J. Steroid Biochem. Molec. Biol. Vol. 37, No. 5, pp. 717–724, 1990
Printed in Great Britain.

0960-0760/90 $3.00 + 0.00

PURIFICATION OF 5β-REDUCTASE FROM HEPATIC CYTOSOL FRACTION OF CHICKEN

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(Received 18 June 1990)

Summary—From the cytosol fraction (supernatant fluid at 105,000 *g*) of chicken liver, 4-en-3-oxosteroid 5β-reductase (EC 1.3.1.23) was purified by ammonium sulfate precipitation, followed by Butyl Toyopearl, DEAE–Sepharose, Sephadex G-75 and hydroxylapatite column chromatographies. The enzyme activity was quantitated from amount of the 5β-reduced metabolites derived from [4-^{14}C]testosterone. During the purification procedures, 17β-hydroxysteroid dehydrogenase which was present in the cytosol fraction was separated from 5β-reductase fraction by the Butyl Toyopearl column chromatography. By the DEAE-Sepharose column chromatography, 3α- and 3β-hydroxysteroid dehydrogenases were able to be removed from 5β-reductase fraction. The final enzyme preparation was apparently homogeneous on SDS–polyacrylamide gel electrophoresis. Purification was about 13,600-fold from the hepatic cytosol. The molecular weight of this enzyme was estimated as 37,000 Da by SDS–polyacrylamide gel electrophoresis and also by Sephadex G-75 gel filtration. For 5β-reduction of 4-en-3-oxosteroids, such as testosterone, androstenedione and progesterone, NADPH was specifically required as cofactor. K_m of 5β-reductase for NADPH was estimated as 4.22×10^{-6} M and for testosterone, 4.60×10^{-6} M. The optimum pH of this enzyme ranged from pH 5.0 to 6.5 and other enzymic properties of the 5β-reductase were examined.

INTRODUCTION

In general, there is intensive activity of 4-en-3-oxosteroid 5β-reductase (EC 1.3.1.23, hereafter abbreviated as 5β-reductase) in organs of aves, such as European starling [1], dove [2], quail [3, 4] and chicken [5–11]. In our previous paper, we reported of intracellular distribution of testosterone-metabolizing enzymes in the hepatic tissue of chicken, and found that testosterone was almost exclusively converted by 5β-reductase to 5β-dihydrotestosterone and other 5β-reduced steroids [12]. Therefore, it was suggested that the 5β-reductase played an important role at the initial stage of testosterone metabolism in the chicken liver. In this paper, we dealt with purification of 5β-reductase from hepatic cytosol fraction of chicken, and some characteristics of the purified enzyme.

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Abbreviations: Testosterone, 17β-hydroxy-4-androsten-3-one; 5β-dihydrotestosterone, 17β-hydroxy-5β-androstan-3-one; androstenedione, 4-androstene-3,17-dione; progesterone, 4-pregnene-3,20-dione.

EXPERIMENTALS

Chemicals

[4-^{14}C]Testosterone, [4-^{14}C]androstenedione and [4-^{14}C]progesterone (S.A. 1.9 GBq/mmol, each) were obtained from New England Nuclear (Mass., U.S.A.). Non-radioactive steroids were offered by Dr H. Inano, National Institute of Radiological Sciences (Chiba-shi, Japan). $NADP^+$ and NADPH were obtained from Oriental Yeast (Tokyo, Japan). NAD^+ and NADH were purchased from Sigma (Mo., U.S.A.). All the solvents used were of analytical grade and used after redistillation.

Enzyme assay

Enzyme to be tested was dissolved in 1.5 ml of the incubation mixture which contained 99 nmol of NADPH and 5 nmol of [4-^{14}C]testosterone (4600 dpm) in the sodium phosphate buffer (10 mM, pH 7.4, hereafter abbreviated as the phosphate buffer). Incubation was performed at 54°C for 10 min. After the incubation, the radioactive metabolites were separated by tlc, identified and quantitated, as previously

reported [12]. Then 5β-reductase activity was expressed as amount in nmol of 5β-reduced steroids per min.

Protein analysis

SDS–polyacrylamide gel electrophoresis (PAGE) was carried out with 12.5% (w/v) polyacrylamide as a separation gel, following the method of Laemmli [13]. Protein in the gel was stained by the method of Oakley *et al.* [14], using a silver staining kit (Wako Pure Chemical, Osaka, Japan). For determination of molecular weight of enzyme, electrophoresis calibration kit (Pharmacia, Uppsala, Sweden) was used. Estimation of molecular weight of 5β-reductase by gel filtration on Sephadex G-75 was performed by using gel filtration calibration kit (Pharmacia, Uppsala, Sweden) by the method of Determann and Michel [15]. Protein was quantitated by the method of Bradford [16], using bovine serum γ-globulin as a standard.

RESULTS

Purification of 5β-reductase from hepatic cytosol fraction of chicken

Hepatic tissues (362 g, male of the Broiler strain, purchased from Nagasaki Keikyo Foods, Nagasaki-shi, Japan) of seven chickens, approx. 60 days of age were used. Cytosol fraction was prepared by centrifugation of the hepatic homogenates in the phosphate buffer at 105,000 *g* as previously described [12].

All the following purification procedures were performed at 4°C. Protein profiles in elution were obtained by measurement of absorbance at 280 nm. An aliquot of each fraction was assayed for 5β-reductase activity.

Precipitation with ammonium sulfate

To the cytosol fraction (200 ml), ammonium sulfate was added up to 30% saturation of the salt. After centrifugation, the precipitate was discarded, as being devoid of 5β-reductase activity. The supernatant fluid (192 ml) was subjected to Butyl Toyopearl (Tosoh, Tokyo, Japan: 3.2 × 45 cm) column chromatography, without salting-out procedure.

Hydrophobic interaction chromatography

Butyl Toyopearl 650 (a hydrophobic absorbent) [17] column was equilibrated with the phosphate buffer which contained ammonium sulfate at 30% saturation. After removing unbound protein by the same buffer from the column, bound protein was eluted with the phosphate buffer, containing ammonium sulfate 30–0% gradient saturation (Fig. 1). Each fraction contained 5.3 ml of the eluate. The fractions (Nos 161–184) eluted from the Butyl Toyopearl column were pooled (114 ml) and were dialyzed overnight against the phosphate buffer containing 20% glycerol (by volume) and 7 mM 2-mercaptoethanol (hereafter, abbreviated as GMP-buffer).

Anion exchange chromatography

The dialyzed fraction was then applied to a DEAE–Sepharose CL-6B column (Pharmacia, Uppsala, Sweden: 3.2 × 15 cm) which had been

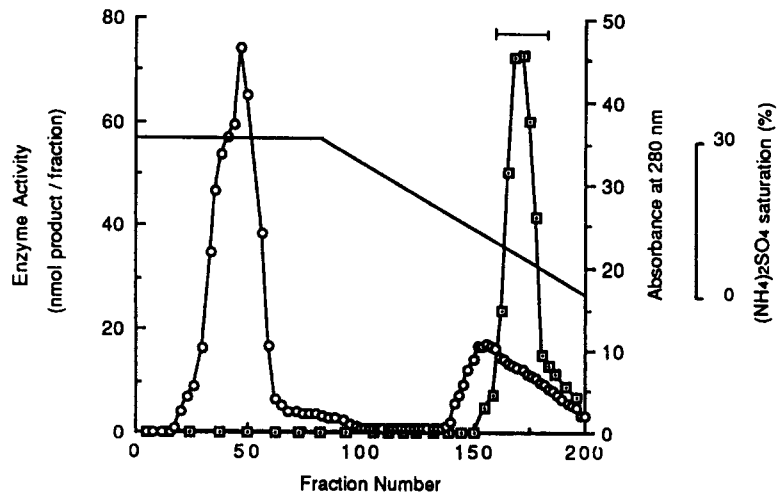

Fig. 1. Butyl Toyopearl chromatography. A linear gradient solution with 30–0% saturation of $(NH_4)_2SO_4$ (——) in the phosphate buffer was used for elution of the enzyme. An aliquot of each fraction (5.3 ml) were analyzed for protein (—○—) and activities of 5β-reductase (—□—). These symbols were also employed in the following figures (Figs 2–5). ⊢——⊣ represents the pooled enzyme fraction which was subjected to further purification.

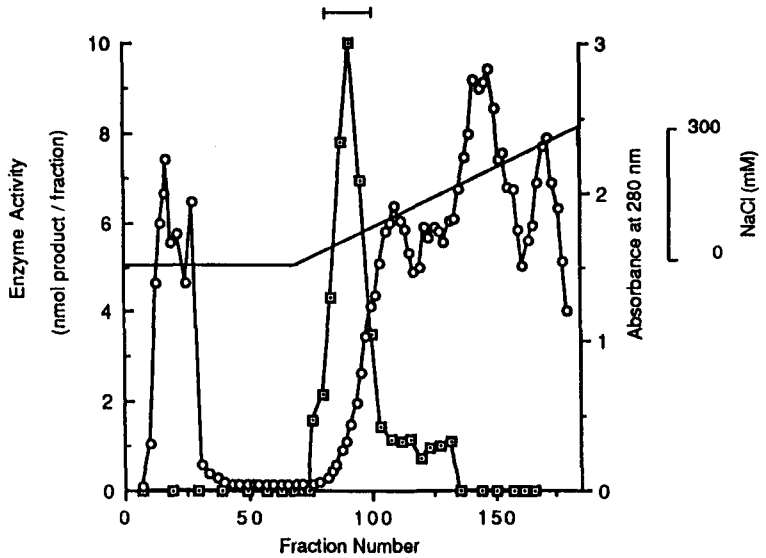

Fig. 2. DEAE–Sepharose chromatography. The enzyme solution which was obtained by the Butyl Toyopearl column chromatography was applied to a DEAE–Sepharose column and was eluted with a linear gradient from 0 to 300 mM NaCl in GMP-buffer (——).

equilibrated with GMP-buffer. After washing out unbound protein with GMP-buffer from the column, bound protein was eluted with a linear gradient (0–300 mM sodium chloride) in GMP-buffer, and every 2.7 ml of the eluate was collected per fraction (Fig. 2). The fractions (Nos 80–91; total volume, 51 ml) which contained 5β-reductase activity were pooled, and subjected to the following procedure.

Gel filtration chromatography

The eluate obtained from the DEAE–Sepharose column chromatography was concentrated up to 2 ml, using a Diaflo Ultrafiltration Membrane (PM-10, Amicon, Mass., U.S.A.) and was applied to the Sephadex G-75 column (1.5 × 97 cm). Then, protein was eluted with GMP-buffer at the speed of 14.3 ml/h, and every 1.9 ml of the eluate was collected per fraction (Fig. 3). The fractions (Nos 49–62; 25 ml in total) that contained 5β-reductase activity were pooled, and the solvent was changed to the phosphate buffer that did not contain glycerol and 2-mercaptoethanol.

Hydroxyapatite chromatography

The fraction in the buffer obtained by the gel filtration as previously described was applied to a Hydroxylapatite Bio-Gel HTP (Bio-Rad, Calif., U.S.A.) column (1.5 × 7.7 cm) which had

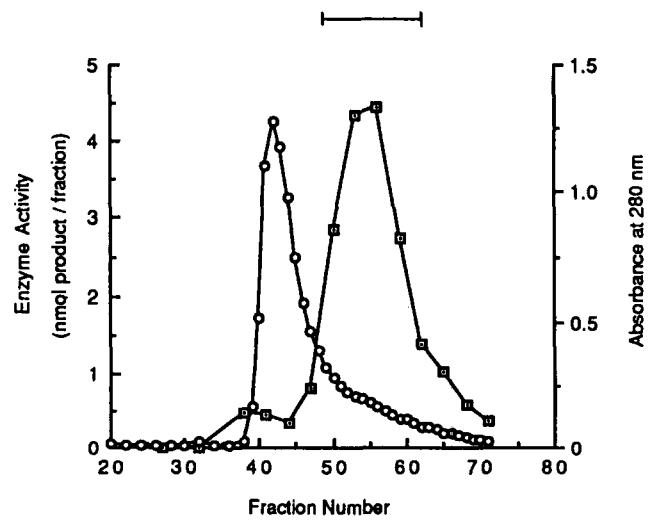

Fig. 3. Sephadex G-75 chromatography. The concentrated enzyme solution (2 ml) which was obtained by the previous chromatography was applied to a Sephadex G-75 column.

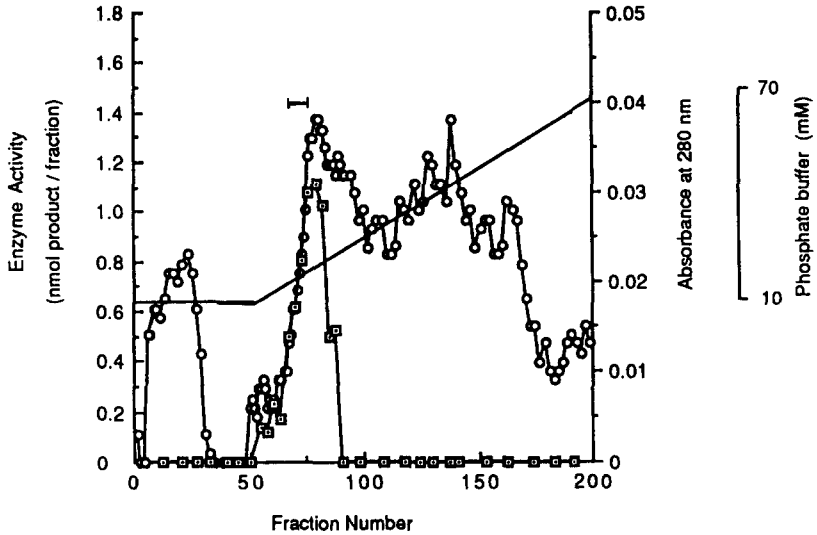

Fig. 4. Hydroxylapatite chromatography. The enzyme preparation which was obtained by the gel filtration was applied to a Hydroxylapatite column. Elution of the enzyme from the column was carried out with a linear gradient from 10 to 70 mM phosphate buffer (——). ⊢—⊣ presents the pooled fractions of the 5β-reductase.

been equilibrated with the phosphate buffer. After unbound protein was washed out from the column, the bound protein was eluted with a linear gradient solution (10–70 mM phosphate concentration). Every 1.4 ml of the eluate was collected per fraction (Fig. 4). The fractions from Nos 66 to 72 were pooled as the final enzyme preparation which was discussed with Fig. 8 in Discussion.

Finally the procedures described above provided approx. 13,600-fold purification of 5β-reductase with an overall yield 2.9% from the cytosol fraction (Table 1). Specific activity of the purified enzyme preparation was found to be about 1220 unit/mg protein under the assay condition of the reductase.

Properties of the purified enzyme

Molecular weight

Electrophoresis of the purified enzyme on SDS–polyacrylamide gel electrophoresis showed a single band corresponding to a M_r of 37,100 Da (Lane 6 in Fig. 5). On the other hand, apparent M_r of the native enzyme was estimated as 37,700 Da by the gel filtration on a Sephadex G-75 column chromatography (Fig. 3), in comparison with those of molecular weight standard proteins.

Cofactor requirement

As shown in Fig. 6, under the condition of the enzyme assay, NADPH enhanced 5β-reduction of testosterone even at a low concentration (5 nmol/1.5 ml/flask), and velocity of 5β-dihydrotestosterone production was logarithmically increased along with arithmetically increased dose of the cofactor and leveled off beyond 100 nmol/1.5 ml/flask. Michaelis constant (K_m) of 5β-reductase for NADPH was estimated as 4.22×10^{-6} M by Lineweaver–Burk plot. On the contrary, in the presence of NADH, 5β-reduction of testosterone was not accelerated at all.

Table 1. Purification of 5β-reductase

Procedure	Total activity (unit*)	Total protein (mg)	Specific activity (unit/mg protein)	Purification (-fold)	Overall yield (%)
105,000 *g* supernatant	1264	13,692	0.09	1	100
$(NH_4)_2SO_4$ fractionation†	1265	12,012	0.11	1.2	100
Butyl Toyopearl‡	306	100	3.06	34	24
DEAE-Sepharose‡	90	2.5	36.0	400	7
Sephadex G-75‡	58	1.5	38.7	430	5
Hydroxylapatite‡	37	0.03	1220	13,560	3

*One unit was defined as the enzyme activity by which 1 nmol of testosterone was 5β-reduced per min under the specified assay condition.
†Supernatant fluid at 30% saturation with $(NH_4)_2SO_4$.
‡Column chromatography.

Fig. 5. SDS–polyacrylamide gel electrophoresis. Aliquots of the pooled enzyme preparations obtained at each chromatographic steps from the cytosol fraction were subjected to analytical gel electrophoresis, using 12.5% (w/v) polyacrylamide gel. The photograph showed the gel in which proteins were stained with silver. Lane 1, the cytosol fraction; lane 2, the supernatant fluid at 30% saturation of $(NH_4)_2SO_4$; lane 3, the eluate (Fraction Nos 161–184) from the Butyl Toyopearl chromatography; Lane 4, the eluate (Fraction Nos 80–91) from the DEAE–Sepharose chromatography; lane 5, the eluate (Fraction Nos 49–62) from the Sephadex G-75 chromatography; lane 6, the preceding eluate (Fraction Nos 66–72) of the hydroxylapatite chromatography; and lane 7, standard proteins for calibration of molecular weight. Details of the employed methods were described in Experimentals.

Dependency of 5β-reductase activity upon pH

Production of 5β-dihydrotestosterone by the enzyme was examined between pH 5.0 and 7.5, under the condition described in the enzyme assay. The velocities of 5β-reduction of testosterone were nearly similar from pH 5.0 to pH 6.5, but, beyond pH 6.5, the velocity was markedly decreased.

Relative velocity of 5β-reduction of different steroids as substrate

As 4-en-3-oxosteroid hormone, progesterone and androstenedione were examined as substrate for 5β-reductase and compared with that of testosterone. Incubations were carried out in the phosphate buffer for 10 min, as described in the enzyme assay. K_m and V_{max} values were calculated from the Lineweaver–Burk plot as 1.73×10^{-6} M and 0.96 nmol product/mg protein/min for androstenedione and as 4.13×10^{-6} M and 5.61 nmol product/mg protein/min for progesterone, respectively, while in case of testosterone, 4.60×10^{-6} M and 5.03 nmol product/mg protein/min were obtained.

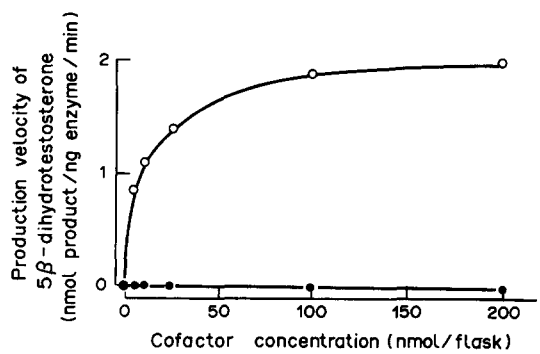

Fig. 6. Effect of cofactor concentration on 5β-reduction of testosterone. The purified enzyme preparation was incubated with testosterone, in the presence of NADPH (○) or NADH (●) (0–200 nmol/1.5 ml/flask, respectively).

Optimal temperature for 5β-reductase

Testosterone (10 nmol, 20,000 dpm) was incubated for 10 min in the presence of NADPH (99 nmol) in the phosphate buffer (1.5 ml). After quantitation of 5β-dihydrotestosterone, 54°C was found as the optimal temperature under the condition examined.

Other characteristics

Production of 5β-dihydrotestosterone was dependent upon length of incubation time up to 90 min, and upon amount of testosterone in the medium up to 20 nmol/flask, but almost independent upon concentration of the phosphate in the medium from 10 to 200 mM.

DISCUSSION

Previously, we reported of the intracellular distribution of testosterone-metabolizing enzymes of the chicken liver, and demonstrated that 5β-reductase activity was exclusively localized in the cytosol fraction [12]. As almost all the metabolites (5 of 6) were derived from 5β-dihydrotestosterone, 5β-reductase seems to be one of the key enzymes related to testosterone metabolism in the chicken liver.

Among the hepatic subcellular fractions of rat, the 5α- and 5β-reductase were clearly separated, due to their different subcellular localizations [18]. A partial purification of the 5β-reductase from rat liver was tried, but 5β-reductase could not be separated from 3α-hydroxysteroid dehydrogenase [19]. In case of porcine liver, 5β-reductase fraction was able to be purified free from 3α-hydroxysteroid dehydrogenase activity, but still 5β-reductase could not be purified to a homogeneous state [20]. Thereafter, purification of 5β-reductase from rat liver cytosol was achieved [21, 22]. However, up to our knowledges, there is no paper concerning to purification of 5β-reductase from other species than the mammalian.

5β-Reductase activity in porcine liver was labile against several purification procedures [20]. In order to stabilize 5β-reductase activity of rat liver, addition of glycerol was found effective [21]. In the case of chicken liver, addition of glycerol to the hepatic reductase fraction was also found to be protective against inactivation (data were not shown). As partially purified 5β-reductase from chicken liver cytosol was inactivated by *p*-chloromercuribenzoate at a low concentration, we employed the buffer which contained 20% (v/v) glycerol and 7 mM 2-mercaptoethanol at the purification step between DEAE–Sepharose to Sephadex G-75 column chromatographies, in order to protect SH-group of the 5β-reductase.

On the enzyme assay, we set the incubation temperature as 54°C, as the peak of apparent 5β-reductase activity was found around this temperature. As a part of the assay procedure, radioactive metabolites of testosterone was separated by tlc and were quantitated. By the detailed analysis of the metabolites, we could confirm separation of other enzymes, i.e. 3α-, 3β- or 17β-hydroxysteroid dehydrogenases from the 5β-reductase fraction. As shown in Fig. 7, by the Butyl Toyopearl column chromatography, 17β-hydroxysteroid dehydrogenase was removed from the reductase, and by DEAE–Sepharose column chromatography, the 3α- and 3β-hydroxysteroid dehydrogenases were separated from the 5β-reductase fraction. In fact, 3α-hydroxysteroid dehydrogenase was purified from the cytosol fraction to apparent homogeneous state in this laboratory, by ammonium sulfate precipitation, followed by column chromatographies with DE 51, DEAE Toyopearl and Sephadex G-100 [23].

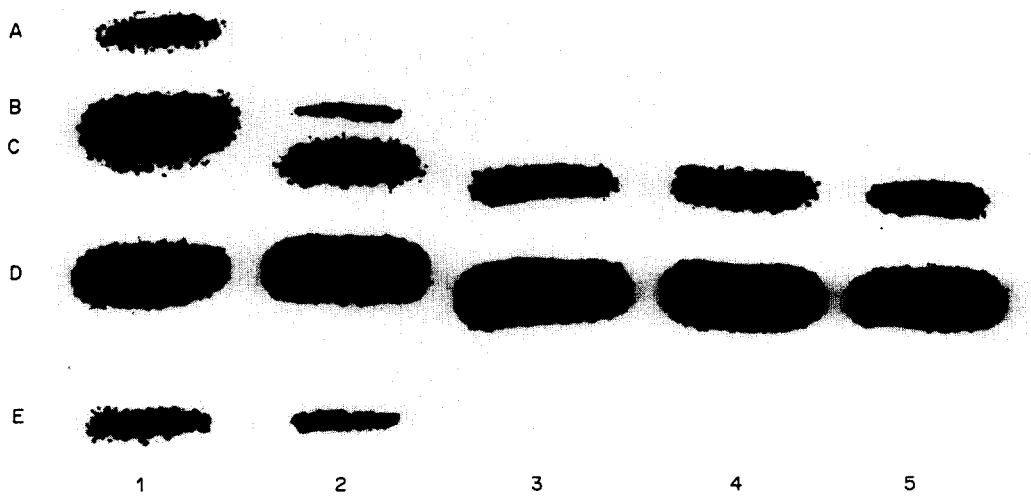

Fig. 7. Autoradiographic demonstration of the radioactive metabolites on tlc of [4-^{14}C]testosterone formed by enzyme preparations obtained at several steps of purification. Lane 1, the cytosol fraction; lane 2, the eluate (fraction Nos 161–184) from the Butyl Toyopearl chromatography; lane 3, the eluate (fraction Nos 80–91) from the DEAE–Sepharose chromatography; lane 4, the eluate (fraction Nos 49–62) from the Sephadex G-75 chromatography; lane 5, the eluate (fraction Nos 66–72) from the hydroxylapatite chromatography. Band A, 5β-androstanedione; band B, 5β-androstane-3β,17β-diol; band C, 5β-dihydrotestosterone; band D, testosterone (substrate), and band E, 5β-androstane-3α,17β-diol.

On the hydroxylapatite column chromatography, the activity of 5β-reductase was detected in the fractions from Nos 66 to 86. As we noticed that the elution profile of protein was not completely in accordance with the profile of the enzyme activity, an aliquot of individual fraction was subjected to SDS–PAGE. As the results shown in Fig. 8, the preceding eluate (the fractions from Nos 66 to 72) showed existence of a single protein (M_r, 37,100 Da) by the electrophoresis. On the other hand, the succeeding eluate (the fractions from Nos 73 to No. 86) showed the presence of two different proteins in the gel, one with M_r of 37,100 and the other, M_r of 33,000 Da (Fig. 8). Due to our analysis of the individual protein, the protein with M_r of 37,100 Da retained 5β-reductase activity, while the other (M_r of 33,000 Da) was devoid of the reductase activity. From the results, we decided not to pool all fractions from Nos 66 to 86, but to pool the fractions from Nos 66 to 72 only as the final preparation. By this simple procedure, we were able to purify the reductase more than 30-fold in term of specific activities from the immediate prior step and to be free from the protein with M_r of 33,000 Da (Table 1 and Fig. 5). The molecular weight determined by SDS–PAGE method was 37,100, which was in accordance with the value obtained by gel filtration on Sephadex G-75 (Fig. 3). This result suggested that the isolated 5β-reductase is a monomer.

The enzyme showed preference of NADPH as cofactor for 5β-reduction (Fig. 6). The similar cofactor requirements of the Δ^4-3-oxosteroid 5β-reductase of rat and porcine livers were reported [19–22]. In the present experiment, optimum pH for 5β-reductase of chicken liver was observed at pH from 5.0 to 6.5 in the phosphate buffer, using testosterone as substrate. In case of 5β-reductase obtained from the cytosol of porcine liver, pH 6.4 was reported as optimal in 0.01 M Tris–HCl buffer [20], while in case of rat liver homogenates, pH 6.5 in 0.1 M potassium phosphate buffer, and pH 7.2–7.5 in 0.1 M Tris–HCl buffer were found as the most preferable [19]. For the purified reductase from rat liver cytosol, pH 7.0 in 10 mM potassium phosphate buffer and pH 7.4 in 200 μM Tris–HCl buffer as optimum pH were reported [21]. Since those results were obtained under different conditions, such as different substrate and incubation temperature employed, those results of optimum pH were not mutually comparable. No significant production of testosterone from 5β-dihydrotestosterone by the purified 5β-reductase was observed in the presence of $NADP^+$. In the presence of EDTA-Na, there was no significant change in activity of the 5β-reductase.

The K_m value for testosterone of 4.6×10^{-6} M is in agreement with the K_m value (5.0×10^{-6} M) of rat hepatic reductase obtained for 7α,12α-dihydrocholest-4-en-3-one [19] and for testosterone (6.4×10^{-6} M) as substrate [20]. The molecular weight of our purified enzyme (37,000 Da) is in agreement with those obtained from rat liver, 37,200 Da [21] and 38,000 Da [22]. 5β-Reductase was found to prefer three different 4-en-3-oxosteroid hormones as substrate to a similar extent, indicating that this enzyme reduces 4-ene of either C-19 C-21 steroids into 5β-reduced steroids with hematopietic activity.

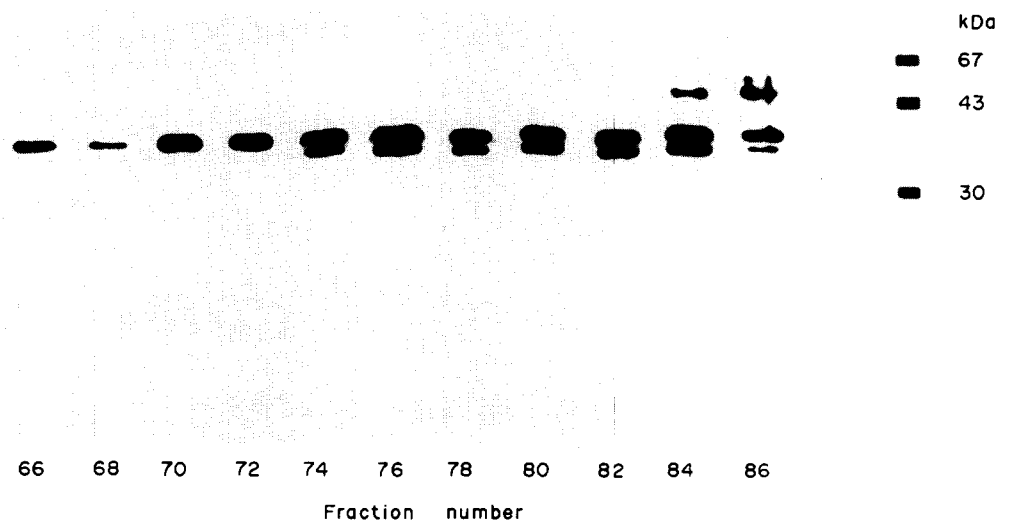

Fig. 8. Electrophoretic patterns on SDS–PAGE of the 5β-reductase fractions (Nos 66–86) eluted from the hydroxylapatite column chromatography.

Acknowledgements—We are indebted to Mr Yoshihiro Ohta, one of our previous coworkers, for his valuable cooperation and discussion and to Drs Hiroshi Inano and Hiroko Ishii-Ohba (National Institute of Radiological Sciences) for their advice on the experimental procedures. We wish to thank Messers Tetsuya Morikawa and Tatsuya Yamashita for their technical assistance. This work was partly supported by special grants-in-aid from Nagasaki University.

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