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PURIFICATION AND CHARACTERIZATION OF 3α-HYDROXYSTEROID DEHYDROGENASE FROM CHICKEN HEPATIC CYTOSOL

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Summary—From the cytosol fraction (supernatant fluid at 105,000 *g*) of chicken liver, 3α-hydroxysteroid dehydrogenase was purified to an apparently homogeneous state by differential precipitation with ammonium sulfate, followed by column chromatographies with DE 51, DEAE–Toyopearl, and Sephadex G-100. Finally the dehydrogenase was purified 103-fold on the basis of the cytosol fraction. Polyacrylamide gel electrophoretic analysis in the presence of sodium dodecyl sulfate (SDS) revealed that molecular weight of the purified enzyme was 66 kDa, while that of the native dehydrogenase in the absence of SDS was estimated as 660 kDa or more from the peak of the enzyme in elution profile from Sephacryl S-200 column chromatography. The dehydrogenase required NADPH specifically for reduction of 3-oxo group of 5β-androstanedione ($K_m = 1.6\ \mu M$). Optimal temperature for 3-oxo reduction was 50°C in incubation for 10 min.

INTRODUCTION

Spencer and Burnet [1] briefly reported that, after the microsomal fraction of chicken liver was solubilized by Triton X-100 and deoxycholate, the supernatant fluid was subjected to Phenyl–Sepharose column chromatography and several fractions of the eluate showed a single band of 3α-hydroxysteroid dehydrogenase, when examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). However, no information on more detailed purification and properties of the purified enzyme was available from the results.

By hepatic cytosol fraction (supernatant fluid at 105,000 *g*) of chicken, radioactive testosterone was almost exclusively reduced to 5β-dihydrotestosterone, which was further converted by 3α- or 3β-hydroxysteroid dehydrogenases to 5β-androstane-3α,17β-diol and 3β,17β-diol [2]. This result indicated that 3α-hydroxysteroid dehydrogenase activity existed in the cytosol fraction. In this paper, we report on the purification of a 3α-hydroxysteroid dehydrogenase from chicken liver homogenates with an attempt to clarify some characteristics of this enzyme.

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MATERIALS AND METHODS

Steroid

As [^{14}C]5β-androstanedione was not commercially available, we prepared it by the following procedures. With the hepatic cytosol fraction of chicken, [4-^{14}C]testosterone (100,000 dpm, 300 nmol) was incubated in the presence of NADPH for 60 min. As the metabolites, 5β-dihydrotestosterone, 5β-androstane-3α(β),17β-diols, 3α(β)-hydroxy-5β-androstan-17-ones, 5β-androstanedione and androstenedione has been firmly identified, as previously reported [2]. Therefore, after incubation, the steroidal extract was subjected to chemical oxidation by 0.5% (w/v) CrO_3 in 90% (v/v) aqueous acetic acid solution, without any further separation. Then, the oxidized steroids were separated by TLC into 5β-androstanedione and androstenedione. The former was identified on the basis of the following criteria [2]. In comparison with authentic preparation of 5β-androstanedione, identical mobility on TLC, the same behavior against chemical oxidation and acetylation, and finally, similar value of R_t in gas chromatography together with identical spectrum by mass spectrometric analysis [2]. Thus prepared [^{14}C]5β-androstanedione was employed as the

substrate for the assay of 3α-hydroxysteroid dehydrogenase.

Cofactors and others

NADPH, $NADP^+$, NADH and NAD^+ were purchased from Sigma (Mo., U.S.A.) and Kohjin (Tokyo, Japan). All the chemicals and solvents used were of analytical grade.

Enzyme assay

In the presence of NADPH, the [^{14}C]5β-androstanedione was incubated at 50°C for 10 min with an aliquot of each fraction eluted from column chromatographies. After the incubation, radioactive metabolites converted by 3α-hydroxysteroid dehydrogenase were subjected to TLC, and their spots were detected by autoradiographic method [2]. The radioactive products were isolated, and quantitated in nmol, after counting with a liquid scintillation spectrometer. Protein was measured by the method of Bradford [3], using bovine serum globulin as standard protein.

Examination of purity of the enzyme preparation and determination of molecular weight of the enzyme

Purity of the final preparation was examined by polyacrylamide (12.5%, w/v) gel electrophoresis in the presence of SDS, according to the method reported by Laemmli [4]. Molecular weight of the dehydrogenase was estimated by SDS–PAGE, using several reference proteins, while that of the native enzyme was determined by the position of the enzyme peak in the elution pattern from Sephacryl S-200 column chromatography.

RESULTS

Purification of 3α-hydroxysteroid dehydrogenase from the cytosol fraction of chicken liver

Fresh liver (500 g) of the chicken (the Broiler strain, male of approx. 60 days of age) was homogenized with 1.5 volume of 10 mM phosphate buffer (pH 7.4), containing 20% (v/v) glycerol and 0.05% (v/v) 2-mercaptoethanol (hereafter, abbreviated as the Buffer). All the procedures of purification of 3α-hydroxysteroid dehydrogenase were performed at 4°C. After the homogenates were centrifuged at 105,000 *g* for 60 min, the supernatant fluid was separated as cytosol fraction from the precipitate. To the supernatant fluid, ammonium sulfate was added to 40% saturation of the salt, and the precipitate was discarded. The precipitate which appeared between 40% and 70% saturation of ammonium sulfate was collected, as this fraction was found to contain most of the activity of the enzyme. The precipitate was dissolved in small volume of the Buffer and dialyzed against the Buffer for 24 h. The dialysate was then subjected to a chromatography with DE 51 column (3.0 × 28 cm) equilibrated with the Buffer. After the enzyme preparation was applied to the column, the column was washed with the Buffer. Then elution started with a linear gradient solution from 0 to 0.4 M NaCl in the Buffer. The fractions in which the enzyme activity was detected were pooled and from the enzyme solution, NaCl was removed by dialysis. The enzyme solution was subjected to a chromatography with DEAE–Toyopearl 650 M column (1.8 × 18 cm) equilibrated with the Buffer. After the enzyme solution was applied to the column, the column was washed with the Buffer and then elution was performed with a linear gradient solution from 0 to 0.3 M NaCl in the Buffer (Fig. 1). The fractions (Nos 82–91) which contained the enzyme activity were pooled and concentrated to 6 ml by ultrafiltration with an Amicon Diaflo membrane. The enzyme solution was applied to Sephadex G-100 column (2.6 × 86 cm) equilibrated with the Buffer and the enzyme was eluted from the column with the Buffer. The fractions which contained the enzyme activity were pooled and concentrated to 3.5 ml by the above ultrafiltration. Then the enzyme solution was subjected again to the same Sephadex G-100 column chromatography. The fractions (Nos 42–50) in which 3α-hydroxysteroid dehydrogenase activity was detected were pooled as the final enzyme preparation (Fig. 2). The molecular weight of the dehydrogenase in the presence of SDS was estimated as 66 kDa by PAGE. On the other hand, the position of the dehydrogenase eluted from Sephacryl S-200 column indicated that molecular weight of the native enzyme would be 660 kDa or more, suggesting that the native enzyme was a polymer which consisted of 10 or more monomers of 66 kDa.

When the final enzyme preparation was analyzed by SDS–PAGE, a single protein band became visible after stained by the silver staining kit (Wako, Tokyo, Japan), as shown in Fig. 3. Purification of 3α-hydroxysteroid dehydrogenase from the hepatic cytosol fraction was summarized in Table 1.

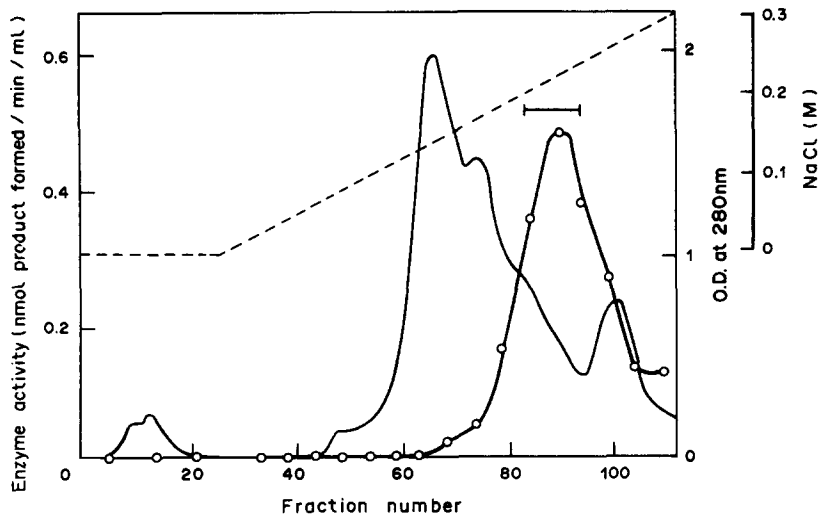

Fig. 1. Elution pattern of 3α-hydroxysteroid dehydrogenase from DEAE-Toyopearl column chromatography. The enzyme fraction which was isolated by DE 51 column chromatography was applied. (o—o) Indicates elution of the enzyme activity, (——) indicates elution of protein (O.D. 280 nm), and the broken line is NaCl concentration. Fractions (Nos 82–91) which corresponded to the range shown by ⊢—⊣ were pooled and subjected to the first Sephadex G-100 column chromatography. Each fraction contained 3.2 ml of the eluate.

The enzyme preparations which were obtained at each step in purification were analyzed of metabolizing activities of testosterone and its metabolites. We found that from 3α-hydroxysteroid dehydrogenase activity, 5β-reductase activity was separated by differential precipitation with ammonium sulfate, and 3β-hydroxysteroid dehydrogenase activity was separable by the DEAE-Toyopearl column chromatography.

Properties of the 3α-hydroxysteroid dehydrogenase

Stability. Activity of 3α-hydroxysteroid dehydrogenase in the cytosol fraction was found to be easily lost, when kept in a refrigerator, but was prevented by addition of glycerol and 2-mercaptoethanol and storage at −20 or −80°C from inactivation to appreciable extent (data not shown).

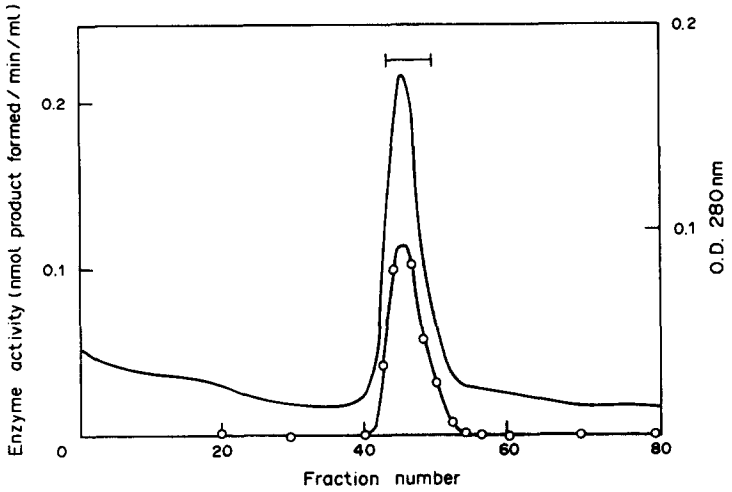

Fig. 2. Elution profile of 3α-hydroxysteroid dehydrogenase from the second Sephadex G-100 column chromatography. (o—o) Indicates elution of the enzyme activity and (——) indicates elution of protein (O.D. 280 nm). Fractions (Nos 42–50) which correspond to the range shown by ⊢—⊣ were pooled and subjected to analysis by SDS-PAGE. Each fraction contained 3.2 ml of the eluate.

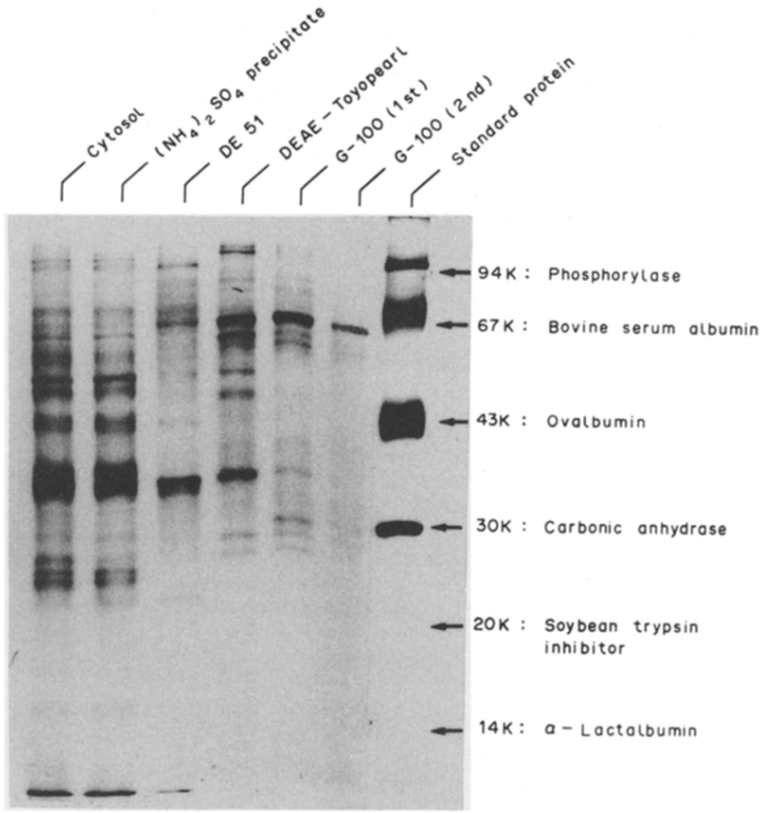

Fig. 3. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS–PAGE) of the enzyme preparations obtained at each step of the purification. Aliquots of the enzyme preparations from the cytosol were subjected to gel electrophoresis, using 12.5% (w/v) polyacrylamide gel. After the electrophoresis, protein on gel were stained by silver staining kit (Wako, Tokyo, Japan).

Optimal temperature. Reduction velocity of the 3-oxo group of 5β-androstanedione (4500 dpm, 0.3 nmol/flask) by the enzyme was examined from 30 to 70°C in the presence of NADPH (100 nmol in 0.1 ml/flask). The amount of the enzyme was fixed at 0.178 mg protein in 0.5 ml/flask. The incubation was performed for 10 min and the highest velocity was observed at 50°C; this temperature was employed for the present enzyme assay.

Cofactor requirement. Incubation of 5β-androstanedione (4500 dpm, 0.3 nmol/flask) with the same amount of the enzyme as stated above were carried out in the presence of 100 nmol of nucleotides at 39°C for 10 min. Total volume of the solution per flask was fixed as 0.6 ml. After the incubation, 3α-hydroxy-5β-androstan-17-one was quantitated as the product. In the presence of NADPH, 0.20 nmol of the product was obtained, whereas 0.01 nmol of the product was found in the presence of NADH. NAD^+ and $NADP^+$ did not enhance 3α-reduction of the substrate at all. This clearly indicated that NADPH was the most preferable cofactor among the nucleotides examined. By Lineweaver–Burk plots, the K_m value of the 3α-hydroxysteroid dehydrogenase to NADPH was calculated as 1.6 μM.

Table 1. Summarized result of purification of 3α-hydroxysteroid dehydrogenase from chicken hepatic cytosol

Procedure	Total activity (nmol product formed/min)	Total protein (mg)	Specific activity (nmol product formed/min/ mg protein)	Recovery (%)	Purification (-fold)
Supernatant fluid at 105,000 *g*	41.29	18,270	0.0023	100	1
Ppt at $(NH_4)_2SO_4$ 40–70% saturation	15.84	5,912	0.0027	38	1.2
DE 51*	8.86	404.6	0.0219	21	9.5
DEAE-Toyopearl*	1.29	34.4	0.0375	3.1	16.3
1st Sephadex G-100*	1.45	12.9	0.112	3.5	48.7
2nd Sephadex G-100*	0.616	2.6	0.237	1.5	103.0

*Column chromatography

DISCUSSION

Activity of 5β-reductase, 3α- and 3β-hydroxysteroid dehydrogenase was exclusively found in the cytosol fraction of the chicken liver, while 17β-hydroxysteroid dehydrogenase activity was localized in the microsomal fraction (10,000–105,000 *g* precipitate) [2]. The concentration of 3α-hydroxysteroid dehydrogenase in chicken cytosol in the present paper was comparable with that of rat [5], but relatively higher than that of chicken which was previously reported [1]. Due probably to a higher concentration of the dehydrogenase in the cytosol, we could achieve purification of the enzyme to an apparently homogeneous state by 103-fold from the cytosol level (Table 1).

It was found that testosterone was efficiently converted by the cytosolic enzymes of chicken liver to 5β-androstane-3α,17β-diol and its 3β-epimer, whereas the intermediate, 17β-hydroxy-5β-androstan-3-one did not accumulate in the incubation medium [2]. This result suggested that activity of the 3α- and 3β-hydroxysteroid dehydrogenases was relatively higher than that of the 5β-reductase, in accordance with the previous observation that in the rat liver cytosol, 3α-hydroxysteroid dehydrogenase was more active than 5β-reductase [5]. In this regard, from the cytosol fraction of chicken liver, 5β-reductase was purified to apparent homogeneity and its molecular weight was determined as 37 kDa in this laboratory [6].

Rat hepatic 3α-hydroxysteroid dehydrogenase has been isolated from the hepatic cytosol [5, 7–10] and also from the microsomal fraction after solubilization [11]. The cytosolic dehydrogenase seemed to be a polymer, as it dissociated by SDS into monomers of 32.5 kDa [8].

Similarly, 3α-hydroxysteroid dehydrogenase was purified from cytosol fraction of mouse liver and its molecular weight was estimated as 34 kDa [12]. In the case of rabbit liver, 3α-hydroxysteroid dehydrogenase was purified from the hepatic cytosol and molecular weight of the monomer was reported as 30–37 kDa [13]. In comparison with the molecular weights of mammalian hepatic 3α-hydroxysteroid dehydrogenase, this study revealed that the molecular weights for the dehydrogenase of chicken liver were 66 kDa for the monomer and 660 kDa or more for its native enzyme.

The cytosolic 3α-hydroxysteroid dehydrogenase was able to reduce mutagenicity of benzo(a)pyrene metabolites transformed by hepatic cytochrome *P*-450-involved enzyme systems, and this enzyme was indistinguishable from dihydrodiol dehydrogenase [14]. 3α-Hydroxysteroid-dihydrodiol dehydrogenase oxidized the hydroxylated polycyclic aromatic carbons regio- and stereo-specifically [15].

As 3α-hydroxysteroid dehydrogenase is involved in hepatic and prostatic metabolism of androgens and also plays a role of detoxification of mutagens related to aromatic hydrocarbons, further biochemical investigation of both endocrinological and pharmacological roles of this enzyme is required.

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