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Transformation of cholesterol to testosterone by *Mycobacterium* sp.

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SUMMARY

The production of testosterone from cholesterol via a single-step microbial transformation process was investigated. A supplement of 4% glucose and 1% peptone into a synthetic medium, pH control at 6.0 and continuous feeding of cholesterol were the most important parameters. Under optimal conditions, the maximum molar conversion rate of testosterone from cholesterol was up to 42.68% in 2.5-L surface-aerated fermentor after 100 h cultivation. The reduction of androst-4-ene-3,17-dione, an intermediate product, to testosterone was catalyzed by 17β-hydroxysteroid dehydrogenase in *Mycobacterium* sp. Testosterone was isolated from the fermentation broth by the addition of Amberlite XAD-7 resin and was further purified by flash chromatography on a silica gel column. After crystallization in acetone, testosterone could be obtained as needle crystals.

INTRODUCTION

Testosterone is a male sex hormone or androgen which is produced throughout life by the testes. The final step in the biosynthesis of testosterone is reduction of androst-4-ene-3,17-dione (AD) to testosterone. This reaction is catalyzed by the microsomal enzyme 17-ketosteroid reductase (17β-hydroxysteroid:NADP 17-oxidoreductase, EC 1.1.1.64) [1]. The enzymatic reduction of AD to testosterone by 17β-hydroxysteroid dehydrogenase (17β-hydroxysteroid:NAD 17-oxidoreductase, EC 1.1.1.63) has also been found in microorganisms such as *Pseudomonas* [9,10], *Saccharomyces* [11,14] and *Marchantia* [4]. Although AD can be produced from the selective side chain cleavage of cholesterol via microbial transformation process [5], the industrial production of testosterone from AD is carried out via four steps of chemical synthesis [3].

Wang [13] reported that when strains of the genus *Mycobacterium* were grown in a nutrient broth supplemented with 40 g L^{-1} of glucose, the major transformation product of cholesterol was shifted from AD to testosterone. We have previously reported a novel process for the production of AD from cholesterol in a synthetic medium using a surface-aerated fermentor as the reactor [8]. The purpose of this study was to investigate a suitable single-step microbial transformation process for the production of testosterone from cholesterol by *Mycobacterium* sp.

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MATERIALS AND METHODS

Microorganism

A cholesterol-assimilating and AD-producing mutant of *Mycobacterium* sp. NRRL B-3805 was used throughout this study.

Materials

Yeast extract, nutrient broth, peptone and agar were obtained from Difco (Detroit, MI, USA). Androst-4-ene-3, 17-dione (AD) (A9630), testosterone (TS) (T1500), Tween 80 (P1754) and Amberlite XAD-7 (A7768) were obtained from Sigma (St Louis, MO, USA). Inorganic salts and other chemicals were all of reagent grade.

Cultivation methods

The stock culture was maintained on an enriched nutrient agar medium which contained (per liter): nutrient broth 8 g, yeast extract 10 g, glucose 10 g, and agar 20 g (pH 6.8). A glucose–nutrient broth medium consisting of 16 g nutrient broth and 40 g glucose per liter of distilled water (pH 6.8), and an AD-producing synthetic medium consisting of 1.5 g ammonium acetate, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.4 g K_2HPO_4, 0.8 g KH_2PO_4, 5 mg $FeSO_4 \cdot 7H_2O$, 2 mg $ZnSO_4 \cdot 7H_2O$, 0.5 mg $MnSO_4 \cdot 4H_2O$ and 0.1 g Tween 80 per liter of distilled water (pH 8.0) [8] were used as seed and basal media for the production of testosterone, respectively. Five milliliters of the seed culture were inoculated into 100 ml of a medium supplemented with 1 g L^{-1} cholesterol in a 500-ml Hinton flask. The culture was incubated at 30 °C on a rotary shaker (120 r.p.m.) for 5 days. Fermentation experiments were carried out in a 2.5-L surface-aerated fermentor (Eyela Model M-100, Tokyo Rikakikai Co., Tokyo) [7].

Analytical methods

Steroids. The culture filtrate was extracted with ethyl acetate. The organic phase was evaporated and the residue was dissolved in methanol. AD and testosterone in the methanol solution were then determined by HPLC using a C_{18} column and a mobile phase composed of acetonitrile and water (50/50, v/v) [7]. Cholesterol was measured according to the Liberman–Burchard color reaction [15]. The molar conversion rate of testosterone was calculated on the basis of cholesterol added to the medium.

Glucose and cell mass. Glucose was determined by the dinitrosalicyclic acid method [2]. Cell mass was determined with a turbidimetric method (OD_{600}) as described in a previous paper [6].

Enzyme assay. 17β-Hydroxysteroid dehydrogenase activity was measured by the rate of NADH formation or reduction monitored at 340 nm as described by Schultz et al. [10]. The oxidation of hydroxysteroid was measured in an assay mixture (total volume of 3 ml) containing 1.2 μmol NAD, 1.04 μmol testosterone, and 100 μmol sodium phosphate or sodium pyrophosphate, pH 6.0–10.0. The reduction of ketosteroid was measured in an assay mixture containing 1.2 μmol NADH, 1.04 μmol androst-4-ene-3,17-dione, and 100 μmol sodium phosphate or sodium pyrophosphate, pH 5.0–9.0.

RESULTS

Optimization of medium composition

The effect of medium composition on the production of testosterone from cholesterol was examined with shaken cultures incubated at 30 °C for 5 days. As shown in Table 1, the main product of the transformation of cholesterol by *Mycobacterium* sp. in both nutrient broth medium and synthetic medium was AD. However, the production of testosterone was promoted by the addition of 4% glucose. Furthermore, the molar conversion rate of testosterone from cholesterol increased up to 52.2% when the transformation was carried out in the synthetic medium supplemented with 4% glucose and 1% peptone.

Production of testosterone in fermentor

The fermentation conditions for production of testosterone from cholesterol were investigated in a 2.5-L surface-aerated fermentor loading 1 L of synthetic medium in which 4% glucose and 1% peptone were added. After the culture was incubated at 30 °C for 24 h, 0.1% cholesterol was added. As shown in Fig. 1, the cell mass increased in parallel with the rapid consumption of cholesterol. On the contrary, the consumption rate of glucose in the medium was much slower

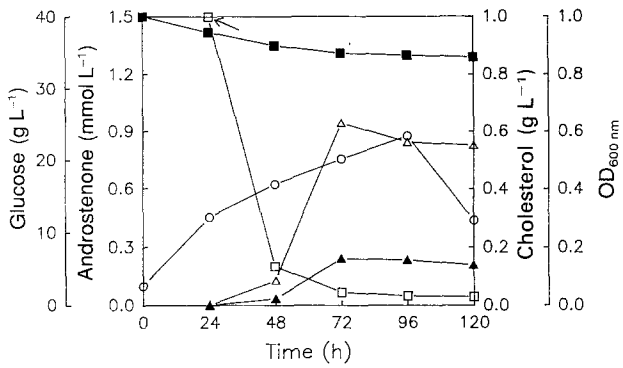

Fig. 1. Production of androstenones from cholesterol by *Mycobacterium* sp. in a 2.5-L surface-aerated fermentor. Fermentation conditions: the composition of peptone-glucose-supplemented synthetic medium was the same as that in Table 1. Operation conditions were as follows: working volume, 1 L/2.5 L fermentor; inoculum size, 5%; surface aeration rate, 1 v.v.m.; agitation speed, 300 r.p.m.; temperature, 30 °C. ▲, TS; △, AD; □, cholesterol; ■, glucose; ○, cell growth ($OD_{660\ nm}$); ↓, 0.1% cholesterol added.

TABLE 1

Effect of medium composition on the production of androstenones[a]

Medium[b]	Androstenones[c] (μM)		Molar conversion rate (%)		
	TS	AD	TS	AD	Total
Nutrient broth (NB)	–	1370	–	52.53	52.53
Glucose + (NB)	629	645	24.33	24.95	49.28
Synthetic medium (SM)	–	1014	–	39.21	39.21
Glucose + (SM)	88	1114	3.40	43.08	46.48
Peptone + Glucose + (SM)	1350	240	52.20	9.28	61.48

[a] Media were supplemented with a 1 g L^{-1} cholesterol and the cultures were incubated at 30 °C on a rotary shaker (120 r.p.m.) for 5 days.

[b] Medium composition: (1) Nutrient broth, 16 g L^{-1}; (2) Synthetic medium, 1.5 g ammonium acetate, 0.4 g K_2HPO_4, 0.8 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 5 mg $FeSO_4 \cdot 7H_2O$, 2 mg $ZnSO_4 \cdot 7H_2O$, 0.5 mg $MnSO_4 \cdot 4H_2O$ and 1000 ml distilled water; (3) Supplemented components, 40 g L^{-1} glucose; 10 g L^{-1} peptone.

[c] Androstenones: TS, testosterone; AD, androst-4-ene-3,17-dione.

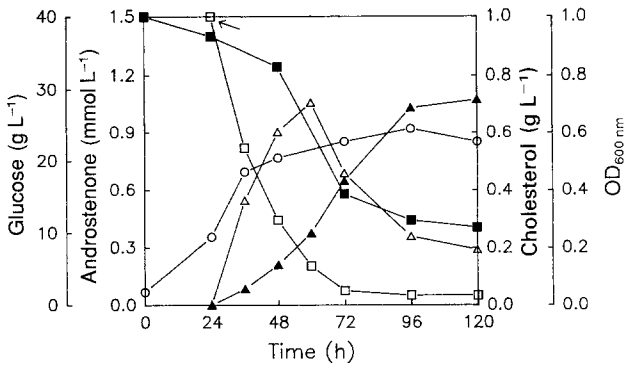

Fig. 2. Production of androstenones from cholesterol by *Mycobacterium* sp. in a 2.5-L surface-aerated fermentor with pH control. Fermentation conditions were the same as in Fig. 1 except the pH was controlled at 6.0. ▲, TS; △, AD; □, cholesterol; ■, glucose; ○, cell growth ($OD_{660\ nm}$); ↓, 0.1% cholesterol added.

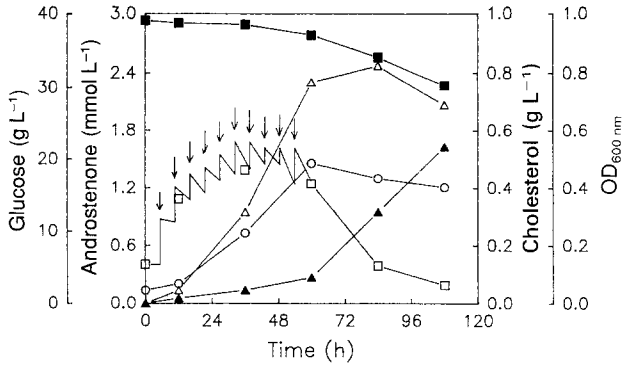

Fig. 3. Production of androstenones from cholesterol by *Mycobacterium* sp. in a 2.5-L surface-aerated fermentor with pH control and continuous feeding of cholesterol. Fermentation conditions were the same as in Fig. 1. ▲, TS; △, AD; □, cholesterol; ■, glucose; ○, cell growth ($OD_{660\ nm}$); ↓, cholesterol feeding rate, 23.5 mg h^{-1}.

than that of added cholesterol. The pH of the medium was maintained between 6.5 to 7.5 during the cultivation period. The maximum accumulation of AD appeared at 72 h cultivation. However, the accumulation of testosterone was very poor. When the pH of the medium was controlled at 6.0 during the cultivation, the rapid consumption rate of added cholesterol was in parallel with that of glucose. The pattern of cell growth, however, was similar to that of the pH-noncontrolled system (Fig. 2). The maximum AD accumulation appeared at 60 h, then most of the AD was converted to testosterone after 120 h cultivation. The total molar conversion rate of AD and testosterone was about 60% whether the pH was controlled at 6.0 or not. However, the molar conversion rate of testosterone in a pH-controlled system was superior to that of the free growing system (Table 2). As shown in Fig. 3, the total molar conversion rate of AD and testosterone could increase up to 90.56% by the continuous feeding of cholesterol after 100 h cultivation when the pH was controlled at 6.0. Although the molar conversion rate of testosterone was reduced to 42.68%, the total productivity of testosterone was superior to that of the batch feeding process under the same cultivation conditions (Table 2).

Interconversion of AD and testosterone by the partially purified enzyme

Cells of the *Mycobacterium* sp. were cultured under the same condition as that in Fig. 1 for 120 h. The bacterial cells were recovered and broken using a French press and lysozyme treatment. 17β-Hydroxysteroid dehydrogenase in the supernatant fluid was then purified by means of ammonium sulfate-precipitation and gel filtration in Sephadex G-100. The enzymatically active fractions were pooled and used to study the effect of pH on the interconversion of AD and testosterone. The optimal pH for the conversion of AD to testosterone by the partial purified enzyme was 6.0 and the conversion activity decreased as the pH increased. In contrast, the oxidation of testosterone to AD was predominant at alkaline pH (Fig. 4).

Isolation and purification of testosterone

Testosterone and AD accumulated were isolated from the fermentation broth by the addition of Amberlite XAD-7 resin (20 g per liter of fermentation broth) as an adsorption carrier. The resin particles were filtered and washed with distilled water, and then testosterone and AD were eluted with acetone (2 ml per gram of resin), dried over sodium

TABLE 2

Effect of fermentation conditions on the production of androstenones in a 2.5-L surface-aerated fermentor

Conditions[a]	Androstenones (μM)		Molar conversion rate (%)		
	TS	AD	TS	AD	Total
Without pH control	227	1337	8.77	51.69	60.46
With pH control	1169	384	45.20	14.85	60.05
With pH control and continuous feeding of cholesterol	1700	1900	42.68	47.88	90.56

[a] Fermentation conditions were the same as in Figs 1, 2 and 3.
Total amount of cholesterol added was 1.54 g.

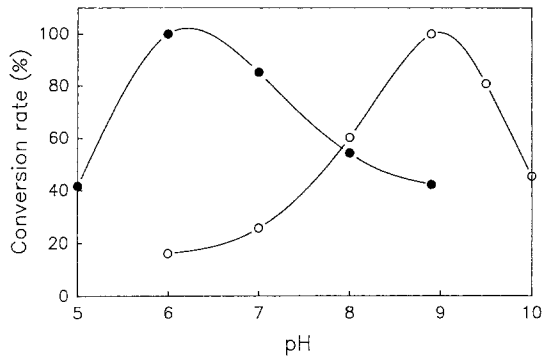

Fig. 4. Effect of pH on the interconversion of testosterone and AD by the partial purified 17β-hydroxysteroid dehydrogenase of *Mycobacterium* sp. Buffer used: pH 5.0–8.0, sodium phosphate; pH 8.9–10.0, pyrophosphate. ●, reduction of AD to testosterone; ○, oxidation of testosterone to AD.

sulfate and evaporated to dryness. The mixture of testosterone and AD was further separated by flash chromatography on a silica gel column (15 × 2.5 cm) eluted with ethyl acetate/n-hexane (40/60, v/v). The fractions containing testosterone were collected and rechromatographed on a silica gel column using ethyl acetate/chloroform (14/86, v/v) as the developing solvent to remove the minor quantity of contaminated AD. The effluent containing testosterone was evaporated under reduced pressure.

Testosterone powder with a purity of 99.9% was obtained. After crystallization in acetone, needle crystals were obtained. The melting point of needle crystals was 153–154 °C which was identical to that of authentic testosterone.

DISCUSSION

The steroid drugs represent only 2.5% of the total value of pharmaceuticals presently on the world market. However, the worldwide pharmaceutical industry needs more than 2000 tons per year of steroid raw materials [12]. The interest in the microbial conversion of sterols such as cholesterol or phytosterol to 17-ketosteroids has been enhanced in the past decade due to the increased demand for steroid drugs and the shortage of diosgenin. Currently the microbial process is used on an industrial scale for the production of AD from sterols. Enzymatic reduction of AD to testosterone has also been found in animals [1] and microorganisms [4,9,11]. However, the information concerned with the production of testosterone from sterols via a single-step microbial transformation process is still very limited.

As shown in Table 1, the main product of the transformation of cholesterol by *Mycobacterium* sp. in a nutrient broth or synthetic medium was AD. However, accumulation of testosterone was enhanced by addition of 4% glucose into both media. The maximum molar conversion rate of testosterone from cholesterol in a synthetic medium supplemented with 4% glucose and 1% peptone was superior to that in a nutrient broth medium supplemented with 4% glucose [13].

In a preliminary experiment it was found that the *Mycobacterium* sp. used in the present study could consume cholesterol in preference to glucose in the presence of these two substrates (data not shown). As shown in Fig. 1, cholesterol was consumed more rapidly than glucose even after 24 h cultivation in glucose-supplemented medium. Although the consumption of cholesterol was in parallel with that of glucose in the pH-controlled system, the pattern of cell growth was similar to that in the pH-noncontrolled system (Fig. 2). Furthermore, AD appeared at first, then most of it was converted to testosterone. Since the reductive biotransformation of AD to testosterone required regeneration of a reduced nicotinamide cofactor, it was suggested that the accumulation of testosterone in the medium supplemented with a higher concentration of glucose was due to an increased supply of reducing power [11].

The optimal pH for the conversion of AD to testosterone by the partial purified 17β-hydroxysteroid dehydrogenase of *Mycobacterium* sp. was confirmed to be 6.0. The conversion activity decreased as the pH increased. On the other hand, the oxidation of testosterone to AD predominated at an alkaline pH. These results were similar to the pH data of 17β-hydroxysteroid dehydrogenase from *Pseudomonas testosteroni* [10] and could explain why the accumulation of testosterone in medium controlled at pH 6.0 (Fig. 2) was superior to that in the medium without pH control (Fig. 1).

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