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THE PURIFICATION OF 3β-HYDROXYSTEROID SULFOTRANSFERASE OF THE HAMSTER EPIDIDYMIS

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Summary—The purification of a hydroxysteroid sulfotransferase from the cytosolic fraction of the hamster epididymis is described using ammonium sulfate precipitation, gel filtration and PAP agarose affinity chromatography. A purification of 360-fold was achieved and resulted in the isolation of one major protein as evidenced by HPLC and SDS gel-electrophoresis. The "native" enzyme is a dimer of mol. wt 106,000 and is composed of subunits having the same molecular weight.

INTRODUCTION

The presence of relatively high concentrations of steroid sulfates in the hamster epididymis [1] and their involvement in the maturational process of spermatozoa within the male tract [2] necessitated the purification of the conjugating enzyme responsible for the production of this class of compound. It has been reported that desmosteryl sulfate represents more than 90% of the sterol sulfate fraction of hamster spermatozoa [3]. Furthermore, maturing spermatozoa exhibit an increasing affinity for sterol sulfates along the epididymis [4] and the concentration of desmosteryl sulfate within the epididymis and spermatozoa increases several-fold along this conduit [1]. The important role of sterol sulfates in the reproductive process is indicated by the finding that desmosteryl sulfate is a potent inhibitor of capacitation *in vitro* [5] and is also a specific inhibitor of acrosin [6, 7].

It is evident, therefore, that the enzyme system responsible for the sulfurylation process within the male tract warranted further investigation. Using a partially purified epididymal extract we have developed a rapid assay system and this partially purified preparation exhibited a high specificity towards 3β-hydroxysteroids such as dehydroisoandrosterone (DHA) and desmosterol (desM) [8]. The present report concerns the purification of this enzyme from hamster epididymis.

EXPERIMENTAL

Reagents

Dehydroisoandrosterone (DHA) was purchased from Steraloids (NH, U.S.A.) and recrystallized from methanol. Radiolabelled 3′-phosphoadenosine 5′-phosphosulfate-^{35}S (PAP^{35}S) (16 Ci/mmol) was supplied by New England Nuclear (Dorval, Quebec) and the lithium salt of PAPS and monothioglycerol was purchased from Sigma Chemical (St Louis, MO, U.S.A.). Pharmacia (Dorval, Canada) was the source of Sephacryl S-300 and the affinity gel PAP-Agarose was obtained from Sigma as adenosine 3′-5′-diphosphate Agarose, 0.8 μmol/ml. All other chemicals were of reagent grade. Protein concentration was assayed according the technique of Bradford[19].

Assay of sulfotransferase

The assay of DHA sulfurylation by hamster epididymal extracts has been previously described [8]. Briefly, the incubation medium consisted of 250 mM THAM, [Tris (hydroxymethyl) aminomethane] 20 mM cysteine hydrochloride, 40 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 100 μM DHA dissolved in 10 μl propylene glycol and 430 μM of PAP^{35}S (500,000 cpm). The pH of the reaction was 8.7 and the final was 150 μl. The DHA^{35}S formed during a 30 min incubation period at 37°C was extracted after stopping the reaction with 600 μl of methanol. Following this procedure, 800 μl of KCl 20% (w/v) was added and the steroid sulfate was recovered by extraction into 2 × 2 ml of ethyl acetate. After evaporation of the organic extract, the radioactivity was assayed using an LKB Rack Beta counter using P-950A (New England Nuclear) as the scintillation fluid.

Purification procedure

Twenty-two hamsters, 11 weeks of age, were sacrificed by cervical dislocation and their epididymides were rapidly removed and placed on ice. The tissues were cleaned of adhering fat and minced in 2 vol of cold homogenization buffer consisting of

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50 mM THAM, 30 mM cysteine hydrochloride and 250 mM sucrose (pH 7.5). The homogenization was carried out with a Brinkmann Polytron using 4 × 10 s periods at an intensity of 6. The homogenate was centrifuged at 105,000 *g* for 90 min at 4°C. The cytosolic fraction obtained between the pellet and the top lipid layer was submitted to a precipitation procedure using ammonium sulfate in solid form to arrive at a 50% saturated solution. The precipitation was allowed to proceed for 12 h at 4°C. The precipitate, recovered by centrifugation, was dissolved in 7 ml of TMMS buffer: (50 mM THAM, 0.25% (v/v) monothioglycerol, 5 mM Mg SO_4, adjusted to pH 7.6 with 5N HCl). The same buffer was used to equilibrate a 37 × 2.6 cm column of Sephacryl S-300 which had a flow rate of 25–30 ml/h. The entire extract (ca. 90 mg of protein) was applied to this column and 5 ml fractions were collected, the optical density was monitored at 280 nm and each fraction was assayed for sulfotransferase activity. The more active fractions were pooled and concentrated with a PM 10 membrane mounted on a Diaflo system (Amicon). The pH of the extract was then adjusted to 8.7 with 5 N HCl prior to chromatography on a 5 ml column (1 cm dia) of PAP-Agarose gel. The flow rate was set at 6 ml/h using TMMS buffer with the pH adjusted to 8.7. The affinity column was washed with five column volumes of buffer and 3.5 ml fractions were collected. The pH of the elution buffer was reset to 7.6 with 5N NCl and a 0.6 mM solution of PAPS was added to elute the enzyme. The fractions collected were of 0.7 ml and the protein concentration was assayed with the method of Bradford since PAPS interferes with the monitoring at 280 nM. Those fractions which contained sulfotransferase activity (specifically eluted by the addition of PAPS) were pooled and analyzed on HPLC as well as with SDS gel-electrophoresis. The Sephacryl S-300 column was calibrated with Biorad gel filtration standards of known molecular weight.

Purification of the enzyme with HPLC

A Waters high pressure liquid chromatographic system was used to analyse those fractions that were purified by affinity chromatography. The system consisted of an 1-250 column and a model 441 u.v. detector fitted with a 280 nm filter. The elution buffer consisted of 50 mM THAM, 0.25% monothioglycerol and 50 mM Na_2SO_4 adjusted to pH 7.4 with 5N HCl and the flow rate was set at 1 ml/min. One-hundred to three-hundred μl of the extract were injected and the fractions eluted were monitored at 280 nm and assayed for sulfotransferase activity. The column was calibrated with the Bio-Rad protein standards used previously.

SDS gel-electrophoresis

The method of Laemmli was used [10] and for the electrophoresis the technique of Studier[11] was employed. The separation gel consisted of 12% acrylamide–0.32% *bis*-acrylamide in 375 mM THAM (pH 8.8), 0.1% (w/s) SDS, 0.05% ammonium persulfate and 0.033% (v/v) *N,N,N,*-tetramethylenediamine. The concentration gel consisted of 5% acrylamide–0.13% *bis*-acrylamide in 125 mM THAM (pH 6.8), 0.1% SDS and 0.5% (w/v) riboflavin. Gel plates of 15 × 16 cm were used and a thickness of 0.75 mm was selected. Denaturation of the protein preparation was carried out for 20 min in boiling water in 100 mM THAM, 4% (v/v) β-mercaptoethanol and 1.5% SDS. The proteins were applied to the gel (1–2 μg) in solution of 10% glycercol and 0.1 mg% bromophenol blue. Biorad "low molecular weight standards for SDS electrophoresis" were used to estimate the electrophoretic mobility of authentic proteins. Silver nitrate staining was adapted from the method of Morrissey[12].

RESULTS

The overall efficiency of the purification procedure, which involved only two chromatographic steps, is presented in Table 1. Most of the initial sulfotransferase activity present in the crude homogenate was recovered in the cytosolic fraction. As reported for the adrenal sulfotransferase [13], ammonium sulfate precipitation of the enzyme is an essential step to stabilize the sulfotransferase and allows the continuation of the purification procedure. In general, the recovery of the enzyme following this treatment was over 80% but in this particular study at 57% recovery was achieved with a 3-fold purification. The fractionation of the latter extract on

Table 1. Purification of 3β-hydroxysteroid sulfotransferase of hamster epididymis

Procedure	Protein (mg)	nmol DHA^{35}S/30 min Total activity	Specific activity (per mg)	Enzyme recovery (%)
Homogenate	1209	1035	0.9	100.0
Cytosol	503	961	1.9	92.8
$(NH_4)_2SO_4$ (50%) precipitate	96.0	541	5.6	52.3
S-300 Gel filtration	26.4	241	9.2	23.4
PAP-Agarose column	0.047	15	327.7	1.5

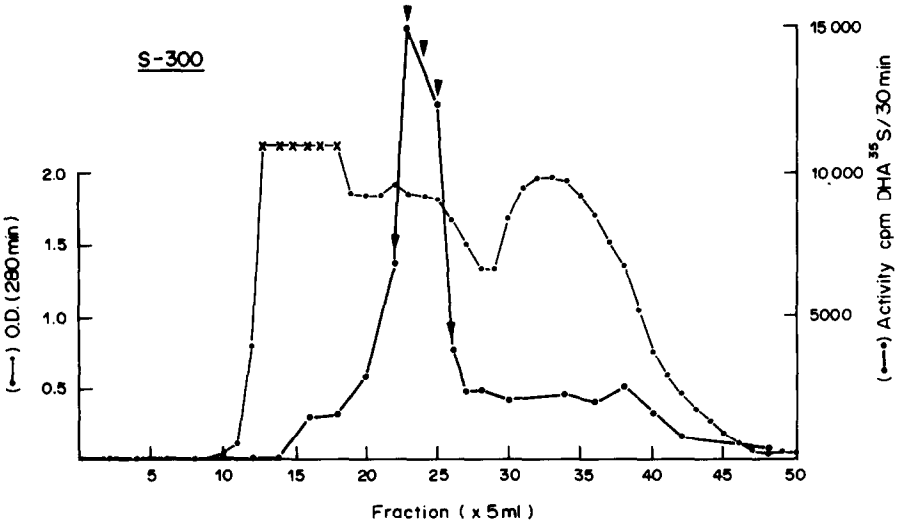

Fig. 1. Elution profile of the epididymal sulfotransferase preparation on Sephacryl S-300. Those fractions indicated with an arrow were combined and concentrated for further purification.

a Sephacryl S-300 column is presented in Fig. 1. Only one main peak of activity was detected and the corresponding fractions were concentrated for further purification by affinity chromatography. As seen in Fig. 2, the application of the latter extract on the PAP-Agarose gel resulted in a large amount of sulfotransferase activity which was not retained by the column but was simply eluted somewhat later than the non-absorbed protein. This low capacity of the affinity column resulted in the large loss of enzyme activity during the final purification step (final yield 1.5%). The appearance of PAPS in the eluate is easily observed since the O.D. increases in the presence of PAPS (m = 254 nm). In the presence of PAPS a high sulfotransferase activity was recovered in 2 fractions. These two fractions were analyzed separately on SDS gel-electrophoresis, but were pooled for the further purification using HPLC.

Enzyme purification by HPLC

The I-250 column used is reported to be efficient for the separation of proteins of mol. wt from 13,000 to 600,000. As observed in Fig. 3, only one symmetrical peak (eluting time = 10 min) was observed at 280 nm which was followed by a large increase in absorption which is due to the high absorbance of PAPS in the purified extract following affinity chromatography.

Those fractions obtained from the affinity chromatography procedure as well as those obtained following HPLC were analyzed by SDS gel-electrophoresis. Figure 4 illustrates the results ob-

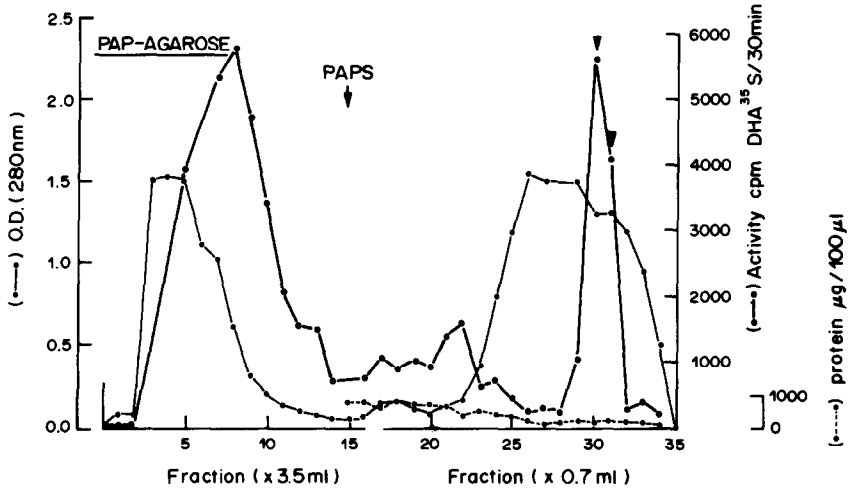

Fig. 2. Elution profile of the epididymal sulfotransferase on the affinity column, PAP-Agarose. A major portion of the activity was eluted during the washing procedure which probably represents an over-loading of the column. A second fractionation was eluted (fraction 30) with the addition of PAPS. Those fractions indication with an arrow were combined and analyzed by HPLC and SDS-electrophoresis.

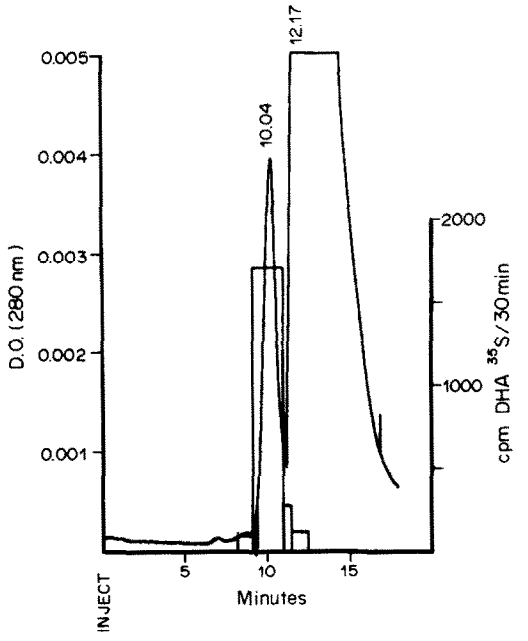

Fig. 3. Elution profile of the enzyme preparation on HPLC using a Waters I-250 column. The enzyme was eluted as a symmetrical peak at an approx retention time of 10 min. The large peak of absorption observed at 12.17 min is due to the presence of PAPS.

tained after silver staining of the gel. Only one major band of protein is visible (HPLC, b and c). The presence of three very minor bands was also observed.

Molecular weight determination

Figure 5 illustrates the calibration curves obtained with the Sephacryl S-300 and I-250 (HPLC) columns with proteins of known molecular weight. Following purification on Sephacryl, The elution volume of the enzyme corresponded to a mol. wt of 106,000. Upon HPLC chromatography, this protein is also eluted according to a mol. wt of 106,000. When analyzed for subunits in a dissociating medium the affinity-purified and HPLC-purified extracts yielded a single major band of protein of mol. wt 52,500–53,000 (Fig. 6).

DISCUSSION

Due to its instability, few procedures for the purification of hydroxysteroid sulfotransferases have been reported. Adams and McDonald[13] however, did succeed in the isolation of this enzyme from the human adrenal by an affinity chromatographic procedure using a DHA-Sepharose gel. Later, the purification of three rat liver sulfotransferases of

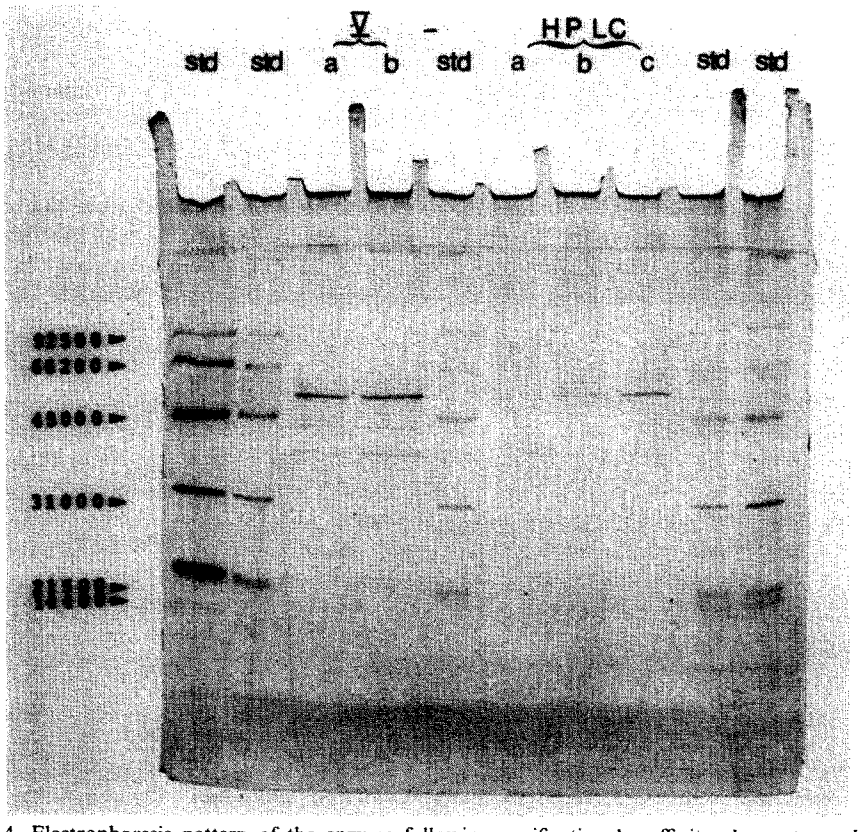

Fig. 4. Electrophoresis pattern of the enzyme following purification by affinity chromatography and HPLC. Silver nitrate was used to increase detection. Authentic proteins (std) were obtained from Bio.Rad; phosphorylase B, 92,500; bovine serum albumin, 66,200; ovalbumin 45,000; carbonic anhydrase, 31,000, soya bean trypsin inhibitor, 21,500 and lysozyme, 14,400. Fractions V a and b represent two fractions obtained from the same enzyme preparation purified by affinity chromatography. HPLC fractions a, b and c represent three different dilutions of the same preparation. Staining was achieved with silver nitrate.

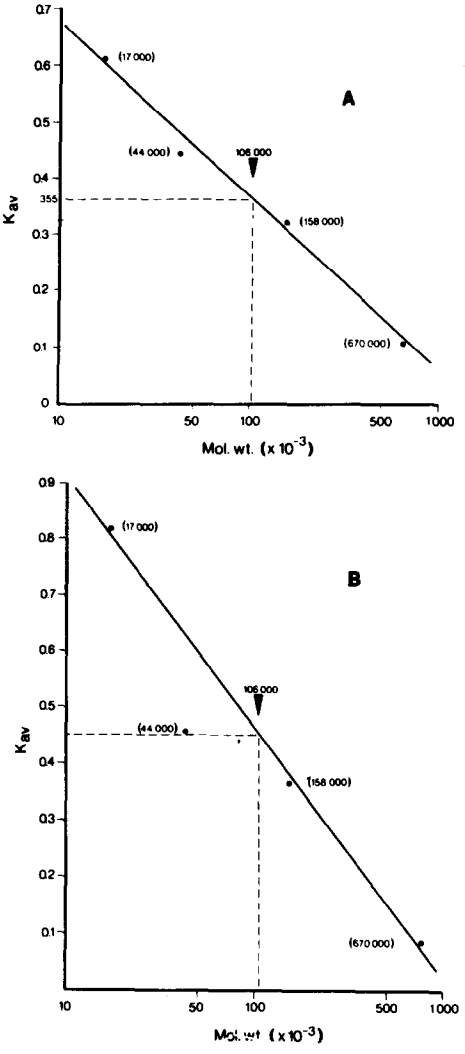

Fig. 5. Molecular weight determination using Bio-Rad "gel filtration standards" as reference proteins; Thyroglobulin 670,000; immunoglobulin, 158,000; ovalbumin, 44,000; myoglobulin, 17,000. The calibration curves are presented as a function of the log mol. wt. The arrows indicate the elution pattern of the sulfotransferase. A-Sephacryl S-300. B- Waters I-250 column.

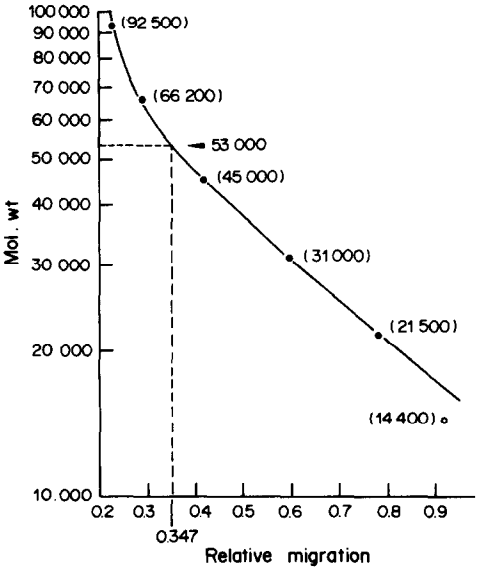

Fig. 6. Molecular weight determination of sulfotransferase following SDS-electrophoresis. After coloration with Coomassie Blue as single band was observed with a mol. wt of 53,000. Authentic standards are identical to those described in Fig. 4.

differing specificity was also reported [14, 15] using conventional chromatographic techniques as well as the affinity gel of Adams and McDonald[13]. The very low recovery of the purified enzyme reported in the present study (1.5%) is of the same order of magnitude as that recorded for certain of the liver sulfotransferases [15]. This low yield precluded any further investigation of enzyme properties. However, with regard to specificity, the partially purified epididymal sulfotransferase would appear to be specific for the 3β-hydroxyl function of the steroid nucleus [8].

The purification of epididymal sulfotransferase reported herein shares certain features with the methods reported above. The enzyme activity is mainly present in the cytosolic extract of the tissue homogenate and ammonium sulfate precipitation resulted in a stabilization of enzyme activity [13]. A gel filtration step was used to eliminate a large amount of protein that could interfere with the function of the affinity column if the ammonium sulfate extract was used directly.

A DHA-Sepharose column has been used previously for the purification of adrenal sulfotransferase. This gel proved to be inadequate for the purification of the epididymal sulfotransferase due to its high instability. In view of the marked stabilization of this type of enzyme by PAPS or PAP [14, 16] which was also observed with the epididymal enzyme [17] a PAP-agarose column was selected. This resulted in an *in situ* stabilization of the enzyme while bound to the affinity gel and then elution was achieved by the addition of PAPS in order to retain enzyme activity. The use of this column resulted in a 35-fold purification of the enzyme recovered after gel filtration which ultimately led to a 360-fold overall purification.

The degree of purity of the enzyme preparation following affinity chromatography was established by HPLC gel filtration and SDS gel-electrophoresis. Here again, due to the high instability of this enzyme and the inability to recover activity after a standard electrophoresis procedure, an HPLC procedure was developed. This system enabled the identification of a single peak of protein, associated with sulfotransferase activity, separated from PAPS and which corresponded to a mol. wt of 106,000. The same value was obtained with the enzyme which was eluted from the gel filtration procedure. Upon denaturation with SDS only one major protein band was observed with a mol. wt of 53,000 on SDS-electrophoresis.

This molecular weight indicates that the "native" epididymal sulfotransferase exists as a dimer composed of subunits of equal molecular weight. This finding is in agreement with the physical characteristics of the adrenal [13] and liver [15] sulfotransferases. The purification of this enzyme will enable us to pursue studies concerning the physiological control of this enzyme in the mammalian epididymis.

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