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The Conversion of Lithocholic Acid into 5β-Androstan-3, 17-dione in the Cell-free System of *Mycobacterium* sp. NRRL B-3805

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Abstract □ In a microbial cell-free extract system, side chain cleavage on various sterols and steroids was tested. The cell-free extracts of *Mycobacterium* sp. NRRL B-3805 showed the side chain cleavage activity on lithocholic acid to form 5β-androstan-3,17-dione. The properties of the activity were examined.

keywords □ *Mycobacterium* sp. NRRL B-3805, cell-free system, side chain cleavage, lithocholic acid, 5β-androstan-3, 17-dione.

Microbial side chain cleavage of plant or animal sterols provides starting materials for various steroidal drugs. Through strain improvement, this process has been employed for the production of 17-ketosteroids such as 4-androsten-3, 17-dione and 1, 4-androstadien-3, 17-dione in fermentation industries[1]. But there has been few report regarding to the cell-free system or purification of an active protein of sterol side chain cleavage activity[2]. Functional protein(s) purified for the activity could be a good starting point for understanding enzymatic reaction mechanisms and cloning.

For these purposes, we investigated the cell-free system for microbial side chain cleavage of sterols. The cell-free extracts of *Mycobacterium* sp. NRRL B-3805 showed side chain cleavage activity on lithocholic acid. In this paper, we describe the characteristics of the activity.

EXPERIMENTAL METHODS

Microorganisms

Since *Arthrobacter simplex* IAM 1660, *Brevibacterium lipolyticum* IAM 1398, *Mycobacterium* sp. NRRL B-3683 and *Mycobacterium* sp. NRRL B-3805 have been known to produce 17-ketosteroids from sterols[3,4], they were used for testing the activities of 17-ketosteroid production and preparation of cell-free extracts. *A. simplex* and *B. lipolyticum* were maintained on nutrient agar slants containing 0.01% cholesterol. *Mycobacterium* sp. NRRL B-3683 was preserved on the KCTC medium 80 (glycerol 1%, sodium citrate 0.1%, NH_4Cl 0.1%, K_2HPO_4 0.05%, $MgSO_4$ 0.05%, urea 0.05%, $FeCl_3$ 0.05%, tryptic soy broth 0.1%, pH 7.0) and *Mycobacterium* sp. NRRL B-3805 on the KCTC medium 129 (glucose 0.1%, cholesterol 0.05%, NH_4NO_3 0.1%, K_2HPO_4 0.025%, $MgSO_4$ 0.025%, $FeSO_4 \cdot 7H_2O$ 0.0001%, yeast extract 0.5%, pH 7.0).

Fermentation

In the case of *A. simplex* and *B. lipolyticum*, the culture was started with inoculation of seed culture by 5% into a fermentation medium (NH_4NO_3 0.1%, $MgSO_4 \cdot 7H_2O$ 0.025%, K_2HPO_4 0.025%, yeast extract 0.5%, pH 7.0). A substrate (0.1% steroid or sterol, final conc.) was suspended with tween 80 (0.01%) and added to the 20-22 hr culture broth. α, α′-Dipyridyl (1 mM, final conc.) was added 1 hr after the substrate addition. *Mycobacterium* sp. was inoculated into nutrient broth containing 0.1% sterol or steroid, and 1.5% glucose with one loopful from the stock slant. Cultivation period was 72 hr for *A. simplex* and *B. lipolyticum*, and 120 hr for *Mycobacterium* sp., at 30℃ and 200 rpm.

Preparation of cell-free extracts

Microorganism was cultivated as in the fermentation section except 0.02% cholesterol addition in the culture medium. Cells were harvested after 48 hr (*A. simplex* and *B. lipolyticum*) or 60 hr (*Mycobacterium*

Table I. Quantitative analysis of 17-ketosteroid from various sterols and steroids

Substrate	17-Ketosteroid production (ADD/AD*) mg/ml (%, conversion yield)			
	B. lipolyticum IAM 1398	*A. simplex* IAM 1660	*M.* sp. NRRL B-3683	*M.* sp. NRRL B-3805
Cholesterol	0.26 (35.3)	0.30 (40.7)	0.15 (20.5)	
			0.23 (31.1)*	0.15 (20.5)*
β-Sitosterol	0.12 (17.3)	0.14 (20.3)	0.20 (29.4)	
			ND*	0.14 (20.5)*
Stigmasterol	0.03 (3.98)	0.04 (5.37)	0.14 (19.9)	
			ND*	0.08 (10.5)*
Lithocholic acid	0.07 (9.71)	0.05 (6.4)	0.03 (3.73)	
			0.17 (22.2)*	0.16 (21.2)*
Pregnenolone	0.21 (22.8)	0.30 (33.0)	ND	
			trace*	trace*
Progesterone	0.19 (21.1)	0.21 (22.9)	ND	
			ND*	trace*

ND: not detected

sp.) incubation, by centrifugation with 6,500 rpm on GSA rotor at 4℃ for 5 min. Cell pellets were washed with 0.05 M phosphate buffer (pH 7.8) and resuspended in the same buffer containing 0.25 mM EDTA and 1 mM DTT, resulting in 50% cell suspension. The cell suspension was subjected to bead beating (30 sec beating − 2 min. standing, 4 times) with equal volume of glass bead (0.1 mm diameter). The broken cell suspension was centrifuged at 20,000×g at 4℃ for 20 min. The supernatant was re-centrifuged at the same condition as before. The resulting supernatant fraction was used as cell-free extracts.

For ultracentrifugation, 20,000×g cell-free extracts was subjected to 37,000 rpm centrifugation on 70.1 Ti rotor at 4℃ for 30 min, equivalent to 100,000×g.

Reaction with cell-free extracts

To the cell-free extracts (20,000×g or 100,000×g), an appropriate substrate (final conc. 0.05%) was added. The mixture was incubated at 30℃ with shaking for 24 hr.

Treatment of cell-free extracts with amberite XAD resin

Amberite XDA 2 or 16 (2 gr prewashed with 50 mM phosphate buffer) which could be employed to adsorb C_{19}-steroids from the cell-free extracts[5] was added to the cell-free extracts obtained from 10 gr cell (wet wt.). With intermittent stirring, the

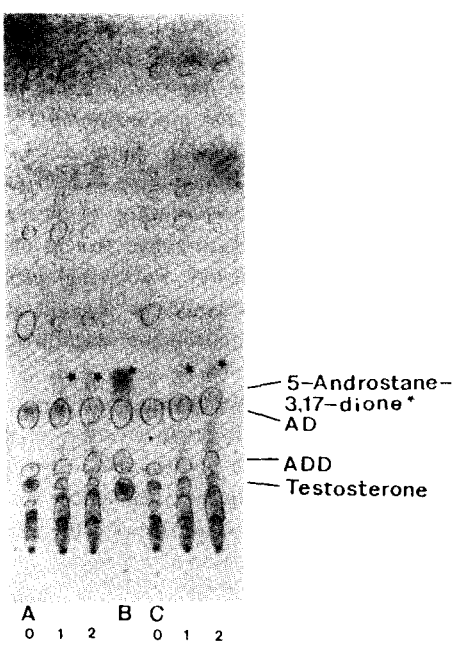

Fig. 1. Effect of filtration (0.2 μm bacteriological filter) of 20,000×g cell-free extracts on 5β-androstan-3, 17-dione formation.

A: Cell-free extracts before filtration and lithocholic acid, B: Standard 4-androsten-3, 17-dione (AD), 1, 4-androstadien-3, 17-dione (ADD), testosterone and 5β-androstan-3, 17-dione, C: Cell-free extracts after filtration and lithocholic acid 0: 0 hr incubation sample, 1: 4 hr incubation sample, 2: 24 hr incubation sample.

Table II. Product formation pattern of reaction mixture of cell-free extracts with various steroidal substrates

Substrate	Product identified on TLC*		
	B. lipolyticum IAM 1398 *A. simplex* IAM 1660	*M.* sp. NRRL B-3683	*M.* sp. NRRL B-3805
Cholesterol	Cholestenone, Cholestadienone	Cholestenone	Cholestenone
Lithocholic acid	–	–	5β-androstan-3, 17-dione Unkown compd.
Pregnenolone	Progesterone dehydroprogesterone	Progesterone	Progesterone
Progesterone	Dehydroprogesterone	–	–

*TLC: Silical gel 60 GF_{254} (Merck)
Solvent system: Chloroform: ethyl ether (10 : 1, v/v)

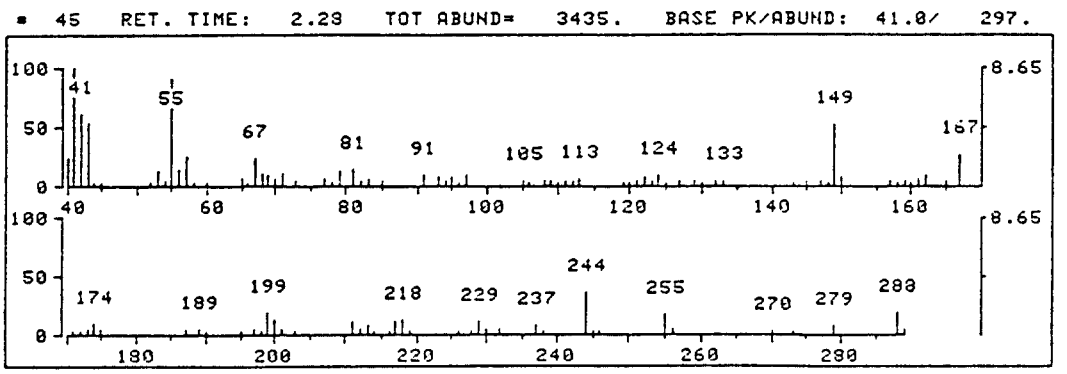

Fig. 2. Mass spectrum of the unkown compound isolated through preparative TLC from incubation mixture of cell-free extracts and lithocholic acid.

suspension was maintained for 45 min in an ice bath and then filtered through a funnel stopped with glass wool. The resin was washed with 20 ml of 50 mM phosphate buffer. The filtrate and the XAD resin were tested for the activity with lithocholic acid at 30℃ for 24 hr incubation.

Analysis of fermentation or reaction products

Samples were extracted with equal volume of ethyl acetate. The fraction of ethyl acetate was applied on TLC plate (GF254, Merk) or GC (OV101, 1/4″ column 10 ft, N_2 flow rate 60 cc/min) directly, or after concentration by nitrogen gas[6]. The solvent system for TLC was chloroform : ethyl ether (10 : 1, v/v). Spots on TLC were identified by UV or 10% H_2SO_4 with heating (80℃, 30 min).

RESULTS AND DISCUSSION

In the fermentation experiments, 4-androsten-3, 17-dione (AD) and/or 1, 4-androstadien-3, 17-dione (ADD) were produced by *A. simplex* IAM 1660, *B. lipolyticum* IAM 1398, *Mycobacterium* sp. NRRL B-3683 and *Mycobacterium* sp. NRRL B-3805 from sterols and steroids as shown in Table I. The ADD production from various sterols and steroids by *A. simplex* ad *B. lipolyticum* decreased in the following sequence: cholesterol>pregnenolone>progesterone>β-sitosterol>lithocholic acid>stigmasterol. *Mycobacterium* sp. NRRL B-3683 utilized β-sitosterol best for ADD production and cholesterol for AD production. *Mycobacterium* sp. NRRL B-3805 produced AD most from lithocholic acid. In contrast to *A. simplex* or *B. lipolyticum*, *Mycobacterium* spp. could not transform pregnenolone or progresterone to ADD or AD.

In the experiments with the cell-free extracts of the above four strains, the products in the reaction mixture were identified by TLC or GC. The results are summarized in Table II. Data in Table II suggested that the cell-free extracts used in the experiments did not indicate any activity to cleave an

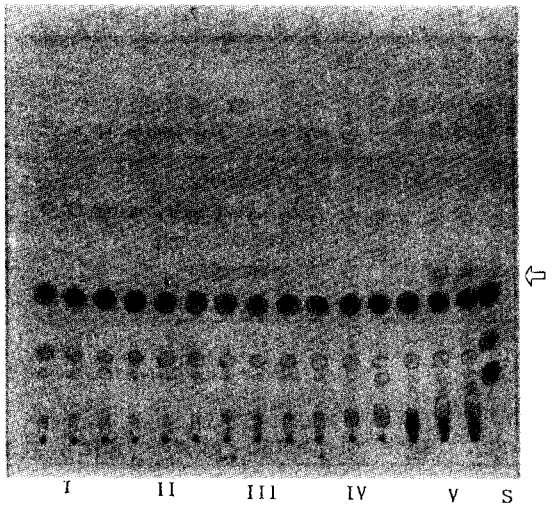

Fig. 3. Boiling effect on lithocholic acid side chain cleavage activity of cell-free extracts of *Mycobacterium* sp. NRRL B-3805.
Activity was assayed at 0, 4, 24 hr incubation time.
I: Cell-free extracts without addition of lithocholic acid, II: Heat treated cell-free extracts, III: Heat-treated cell-free extracts and 3β-OH-bisnor-5-cholenic acid, IV: Cell-free extracts and 3β-OH-bisnor-5-cholenic acid, V: Cell-free extracts and lithocholic acid, S: Standard; from top to bottom, AD, ADD, testosterone.

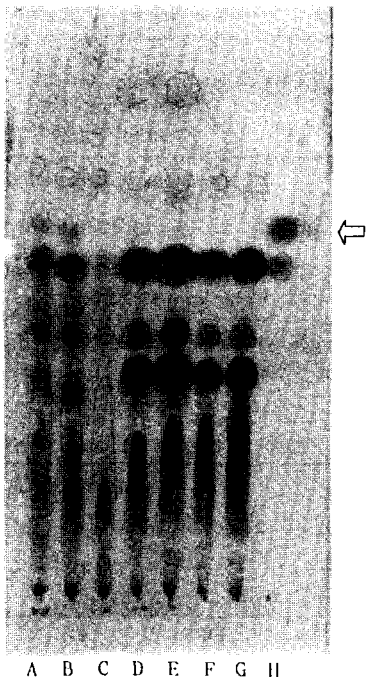

Fig. 4. Effect of Amberite XAD resin on the side chain cleavage activity of cell-free extracts.
A: Cell-free extracts, B: Cell-free extracts after protamine sulfate treatment, C: Filtrate after XAD-2 treatment, D,E,: XAD-2 resin adsorbed activity, F,G:XAD-16 resin adsorbed activity, H: Standard from top, 5β-androstan-3, 17-dione, AD.

intact side chain of sterols. But the cell-free system of *Mycobacterium* sp. NRRL B-3805 showed the accumulation of a possible cleavage product from lithocholic acid. The TLC pattern of the reaction mixture with lithocholic acid as a substrate is shown in Fig. 1. Regardless of filtration through bacteriological filter (Milex-GV, 0.2 μm), yellow to blue spot just above AD was observed. This indicates that the cell-free extracts contained the conversion activity.

The corresponding compound was identified as 5β-androstan-3, 17-dione by isolation through preparative TLC, GC and mass spectrometry analysis. The isolated compound has 0.35 R_f value in chloroform : ethyl ether (10 : 1, v/v) solvent system and 8.37 min retention time in GC analysis (OV101, 1/4″ 10 ft column, N_2 flow rate 60 cc/min, 300-280-320℃). It also showed 288 m/e species in mass spectrometry as shown in Fig. 2. These properties were compared with those of a commercially available standard compound : 5β-androatan-3, 17-dione (Steraloids. INC).

To confirm the conversion activity, 20,000×g cell-free extracts were subjected to 100,000×g ultracentrifugation, heat treatment (10 min) in a boiling water bath and Amberite XAD resin treatment. In ultracentrifugation, the supernatant fraction showed the accumulation of 5β-androstan-3, 17-dione but the precipitate did not (data not shown). As shown in Fig. 3, the boiled cell-free extract did not have any activity. Also we could not observe any particular spot from the incubation mixture of the cell-free extracts and 3β-OH-bisnor-5-cholenic acid. This may indicate the substrate specificity to stereochemistry. The activity in the cell-free extracts showed the tendency to be bound to Amberite XAD resin. Fig. 4 indicates that phenomenon. The filtrate after Amberite XAD treatment did not accumulate androstan-3, 17-dione, while the treated Amberite XAD showed the activity.

These results demonstrated clearly the side chain

cleavage activity of the cell-free system of *Mycobacterium* sp. NRRL B-3805 upon lithocholic acid. The previous report by Fujimoto *et al.*, suggested that the cell-free extracts converted 26-hydroxy-2-ethyl cholest-4-en-3-one or 3-oxo-24-ethyl cholest-4-en-26-oic acid to 3-oxo-chol-4-en-24-oic acid[2]. If the cell-free extract was supplemented with ATP, coenzyme A, $MgCl_2$ and phenazine methosulfate, 3-oxo-chol-4-en-24-oic acid was transformed to 4-androsten-3, 17-dione[2]. But in our results, the cell-free extract itself showed the conversion activity of lithocholic acid to 5β-androstan-3, 17-dione without any addition of cofactors.

CONCLUSION

The cell-free extracts of *Mycobacterium* sp. NRRL B-3805 showed the side chain cleavage activity of lithocholic acid to 5β-androatan-3, 17-dione. In contrast to the report by Fujimoto *et al.*[2], the activity in the cell-free extracts did not require any cofactor additions. The activity was lost upon heat treatment and bound to Amberite XAD resin. The system utilized lithocholic acid as substrate but neither 3β-OH-bisnor-5-cholenic acid, cholesterol, pregnenolone nor progesterone. Based on these results, it might be possible to purify the active fraction from the cell-free extracts of *Mycobacterium* sp. NRRL B-3805.

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