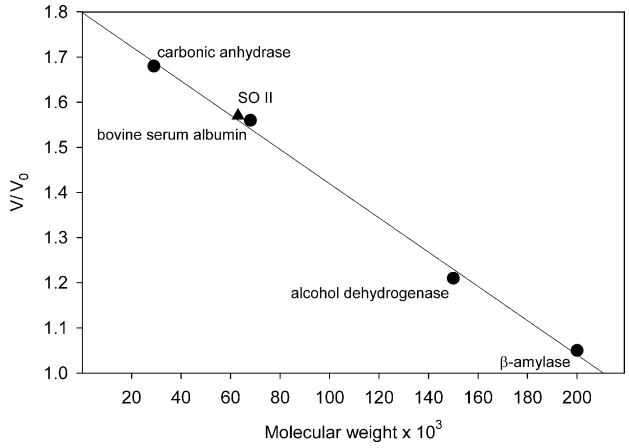

Fig. 2. Gel-filtration on Bio-Gel A-0.5 M of SO from *M. vaccae*. HA-I preparation was applied to a Bio-Gel A-0.5 M column (0.8 cm × 55 cm) equilibrated with buffer A. The column was developed with 70 ml of buffer A at a flow rate of 7 ml/h and volumes of 2.7 ml were collected. Standard proteins (●), SO-II (▲) activity are indicated.

3.2. Transformation of steroids by enzyme preparations

Steroid transforming activities of the enzyme preparations obtained at the different purification steps are presented in Table 2. It should be noted that SO and other activities were revealed only in the presence of exogenic cofactors. As shown, preparation D-I obtained after first purification step actively oxidized sitosterol and DHEA to the corresponding 3-keto-4-ene-steroids thus indicating 3-hydroxysteroid/5-ene-isomerase activities.

Along with 3-keto-4-ene-steroids, the metabolites (Rf 0.24 and 0.16) from sitosterol and DHEA were isolated by preparative TLC and identified by MS as 6-hydroxy-4-sitosten-3-one and 6-hydroxy-4-androsten-3,17-dione (6-OH-AD), respectively.

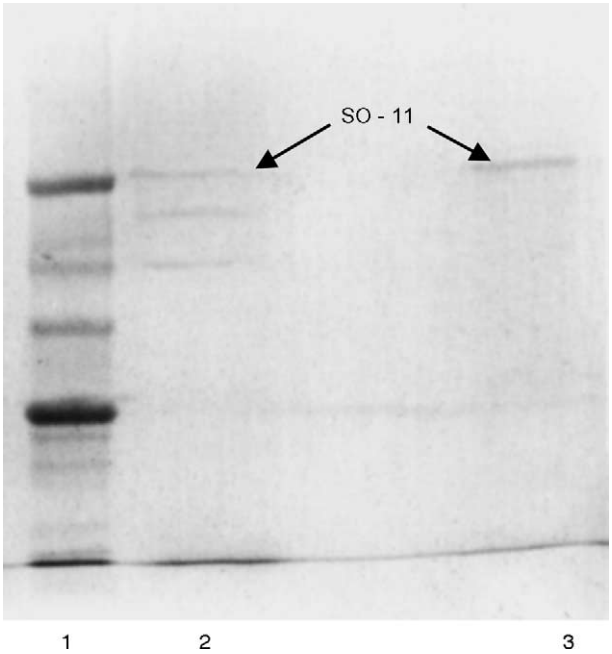

Fig. 3. SDS–PAGE of SO from *M. vaccae*. Aliquots of SO preparations were subjected to electrophoresis on 7.5% gel containing 0.1% SDS. The gel was stained with Coomassie blue. Tracks are the followings: (1) molecular weight marker sizes of 29 000, 36 000, 45 000, 66 000; (2) HA-I preparation before gel-filtration (42 μg); (3) SO-II (38 μg).

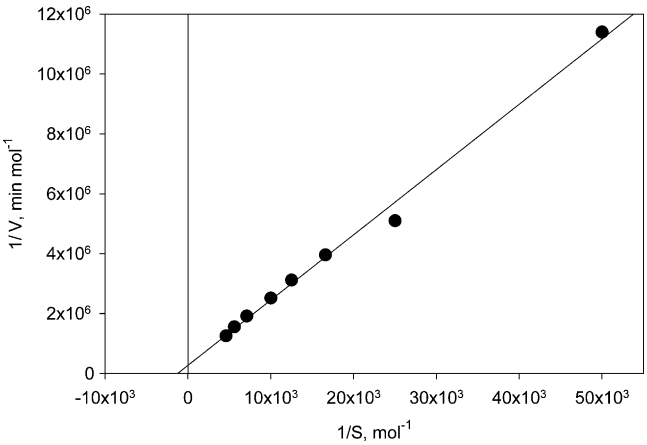

Fig. 4. Double-reciprocal plots of SO activity as a function of DHEA concentration. The assay mixture (1 ml) contained: 20 μM Tris–HCl buffer (pH 6.8); 0.2 μm NADH, 0.5 μg enzyme, 1.99×10^{-5} to 22×10^{-5} M DHEA.

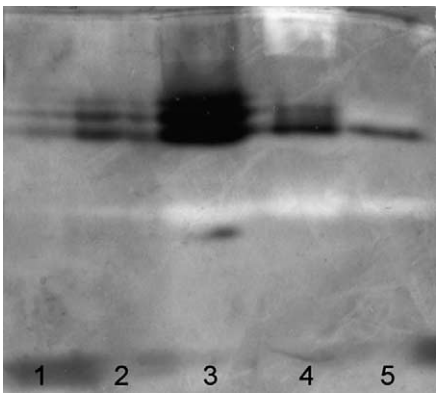

Fig. 5. PAGE of 17-OH SDH intra- and extracellular preparations from *Mycobacterium* spp. followed by dehydrogenase staining. Enzyme preparations were subjected to electrophoresis on 7.5% gel. The gel was stained in accordance with [16]. Tracks are the followings: (1–3) intracellular 17-OH SDH (36, 48, 178 μg, respectively); (4) Triton X-100 eluate (T-I) (130 μg); (5) preparation K-I (concentrate of cell-free cultivation broth) (90 μg).

Similar to the preparations from cell-free cultivation broth, the preparation T-I obtained by Triton X-100 treatment of cells also transformed DHEA to AD and 6-OH-AD thus expressing SO activity (Table 2).

The formation of ADD at the incubation of the enzyme preparations with DHEA indicated the presence of 3-ketosteroid-1(2)-dehydrogenase activity (1(2)-SDH) (Table 2). This activity accompanied SO activity during purification procedure and did not appeared at the final purification step (preparation SO-II).

3.3. 17-OH SDH activity of the preparations

At the incubation of the enzyme preparations with T in the presence of NAD, the formation of AD was observed thus indicating 17-OH SDH activity (Table 2). The presence of 17-OH SDH both in cell-free cultivation broth (K-I) and cell Triton X-100 eluate (T-I) was confirmed by specific gel staining on dehydrogenase activity (Fig. 5). The extracellular 17-OH SDH from K-I appeared as a single dark-stained band while the intracellular enzymes as well as 17-OH-SDH from T-I gave three to four bands. The band of 17-OH SDH from K-I corresponded to the major band of the intracellular enzyme.

As shown in Fig. 6, AD and ADD were formed as the major products of DHEA conversion by the preparation HA-I at the low (less than 30 μg/ml) concentrations of the substrate, while preferable accumulation of T and DHT was observed in a range of DHEA concentrations from 30 to 80 μg/ml.

Table 2
Steroid transforming activity of enzyme preparations

Substrate	Purification step	Cofactor	Product	Putative enzyme
β-Sitosterol	$(NH_4)_2SO_4$ dialysate (D-I)	NAD(P)H	4-Sitosten-3-one; 6-OH-4-sitosten-3-one	SO
DHEA	$(NH_4)_2SO_4$ dialysate (D-I)	NAD(P)H	AD; ADD; 6-OH-AD	SO; 1(2)-SDH
	DEAE-Toyopearl chromatography (D-II)	NAD(P)H	AD; ADD; 6-OH-AD	SO; 1(2)-SDH
	Hydroxyapatite chromatography (HA-I)	NAD(P)H	AD; ADD; T[a]; DHT[a]; 6-OH-AD	SO; 1(2)-SDH; 17-OH SDH
	Hydroxyapatite chromatography (HA-II)	NAD(P)H	AD; ADD; 6-OH-AD	SO; 1(2)-SDH
	Gel-filtration on Bio-Gel A 0.5 M (SO-I)	NAD(P)H	AD; ADD; 6-OH-AD	SO; 1(2)-SDH
	Gel-filtration on Bio-Gel A 0.5 M (SO-II)	NAD(P)H	AD; 6-OH-AD	SO
	Triton X-100 eluate of cells (T-I)	NAD(P)H	AD; 6-OH-AD	SO
Testosterone	DEAE-Toyopearl chromatography (D-II)	NAD	AD; ADD; DHT	17-OH SDH; 1(2)-SDH
	DEAE-Toyopearl chromatography (D-II)	NAD(P)H	No metabolites	–
	Hydroxyapatite (HA-I)	NAD	AD	17-OH SDH
	Hydroxyapatite (HA-I)	NAD(P)H	No metabolites	–
	Gel-filtration on Bio-Gel A 0.5 M (SO-II)	NAD(P)H	No metabolites	–
	Triton X-100 eluate of cells (T-I)	NAD	AD	17-OH SDH
ADD	Hydroxyapatite chromatography (HA-I)	–	AD	Steroid 1-ene reductase

[a] Appeared at initial concentration of the substrate (DHEA): 30–90 μg/ml.

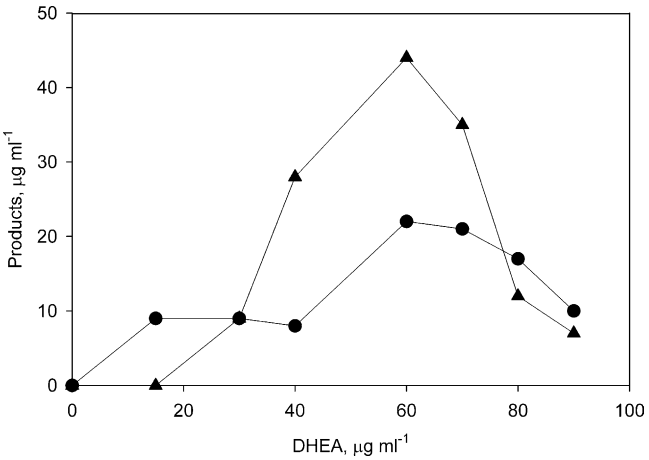

Fig. 6. The effect of DHEA concentration on metabolites formation by HA-I preparation. The assay mixture (2 ml) contained: 20 μM Tris–HCl buffer (pH 6.8); 0.2 μM NAD, 150 μl of enzyme (HA-I); 14.4–115 μg/ml DHEA; (●) ADD + AD; (▲) T + DHT.

4. Discussion

To our knowledge, this is a first publication on the extracellular sterol oxidase purification from the fast-growing mycobacteria.

As shown in this work, the enzyme from *M. vaccae* responsible for modification of 3-hydroxy-5-ene to 3-keto-4-ene moiety of steroids—later identified as SO, was weakly associated with mycobacterial cell wall and located outside the cell. The soft treatment of biomass with nonionic detergent—Triton X-100 allowed to obtain the preparation effectively converting DHEA to AD.

Steroid transforming activities were also found in cell-free cultivation broth obtained without any detergent application at the cultivation of strain on glycerol mineral medium supplemented with sitosterol. These activities were revealed after the concentration and ammonium sulphate precipitation of the enzymes.

Along with 3-keto-4-ene steroids, 6-hydroxy derivatives were revealed at the incubation of the preparations with 3-hydroxy-steroids. Probably, extracellular SO from *M. vaccae* catalyzed not only 3-OH-dehydrogenation and $\Delta^{5\rightarrow}\Delta^{4}$ isomerization, but also 6-hydroxylation. The formation of 6-hydroxy derivatives was described for ChO from different microorganisms. For example, along with dehydrogenation of 3-OH-function and $\Delta^{5\rightarrow}\Delta^{4}$ isomerization ChO from *Pseudomonas* sp. hydroxylated cholesterol to 6β-hydroxyperoxycholest-4-ene-3-one followed by its spontaneous conversion to 6-hydroxycholest-4-ene-3-one and cholest-4-ene-3,6-dione with uptake of 2 mol O_2 and formation of 1 mol H_2O_2 per every molecule of utilized cholesterol. Oxidized ChO isoform dehydrogenated cholesterol forming 5-ene-3-one derivative, while reduced isoform of the enzyme oxidized cholest-5-ene-3-one to 6β-hydroxyperoxycholest-4-ene-3-one [22]. However, the formation of 6-OH-derivatives by ChO from fast-growing mycobacteria was unreported earlier.

The purification scheme applied in this work provided almost 300-fold purification of the extracellular SO of *M. vaccae*. Enzyme molecular weight (62 ± 4 kDa), properties and multifunctional action corresponded to ChO from the relative organisms, rather than short-chain dehydrogenases [9–11].

Along with extracellular SO, 17-OH SDH and 1(2)-SDH activities were revealed in the preparations from the cell-free cultivation broth of *M. vaccae*. These enzymes probably tightly associated with SO since the effective separation was achieved only at the final purification step.

Recently, we isolated and purified two intracellular 17-OH SDH from *M. vaccae* [16]. In this work we showed the presence of extracellular 17-OH SDH in this organism actively converting T to AD in the presence of exogenic NAD. Unlike cytosol ones, this enzyme appeared as one dark-stained band at PAGE-electrophoresis staining.

Combined action of the extracellular enzymes was demonstrated using HA-I preparation. Thes formation of

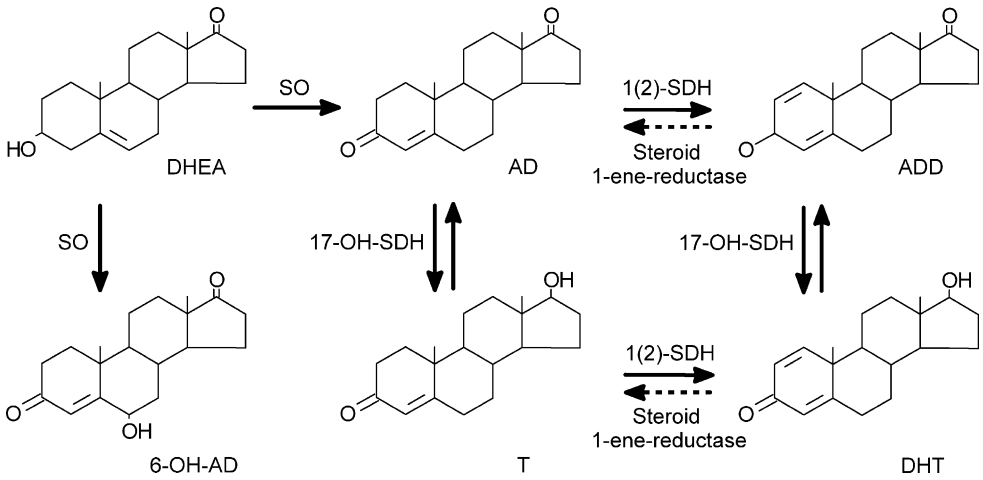

Fig. 7. Putative route of DHEA transformation by extracellular enzymes of *M. vaccae*.

AD from DHEA is attributed to SO activity. Evidently, AD then underwent to 1(2)-SDH and 17-OH-SDH action forming ADD, T, and DHT.

Schematically, the interconversions of steroids by extracellular enzymes of *M. vaccae* are presented in Fig. 7. Interestingly, no 17-hydroxy derivatives of DHEA were observed in detectable amounts thus confirming that 17-OH-SDH has lower affinity to 3-OH-steroids compared with SO.

Two SO activities—SO-I and SO-II were found at the gel-filtration of HA-I preparation. The first fraction eluted in free volume and probably represented aggregated SO, while the second one corresponded to 64 kDa.

The existence of different ChO isoforms was described for related organisms. For example, in *Nocardia rhodochrous* three ChO isoforms were found differed by the presence or absence of hydrophobic part that responsible for the enzyme affinity to the cell wall [5]. The enzyme with dehydrogenase and isomerase activities acquired amphipathic properties thus allowing adsorption on hydrophobic surfaces, aggregation in the absence of detergent and formation of mixed micelle with detergent and sterol.

Thus, extracellular 3-hydroxysteroid oxidase (M_r = 64 kDa) has been isolated from *M. vaccae*. The further investigations propose to study extra- and intracellular SO forms, correlations between steroid-transforming enzymes in bacterial cell, as well as to analyse amino acid sequences and the gene encoding. The results will expand the imagination on the mechanism of hydrophobic sterol substrate uptake by fast-growing mycobacteria.

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