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PURIFICATION OF α- AND β-HYDROXYSTEROID DEHYDROGENASES FROM *PSEUDOMONAS TESTOSTERONI* BY GEL FILTRATION

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SUMMARY

A simple and efficient method for purification of α- and β-hydroxysteroid dehydrogenases from *Pseudomonas testosteroni* has been described. These enzymes are probably of small molecular size.

INTRODUCTION

TALALAY introduced the use of stereospecific hydroxysteroid dehydrogenases in analysis of steroids in biological fluids[1]. Such methods can be applied to crude extracts and exudates, but enzymes free of interfering substances are required.

Pseudomonas testosteroni produces, when cultured on testosterone as the sole source of carbon, an enzyme that catalyzes the dehydrogenation of 3α-hydroxysteroids and another enzyme that oxydizes 3β- and 17β-hydroxysteroids. To be useful tools for analysis of such steroids the enzymes must be separated from each other. TALALAY has described a lengthy method that involves repeated precipitation with acetone and ammonium sulfate. The fractionation is incomplete besides being tedious. We have therefore devised a simpler and more rapid method which yields enzyme preparations of very high stereospecifity.

According to TALALAY the following two types of reactions can be distinguished.

I. 3α-hydroxysteroid dehydrogenase (α-enzyme):

Androsterone + NAD $\rightleftarrows$ androstan-3,17-dione + NADH + H$^+$

II. 3,17β-hydroxysteroid dehydrogenase (β-enzyme):

Testosterone + NAD $\rightleftarrows$ 4-androstene-3,17-dione + NADH + H$^+$

MATERIAL AND METHODS

Preparation of starting material

Pseudomonas testosteroni (ATCC No 11996) was grown in Fernbach flasks, in a medium of the following composition: $(NH_4)_2HPO_4$, 1.0 g; $(NH_4)H_2PO_4$, 1.0 g; KH_2PO_4, 2.0 g; $MgSO_4$, 0.001 g; 10 g yeast extract (Difco); distilled water to 1000 ml.

800 ml of this medium were sterilized, inoculated and incubated 18 h on a shaker incubator at 30°. This culture was used to inoculate 7.2 l of the same medium.

4 g of testosterone* were sonicated in a Raytheon sonic oscillator together with 48 ml of water, for 30 min at 9 kc. The emulsion was added to the flasks after 9 h growth. After another 16 h the cells were harvested by centrifugation at 3000 × *g* for 30 min and washed with cold 0.03 M potassium phosphate buffer, (pH 7.2). 8 l of medium gave about 50 g of cells (wet wt.). The cells were then resuspended in the same buffer, 100 ml/100 g of cells (wet wt.), and sonicated in 40-ml portions, 20 min each and stored at −30°.

Enzyme assay

Reagents: 0.1 M pyrophosphate buffer (pH 8.9); 1.2 mM aq. solution of NAD* neutralized to pH 7 with solid $NaHCO_3$; steroid solutions in dioxane, analytical grade; 10 mg testosterone*/ml for the β activity; 1.5 mg 17β-estradiol*/ml for the β activity; 3.0 mg androsterone*/ml for the α activity.

NADH has a strong broad absorption band around 340 mμ, which is absent in NAD. This makes it possible to follow the reduction of NAD in an Eppendorff

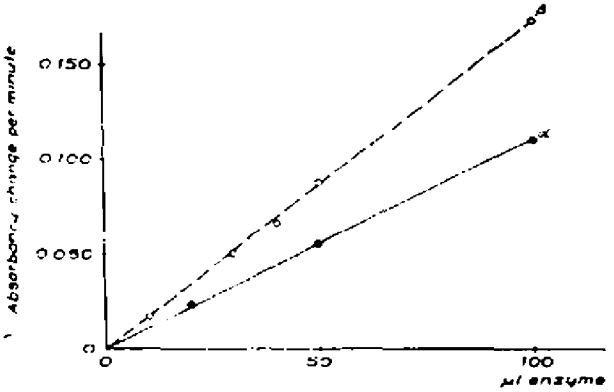

Fig. 1. The proportionality between enzyme concentration and initial reaction rate.

photometer at 334 mμ. The reaction was followed in 1-cm pyrex cuvettes. In these were pipetted 500 μl pyrophosphate buffer, 10 μl steroid solution, water and a final addition of 10–50 μl enzyme solution to a total volume of 1 ml. The blank contained the same reagents except for the steroid. Instead 10 μl dioxane was added. The absorbancy was determined every 15 sec. Before each reading the blank was adjusted to zero. The initial reaction rate was taken as a measure of enzyme activity. One unit of enzyme is defined as the amount which causes a change in absorbancy of 0.001 per min under the above conditions. The initial rate of reduction of NAD was shown to be proportional to the enzyme concentration .See Fig. 1.

Purification

All operations were carried out at 5° unless otherwise stated.

100 ml of sonicate (equivalent to 50 g of cells), was centrifuged at 10 000 × *g*

* Obtained from Sigma Chemical Co., St. Louis, Mo. (U.S.A.).

for 30 min. About 75 ml clear supernatant was obtained. The residue was washed twice with the same volume distilled water. 100 ml distilled water and 30 ml neutralized streptomycin sulphate solution (0.06 g/ml water) was added. (pH should be 7.) After 2 h the precipitate was removed by centrifugation and $(NH_4)_2SO_4$ to 25% saturation was added to the supernatant and dissolved under gentle stirring. The pH was thereby lowered to 5.5. The solution was slowly readjusted back to pH 7.2 with 0.1 M NaOH. After standing for at least 2 h the precipitate was removed by

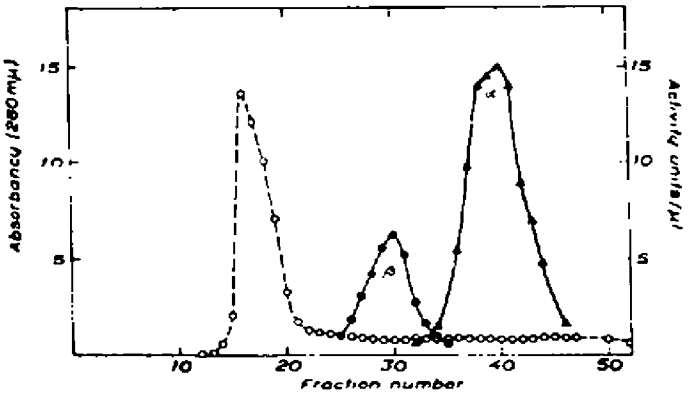

Fig. 2. The separation of the components after passage through the column. ○—○, absorbancy at 280 mμ; ●—●, the β activity; ▲—▲, the α activity.

centrifugation. The supernatant was concentrated from 375 ml to 62.5 ml by dialysis under reduced pressure, according to VON HOFSTEN AND FALKBRING[2]. 4 ml of this concentrated solution was passed through a column packed with Sephadex[3] G 100, 140–400 mesh. Sephadex was obtained from Pharmacia, Uppsala, Sweden. The column was eluated with 0.03 M potassium phosphate buffer (pH 7.2) containing 0.001 M EDTA. The rate of elution was 17 ml/h. Each fraction contained 5.7 ml. The washing was finished within 2 days.

TABLE I

THE SPECIFIC ACTIVITIES AND TOTAL RECOVERIES IN EACH STEP DURING THE PREPARATION

	Volume (ml)		Total activity (units)		Protein (mg/ml)		Specific activity of pooled fractions (units/mg protein)		Activity recovery	
			α	β			α	β	α	β
Sonicate	340		$1.60 \cdot 10^7$	$4.49 \cdot 10^6$	18.5		$2.54 \cdot 10^3$	$0.71 \cdot 10^3$	100	100
Sonicate + streptomycin sulfate	360		$1.58 \cdot 10^7$	$4.46 \cdot 10^6$	—		—	—	99	99
As above + $(NH_4)_2SO_4$	375		$1.58 \cdot 10^7$	$4.36 \cdot 10^6$	7.7		$5.5 \cdot 10^3$	$1.5 \cdot 10^3$	99	99
After concentration	62.5		$1.36 \cdot 10^7$	$3.84 \cdot 10^6$	40.5		$5.4 \cdot 10^3$	$1.5 \cdot 10^3$	85	86
After passage through column	α 86	β 51	$7.40 \cdot 10^5$	$2.30 \cdot 10^5$	α 0.27	β 0.44	$3.2 \cdot 10^6$	$1.0 \cdot 10^4$	76	83

The nitrogen content was determined according to Kjeldahl on material dialyzed 18 h against distilled water. A conversion factor of 6.25 was used to calculate the protein content.

RESULTS

The fractionation obtained by gel filtration on Sephadex G 100 is shown in Fig. 2. The specific activity and total recovery in each step is presented in Table I.

A number of preparations have been made. The distribution of ultraviolet-absorbing material has varied from batch to batch but the enzyme activities have been found in the same place. These preparations therefore may serve as excellent stereospecific group reagents for hydroxysteroids.

The relatively strong retardation on the column indicates that the enzymes are small-size proteins.

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