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PURIFICATION AND PROPERTIES OF THE 5α-DIHYDROTESTOSTERONE 3α(β)-HYDROXYSTEROID DEHYDROGENASE FROM HUMAN PROSTATIC CYTOSOL

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Summary—5α-Dihydrotestosterone 3α(β)-hydroxysteroid dehydrogenase [3α(β)-HSDH] [EC 1.1.1.50/EC 1.1.1.51] which catalyses the conversion of 5α-dihydrotestosterone (5α-DHT) to both 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol was purified to an apparent homogeneous state using cytosol of three human hyperplastic prostates by a 4-step purification procedure. After each purification step 3α-HSDH activity was coincident with 3β-HSDH activity. On average, specific 3α-HSDH activity was enriched 856-fold, specific 3β-HSDH activity 749-fold compared to human prostatic cytosol using anion exchange, hydrophobic interaction, gel filtration and affinity chromatography. Examination of the purified enzyme by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) revealed a single protein band with silver staining. The molecular weight of the enzyme was estimated as 33 kDa by SDS-polyacrylamide gel electrophoresis and as 28 kDa by Sephacryl S-200 gel filtration indicating that the native 3α(β)-HSDH is a monomer. In the presence of the preferred co-factor, NADPH, the purified enzyme had a mean apparent K_m for 5α-DHT of 3.9 μM and a V_{max} of 93.3 nmol (mg protein)$^{-1}$ h^{-1} with regard to 3α-HSDH activity, and a K_m of 6.3 μM and a V_{max} of 20.6 nmol (mg protein)$^{-1}$ h^{-1} with regard to 3β-HSDH activity.

INTRODUCTION

The prostate as a classical androgen target organ is able to convert testosterone to the most potent androgen 5α-dihydrotestosterone (5α-DHT) by 5α-reductase and to further convert 5α-DHT to 5α-androstane-3α,17β-diol (3α-diol) and 5α-androstane-3β,17β-diol (3β-diol), respectively, by 3α(β)-hydroxysteroid dehydrogenase [3α(β)-HSDH] [1]. Thus, not only 5α-reductase but also 3α(β)-HSDH may regulate the 5α-DHT concentration in the prostate. This is of great importance because 5α-DHT seems to play an important role in development of benign prostatic hyperplasia (BPH) [2]. In human prostate, 3α(β)-HSDH exists in a soluble as well as in a membrane associated form [3].

In whole cell homogenates, we reported previously [4] about significantly different K_m-values of both the 3α- and 3β-HSDH between epithelium and stroma in human prostate. This prompted us to investigate by enzyme purification whether different enzymes or merely differences of the cellular environment are responsible for the aforementioned different K_m-values seen in epithelium and stroma. On the other hand, we could not find significant differences in the K_m between 3α- and 3β-HSDH [4]. Thus, enzyme purification is necessary in order to clarify whether or not different enzymes are responsible for both reactions.

This paper describes the co-purification of 3α- and 3β-HSDH activity as well as other enzyme properties in human prostatic cytosol.

EXPERIMENTAL

Chemicals

[1α,2α-^{3}H]DHT (sp. act.: 50.9 Ci/mmol) was purchased from Amersham Buchler (Braunschweig, Germany). Unlabeled steroids were obtained from Sigma (St Louis, MO, U.S.A.) and the eluants for HPLC and the scintillation solution Rialuma from Baker (Groß Gerau, Germany). DEAE Sepharose CL-6B, Phenyl Sepharose CL-4B, Sephacryl S-200, Blue Sepharose CL-6B, PD-10 columns and the molecular weight markers for electrophoresis and gel filtration chromatography were purchased from Pharmacia LKB (Freiburg, Germany). All other

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chemicals were obtained from Merck AG (Darmstadt, Germany), Serva (Heidelberg, Germany) and Boehringer (Mannheim, Germany).

Enzyme assay

5α-DHT 3α(β)-HSDH activity was measured using radiolabeled substrate and high performance liquid chromatography to separate 5α-DHT, 3α-diol, and 3β-diol. Enzyme activity of cytosol or of an aliquot of each fraction eluted from column chromatography was assayed in 202 μl reaction mixture containing 1.5 mM NADPH, 30 nM [1α,2α-^{3}H]DHT, 1800 nM unlabeled 5α-DHT and a NADPH-regenerating system (5 mM glucose-6-phosphate and 0.6 U glucose-6-phosphate dehydrogenase) in buffer A [50 mM Tris, 2 mM EDTA, 2 mM NaN_3, 1 mM dithiothreitol (DTT), 20% (v/v) glycerol, pH 7.5 at 4°C]. The reaction was started at 37°C by the addition of NADPH. Control incubations contained all assay components except enzyme. Incubations were carried out for 20 min in a shaking water bath at 37°C and terminated by adding 3 ml ether. All experiments were performed in duplicate. The metabolites were extracted twice with ether (2·60 s). The ether phases were evaporated to dryness (Vortex Evaporator, Haakle Buchler, Saddle Brook), redissolved in 500 μl ether and again evaporated to dryness. The evaporated steroids were redissolved in 50 μl acetonitrile containing 100 μg of the following steroids: DHT, 3α-diol and 3β-diol. The steroids were separated by high performance liquid chromatography (refractive index detector, fraction collector with peak slope detector, reversed phase column "Lichrosorb RP 18", Pharmacia LKB, Freiburg, Germany), using filtered and helium degassed solutions of acetonitrile–H_2O (50:50, v/v) as eluant [4]. Enzyme activity was calculated from the percentage of radioactively labeled metabolites (3α-diol or 3β-diol) and expressed as nmol of metabolite formed per mg protein per hour. Assay for NADH-linked 3α(β)-HSDH activity employed the same conditions as described above except that NADPH was replaced by NADH.

Purification of the 3α(β)-HSDH

Preparation of human prostatic cytosol. BPH tissue was obtained by suprapubic prostatectomy from three patients, aged 64, 68, and 71 years. After surgical extirpation the tissue was immediately chilled in ice-cold 0.9% NaCl, transported to the laboratory and after taking a sample for histological examination the remainder was cut into small pieces and stored at −196°C.

All procedures were carried out as near as possible to 0°C. Thawed tissue was homogenized within 2 vol buffer A. The homogenate was centrifuged at 800 *g* for 15 min and the resulting supernatant at 10,000 *g* for 10 min. After centrifugation of the 10,000 *g* supernatant at 105,000 *g* for 60 min the resulting supernatant fluid was used as the source of cytosolic 3α(β)-HSDH.

Anion exchange chromatography. The 105,000 *g* supernatant was applied to a DEAE Sepharose CL-6B column (2.6 × 22.5 cm) which had been equilibrated with buffer B (50 mM Tris, 2 mM EDTA, 2 mM NaN_3, 1 mM DTT, pH 7.5 at 4°C). Unbound protein was eluted with equilibrating buffer, and fractions of 2.5 ml were collected and assayed for enzyme activity and protein. The fractions which contained 3α(β)-HSDH activity were pooled and subjected to the following procedure.

Hydrophobic interaction chromatography. To the eluate obtained from DEAE Sepharose column chromatography sodium chloride was added to a final concentration of 2 M. This solution was applied to a Phenyl Sepharose CL-4B column (1.6 × 12 cm) which had been equilibrated with buffer C (50 mM Tris, 2 mM EDTA, 2 mM NaN_3, 1 mM DTT, 2 M NaCl, pH 7.5 at 4°C). After unbound protein had been washed out from the column, bound protein was eluted with a linear salt gradient (2–0 M NaCl) in buffer B. Fractions (3.3 ml) were collected and assayed for 3α(β)-HSDH activity and protein. The fractions containing enzyme activity were pooled and concentrated up to 10% of the initial volume using a Diaflo membrane (PM-10, Amicon, Witten, Germany).

Gel filtration chromatography. The concentrated pool of the hydrophobic interaction chromatography was subjected to a column of Sephacryl S-200 (1.6 × 65 cm) equilibrated with buffer A. Elution of the protein was performed with the equilibrating buffer, collecting fractions of 1.2 ml which were assayed for enzyme activity and protein.

Affinity chromatography. Active fractions from the gel filtration step were pooled and applied to a column packed with Blue Sepharose CL-6B (1.6 × 10 cm) equilibrated with buffer A. The column was washed with buffer A and elution was performed with a linear gradi-

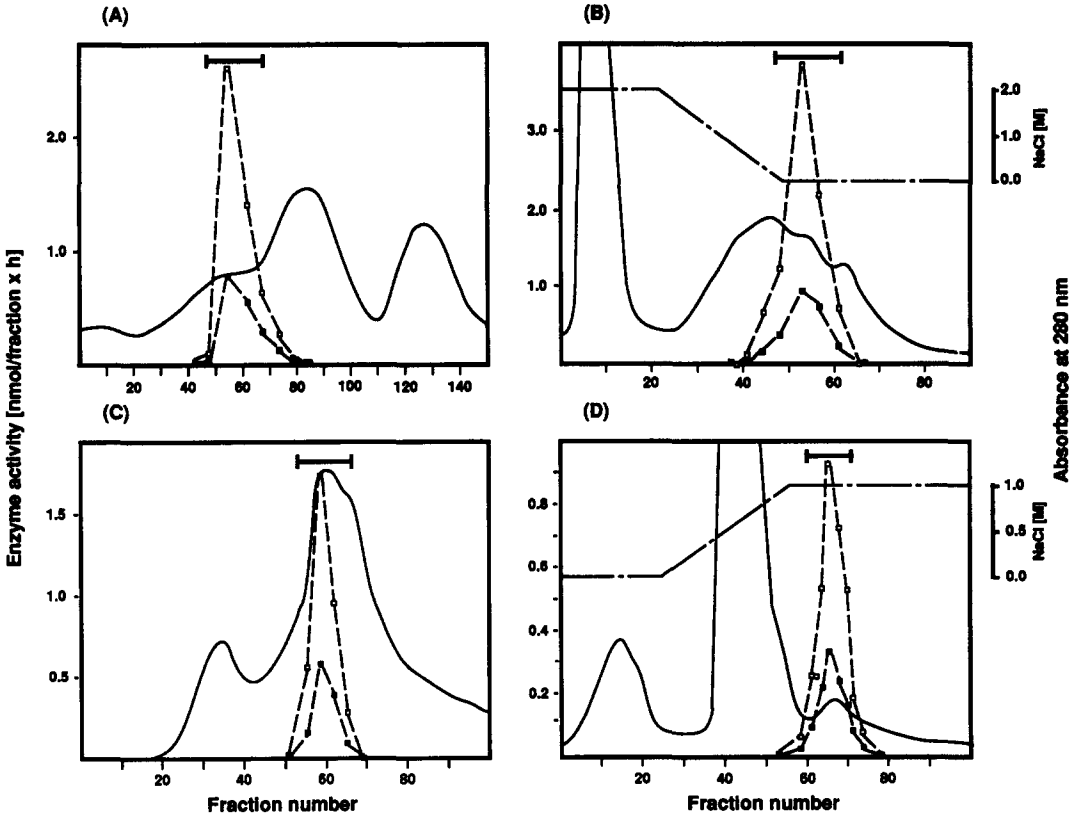

Fig. 1. Chromatograms of different chromatography steps, examplarily shown for enzyme purification from BPH of a 64-year-old male. (A) DEAE Sepharose chromatography, (B) Phenyl Sepharose chromatography, (C) gel filtration chromatography, (D) Blue Sepharose affinity chromatography. (——) Protein (o.d. 280 nm), (– –□– –) 3α-HSDH activity, (– –■– –) 3β-HSDH activity, (— – —) concentration of NaCl, pooled enzyme fractions.

ent from 0 to 1 M NaCl in buffer A. Fractions (1.5 ml) were collected and those containing 3α(β)-HSDH activity were pooled. The buffer of the pool was exchanged with buffer A using prepacked Sephadex G-25 columns (PD-10) equilibrated with buffer A. The resulting enzyme preparation was used as the source of the purified cytosolic 5α-DHT 3α(β)-HSDH in subsequent studies.

Protein determination

Protein content in cytosol and various column chromatography fractions was quantitated using crystalline bovine serum albumin as a standard [5].

Molecular weight estimation by gel filtration

The molecular weight of the native enzyme was determined by the position of enzyme activity in the elution pattern from Sephacryl S-200 column chromatography. As molecular weight markers the following proteins were used: ribonuclease A (13.7 kDa), chymotrypsinogen A (25.0 kDa), ovalbumin (43.0 kDa), and albumin (67.0 kDa).

Polyacrylamide gel electrophoresis (PAGE)

Horizontal sodium dodecyl sulfate (SDS)–PAGE of the various enzyme preparations was performed using a 0.5-mm-thin polyacrylamide gradient gel (4–22.5%) without stacking gel (LKB Application Note 348). Prior to electrophoresis, samples were heated for 10 min in a boiling water bath with 1% SDS, 10 mM DTT and 0.01% bromophenol blue. Protein in the gel was silver stained by the method of Ansorge [6]. The molecular weight markers used for SDS–PAGE were phosphorylase *b* (94.0 kDa), bovine serum albumin (67.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (30.0 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

Enzyme analysis

K_m and V_{max} of cytosolic and purified enzyme were determined by Lineweaver–Burk plots [7] which were obtained by plotting reciprocals of the apparent enzyme activity against reciprocals of substrate concentrations (80, 130, 280, 480

Table 1. Enrichment of 3α-HSDH activity from the prostate of a 64-year-old male

Procedure	Volume (ml)	Total protein (mg)	Total activity (nmol product/h)	Specific activity (nmol product/ mg protein × h)	Overall yield (%)	Purification (-fold)
Supernatant fluid at 105,000 *g*	32.0	322	35.80	0.11	100	1
DEAE Sepharose CL-6B[a]	61.0	40.8	27.10	0.67	76	6
Phenyl Sepharose CL-4B[a]	4.6	7.78	19.05	2.45	53	22
Sephacryl S-200[a]	16.0	3.25	9.03	2.78	25	25
Blue Sepharose CL-6B[a]	16.0	0.049	3.33	67.91	9	617

[a]Column chromatography.

and 1830 nM). K_m- and V_{max}-values were computed through regression lines by the method of least squares.

RESULTS

Purification of 5α-DHT 3α(β)-HSDH from human prostatic cytosol

The 4-step purification procedure revealed a successful purification of 5α-DHT 3α(β)-HSDH from human prostatic cytosol (Fig. 1, Tables 1 and 2). DEAE Sepharose chromatography of the cytosol resulted in a 6.1-fold purification of 3α-HSDH activity with a 76% recovery and a 5.0-fold purification of 3β-HSDH activity with a 68% recovery. Most of the total protein was retained by the DEAE Sepharose column while 3α(β)-HSDH was eluted. In the second step Phenyl Sepharose chromatography gave a 3.7-fold purification and 70% recovery of 3α-HSDH activity, and a 3.3-fold purification and 62% recovery of 3β-HSDH activity. Gel filtration chromatography being necessary for buffer exchange and desalting gave a 1.1-fold (3α-HSDH) and 1.7-fold (3β-HSDH) purification and 47 and 70% recovery, respectively. The final purification procedure using Blue Sepharose affinity chromatography was the most effective purification step resulting in 24.4-fold purification and 37% recovery of 3α-HSDH activity, and 26.2-fold purification and 40% recovery of 3β-HSDH activity. 3α-HSDH activity was enriched 617-fold with a 9% recovery, and 3β-HSDH activity was enriched 717-fold with a 12% recovery (Tables 1 and 2).

3α-HSDH activity was coincident with 3β-HSDH activity during the whole purification procedure (Fig. 1).

The enzyme purification was repeated with BPH tissue of two other patients.

A 492- and 1459-fold purification (5 and 8% recovery, respectively) regarding 3α-HSDH activity, and a 390- and 1139-fold purification (4 and 7% recovery, respectively) regarding 3β-HSDH activity were obtained. Thus, from three experiments the enrichment of 3α- and 3β-HSDH activity from human BPH is on average 856- and 749-fold, respectively.

The ratio of NADPH/NADH-linked activity of the purified enzyme in the presence of equal molar concentrations of NADPH and NADH (1.5 mM) was 12 (3α-HSDH) and 3 (3β-HSDH), respectively.

Purity and molecular weight of the purified enzyme

Samples of the enzyme preparation after each purification step were analyzed for purity by SDS–PAGE (Fig. 2). Silver staining of the purified enzyme (Fig. 2, lane F) showed a single protein band indicating that the 3α(β)-HSDH was purified to apparent homogeneity. The molecular weight was determined to be approx. 33 kDa by comparison to molecular weight standards. By gel filtration chromatography on a Sephacryl S-200 column the molecular weight of the native enzyme was determined to be 28 kDa. These values indicate that the native 3α(β)-HSDH from human prostatic cytosol is a monomeric protein.

Table 2. Enrichment of 3β-HSDH activity from the prostate of a 64-year-old male

Procedure	Volume (ml)	Total protein (mg)	Total activity (nmol product/h)	Specific activity (nmol product/ mg protein × h)	Overall yield (%)	Purification (-fold)
Supernatant fluid at 105,000 *g*	32.0	322	9.03	0.03	100	1
DEAE Sepharose CL-6B[a]	61.0	40.8	6.16	0.15	68	5
Phenyl Sepharose CL-4B[a]	4.6	7.78	3.84	0.49	43	16
Sephacryl S-200[a]	16.0	3.25	2.67	0.82	30	27
Blue Sepharose CL-6B[a]	16.0	0.049	1.06	21.51	12	717

[a]Column chromatography.

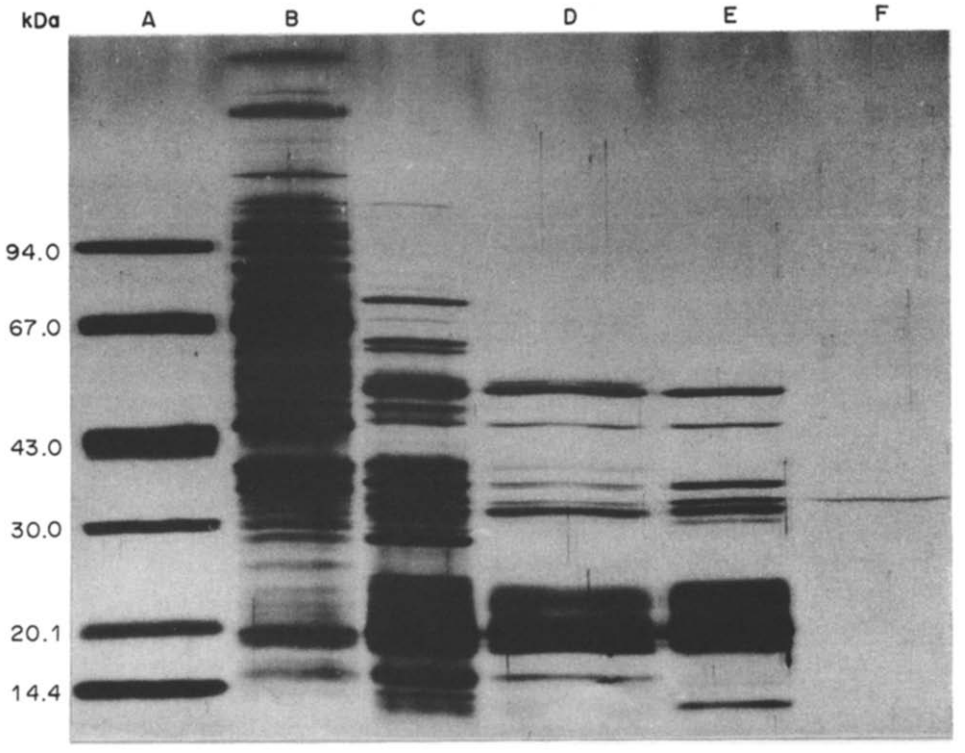

Fig. 2. SDS–PAGE. Samples of the pooled enzymatic preparations from each chromatographic step, as well as the cytosol were subjected to gel electrophoresis, using a polyacrylamide gradient gel (4–22.5%). After electrophoresis protein in the gel was stained by the silver staining procedure. Samples: lane A, molecular weight markers; lane B, cytosol; lane C, pooled fractions from DEAE Sepharose chromatography; lane D, pooled fractions from Phenyl Sepharose chromatography; lane E, pooled fractions from gel filtration chromatography; lane F, purified protein after affinity chromatography and buffer exchange.

Enzyme parameters

K_m- and V_{max}-values were determined in both the cytosols and purified fractions of three BPH (Fig. 3). In the presence of the preferred cofactor, NADPH, the purified enzyme had a mean apparent K_m (μM) for 5α-DHT of 3.9 (range: 3.6–4.2) with regard to 3α-HSDH activity, and a mean apparent K_m (μM) of 6.3 (range: 5.6–7.7) with regard to 3β-HSDH activity. Using cytosol as the source of 3α(β)-HSDH activity, mean K_m-values (μM) of 2.1 (range: 2.1–2.2) with regard to 3α-HSDH activity, and 2.7 (range: 2.0–3.2) with regard to 3β-HSDH were obtained. Thus, the steady state affinity of both enzyme activities is slightly diminished by the purification procedure.

Mean V_{max}-values [nmol (mg protein)$^{-1}$ h^{-1}] of the purified enzyme was 93.3 (range: 87.0–102.0) with regard to 3α-HSDH activity, and 20.6 (range: 11.1–26.3) with regard to 3β-HSDH activity. Those of the cytosolic 3α(β)-HSDH were 0.2 with regard to 3α-HSDH activity, and 0.1 with regard to 3β-HSDH activity.

DISCUSSION

To the best of our knowledge, this is the first report on purification of the cytosolic 5α-DHT 3α(β)-HSDH from human prostatic tissue.

Our results clearly indicate that the purified 5α-DHT 3α(β)-HSDH is a monomeric enzyme with a clear co-factor preference for NADPH compared to NADH. The molecular weight estimated by SDS–PAGE and gel filtration chromatography is 33 and 28 kDa, respectively. In purified preparations as well as in crude cytosols 3α-diol was the major product of 3-keto reduction of 5α-DHT in comparison to 3β-diol. The ratio of 3α-diol to 3β-diol is in the range of 3–4. Finally, according to our study the K_m-values of 3α(β)-HSDH for 5α-DHT in crude cytosol are slightly lower than in purified fractions, indicating only a small loss of substrate affinity due to purification procedure.

Surprisingly, only one protein could be found after purification although (a) two different reactions are catalysed, i.e. 3-keto reduction of 5α-DHT to 3α-diol and 3β-diol, and (b) significantly different K_m-values of 3α(β)-HSDH for

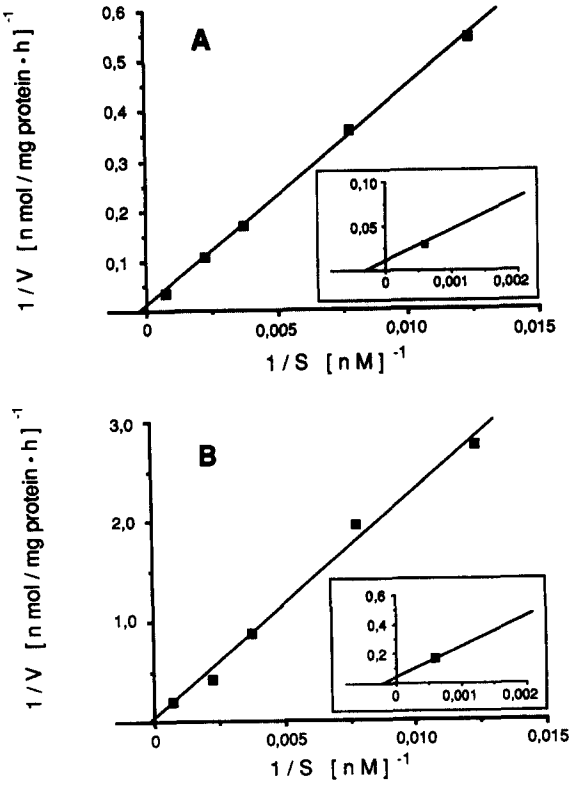

Fig. 3. K_m and V_{max} determination of purified 3α(β)-HSDH. (A) 3α-HSDH activity was assayed as described in Experimental with varying amounts of 5α-DHT (80, 130, 280, 480 and 1830 nM) and constant amounts of NADPH (1.5 mM). Each point was assayed in duplicate and K_m and V_{max} were fitted as described in Experimental. (B) The assays for 3β-HSDH activity and determination of K_m and V_{max} were performed in the same way. The inserts are magnifications of the plot.

5α-DHT in epithelium and stroma of human prostatic whole cell homogenate have been demonstrated previously [4]. However, in this study, during the whole purification procedure including anion-exchange, hydrophobic interaction, gel filtration and Blue Sepharose affinity chromatography 3α- and 3β-HSDH activities always coincided. Even after the final step of enzyme purification leading to homogeneity in SDS–PAGE, 3α- and 3β-HSDH activity were not separable. In other words, the results suggest that either a single protein contains both activities or cytosolic 3α- and 3β-HSDH are different proteins of nearly the same molecular weight being not separable from each other with the experimental conditions used. Furthermore, based on this study the possible existence of different epithelial and stromal 3α(β)-HSDH in cytosol of human prostate [4] could not be confirmed due to our observation that the whole 3α(β)-HSDH activity is eluted in one distinct peak (Fig. 1) at all stages of purification. Nevertheless, again it cannot be ruled out for sure that different 3α(β)-HSDHs might be present in epithelium and stroma of human BPH which, however, are so similar that a separation is not possible under the experimental conditions used. In addition, the differences in K_m measured in whole cell homogenates [4] may be restricted to differences of the microsomal 3α(β)-HSDH. Therefore, instead of cytosol studies on solubilization, purification and characterization of 3α(β)-HSDH from human prostatic microsomes are currently underway in our laboratory to answer the question of possible differences in regard to cofactor requirement as well as K_m- and V_{max}-values between microsomal and cytosolic 3α(β)-HSDH in human prostate.

Although there are several studies in 3α- and 3β-HSDH in human prostate [4, 8–15], properties of the purified enzyme are not known. Cytosolic 3α-HSDH has been partially or fully purified from various tissues of mammalian species [16–19] and the molecular weight of the 3α-HSDH has been reported to be similar to our data on the purified 5α-DHT 3α(β)-HSDH in human prostate. Furthermore, the 3α-HSDH always prefers the cofactor NADPH when compared to NADH. Some of the purified 3α-HSDHs possess broad substrate specificity, e.g. for 5α-dihydroprogesterone, androstanedione and 5α-DHT [17].

In comparison to the partially purified 5α-DHT 3α-HSDH from rat ventral prostate [17], which shows a K_m for 5α-DHT in the range of 0.6–0.8 μM, purified 3α-HSDH from human prostatic cytosol possesses higher K_m-values, i.e. 3.6–4.2 μM, indicating that the steady state affinity of the human prostatic 3α-HSDH for 5α-DHT is lower as compared to the 3α-HSDH of the rat prostatic cytosol. Thus, in human prostate this enzyme is hypothetically less efficient in metabolizing 5α-DHT than in rat prostate. In this context it is interesting to note that only in man, but not in rat, BPH develops spontaneously.

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