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ESTROGEN SULFOTRANSFERASE IN HUMAN PLACENTA

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Summary—Human placental estrogen sulfotransferase (ESFT) was partially purified from the term placental cytosol by $(NH_4)_2SO_4$ precipitation and agarose gel chromatography. Additional purifications caused a rapid loss of the enzyme activity. The activity was abolished by isoelectrofocusing but partially retained by chromatofocusing. The value of pI of human placental ESFT is 5.8 and the same value was obtained for bovine adrenal ESFT. The enzyme protein was able to bind to the affinity resin, estradiol-17-hemisuccinyl-1,2-diaminododecane sepharose 4B, but difficult to be extracted by estradiol (E_2). The extract of the affinity resin showed one major protein band at 68,000 dalton on SDS–polyacrylamide gel electrophoresis. Kinetic studies using partially purified ESFT revealed that E_2 is the best substrate for this enzyme. The relative rate of sulfurylation of E_2, estrone, estriol and dehydroepiandrosterone at 4 μM (K_m for E_2) is 1, 0.3, 0.08 and 0.08, respectively.

INTRODUCTION

Estrogen sulfotransferase (ESFT) has been identified in endocrine organs of various species [1–10]. However, only the bovine adrenal and placental ESFT have been purified and characterized [1–5]. Human placenta contains a substantial amount of ESFT which was found to be able to convert free estrogen to estrogen sulfate when the placental cotyledon was superfused with the radioactive testosterone at concentration in the order of nM [11]. However, the placental ESFT has not been characterized because the activity in tissue homogenate or in cytosol is virtually devoid due to the high level of estrogen sulfatase. Recently, we have partially purified the human placental ESFT and studied the characteristics and the steroid specificity of this placental enzyme.

EXPERIMENTAL

Isolation and purification of estrogen sulfotransferase from human term placenta

Human term placenta was obtained from Cesarian section and immediately transported to the cold room. The villi were disected from the intact placenta and repeatedly rinsed with cold saline to remove excessive amounts of blood. Approximately 200 g of tissue fragments were homogenized in 2 vol of buffer A (0.05 M Tris–HCl, 25 mM EDTA, 0.25 M sucrose, 2 mM phenylmethyl sulfonyl fluoride (PMSF), 3 mM mercaptoethanol, pH 8.0) in a Warren blender, 7 times 10 s burst, at the setting for the high speed. The homogenate was centrifuged at 1000 g for 20 min and then 100,000 g for 30 min to obtain the soluble fraction. The cytosol was treated with increased concentration of $(NH_4)_2SO_4$ to fractionate the cytosolic proteins. The enzyme was found to be precipitated between 0.35–0.50 saturated $(NH_4)_2SO_4$ fraction.

Bio-Gel agarose chromatography

The $(NH_4)_2SO_4$ precipitated protein, 1.8 g, was dissolved in 15 ml buffer B (0.05 M Tris–HCl, 15 mM $MgCl_2$, 1 mM PMSF, 3 mM mercaptothanol, pH 8, ~120 mg protein/ml) and applied to Bio–Gel A-1.5 m (Bio-Rad) column (2.5 × 50 cm). The protein was eluted from the column with buffer B at the rate of 14–15 ml/h. The ESFT was separated from 90% of estrogen sulfatase in this column, Fig. 1. The fractions containing ESFT were concentrated in Amicon B15 and then applied to a second Bio-Gel A-1.5 m column (1.5 × 100 cm) in which the ESFT was separated from the remaining estrogen sulfatase. The

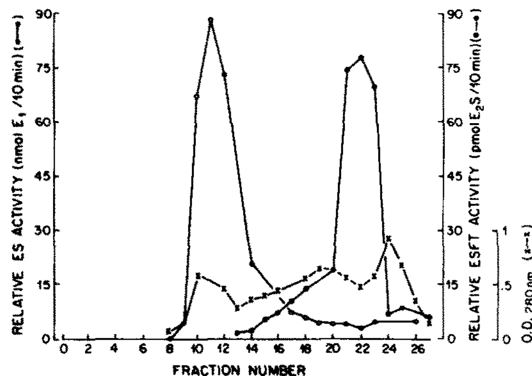

Fig. 1. Separation of human placental ESFT and estrogen sulfatase (ES). 1.8 g of $(NH_4)_2SO_4$ (0.35 to 0.5 saturation) precipitate of the human placental cytosol in 15 ml buffer B was applied to Bio gel A-1.5 m column (2.5 × 50 cm) and eluted with buffer B, 10 ml each fraction, as described in the text. Optical density at 280 nm was measured in 10× dilution of the effluent. The enzyme activities were measured in 0.1 ml aliquots and expressed as the relative activity/each fraction.

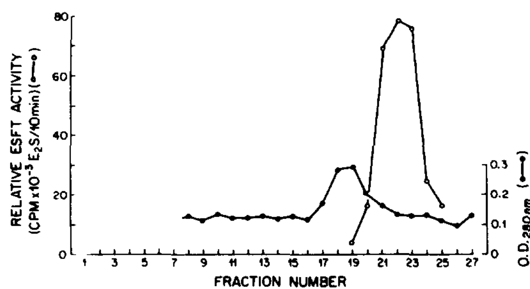

Fig. 2. Protein concentration and ESFT activity from the 2nd agarose gel filtration column (1.5 × 150 cm) The concentrated effluent from the 1st agarose column, 150 mg/5 ml, was applied to the column and eluted with buffer B at 8 ml/min. ESFT activity and protein concentration was determined as described in Fig. 1.

specific activity of the enzyme increased 10–15-fold after the 2nd agarose column, Fig. 2.

Isoelectrofocusing and chromatofocusing

Both isoelectrofocusing and chromatofocusing [10] were used to determine the pI of placental ESFT. The protein solution from the second Bio-Gel A column was concentrated with Amicon B15 and applied to the LKB 8100 ampholine electrofocusing column. The enzyme solution was scanned through the pH gradient from 5–7 in a continuous glycerol gradient (23–58% in buffer B). In chromatofocusing, the sample was applied to a column packed with 20 ml (bed volume) poly buffer ion-exchange resin (PBE 94, Pharmacia). The pH gradient was produced from 7.4 to 5 by eluting the sample with 200 ml polybuffer 74 (Pharmacia, pH 4) containing 10 mM monothioglycerol (MTG), 0.25 M sucrose and 1 mM PMSF, Fig. 3. For the purpose of comparison, pI of bovine adrenal glands ESFT, partially purified by DEAE cellulose concave gradient chromatography

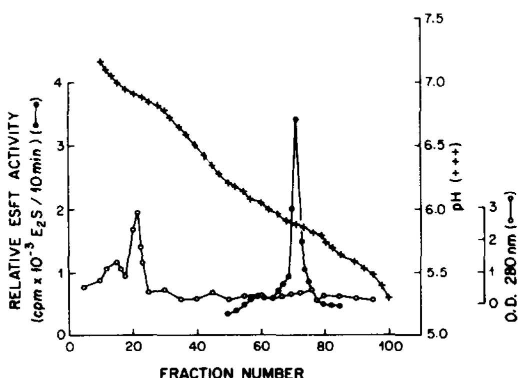

Fig. 3. Chromatofocusing of human placental ESFT. The 100,000 *g* placental cytosol, 60 mg protein in 2 ml of imidazole buffer (0.025 M, pH 7.5 containing 0.25 M sucrose, 10 mM MTG and 1 mM PMSF), was applied to the chromatofocusing column as described in the text. The sample was eluted with polybuffer 74 (1:8 dilution containing 0.25 M sucrose, 10 mM MTG and 1 mM PMSF) at a rate 0.5 ml/min. pH value and O.D. at 280 nm were measured in each fraction. ESFT activity (1 ml aliquot) was measured after neutralized the effluent to pH 7.2.

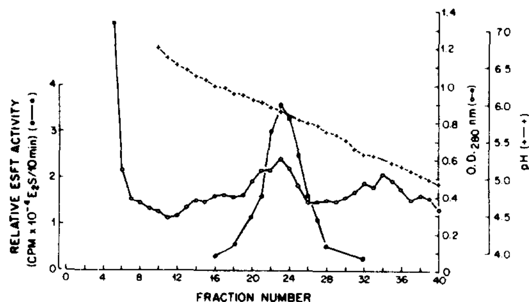

Fig. 4. Isoelectrofocusing of bovine adrenal ESFT. The partially purified bovine adrenal ESFT from DEAE cellulose gradient column, collected at 0.15 M phosphate region, was dialyzed in buffer B. Twenty ml of the sample solution was mixed with 30 ml of 87% glycerol in buffer B, 2 ml 40% ampholine (pH 4–7, LKB) to form the dense gradient solution. The light gradient solution contained 2 ml 40% ampholine in 50 ml buffer B. Anode; 50 ml 0.1 M H_3PO_4 (top) cathode; 50 ml 60% glycerol in 0.1 M NaOH. Electrofocusing was initiated with 0.9 KV, 4.8 mA and ended with 1.7 KV, 2.3 mA from a constant power supply; LKB 2103, 17 h at 4°C. The fractions were collected as 2 ml/fraction. The relative ESFT activity (in 0.05 ml aliquot), pH value, and O.D. at 280 nm were measured in each fraction.

described by Adams and Poulos[1], were also determined, Fig. 4.

Affinity chromatography

Synthesis of 17β-estradiol-17-hemisuccinyl-1,2-diaminododecane sepharose 4B (E2HDODS). The affinity resin was prepared by the method described by Sica *et al.*[12] with modification. Ten g CNBr sepharose 4B (Pharmacia) was freshly activated by soaking the resin in 1 mM HCl for 15 min before use. The resin was then washed with 1 l 1 mM HCl by filtering through a sintered glass at 4°C. The activated CNBr-sepharose 4B was incubated with 25 ml 0.6 mM, 1,2-diaminododecane (DOD) in 0.2 M $NaHCO_3$ at 4°C overnight with gentle shaking. The substituted sepharose was washed with 1 l 1 M NaCl to remove free DOD. The remaining CNBr activated group on the resin was blocked by incubating the resin with 50 mmol ethanolamine in 25 ml 0.2 M $NaHCO_3$ at 4°C overnight and then washed with 1 l each 1 M NaCl and H_2O. The substituted resin was incubated with 3 mg 17β-estradiol-17- hemisuccinate (E_2HS) in 10 ml dioxane at room temperature for 5 h with gentle shaking. The coupled sepharose was washed with 1 l dioxane, 2 l each of H_2O and 70% MeOH to remove any unreacted E_2HS. The amount of E_2 covalently coupled to the resin was estimated by hydrolyzing a small portion of the resin in 0.5 N NaOH at room temperature overnight and measuring the E_2 content by RIA, about 400 ng E_2/ml resin pellet.

Affinity chromatography of placental ESFT solution

Five ml of the effluent of the agarose column at the fraction No. 22 (Fig. 2) was incubated with 0.5 ml of E_2HDODS resin pellet at 4°C overnight with constant shaking. The supernatant was removed and the

resin was washed once with 5 ml 0.2 M KCl in buffer B and then washed 3 times with buffer B alone.

No ESFT activity was detected in the supernatant and washing. The resin was extracted with buffer B containing 1 ml [^{3}H]E_2 (10 μg/ml, same sp. act as the substrate used for the enzyme assay) and adenosine 3′-phosphate 5′ phosphosulfate (PAPS, 0.16 mM, 95% pure, P.L. Biochemical) at 37°C for 30 min or 1 ml [^{3}H]E_2 alone at 4°C overnight.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

The purity of extracted protein from the affinity chromatography was monitored by analyzing the extracts on SDS–PAGE by the method of Weber and Osbon[13]. The samples were prepared in 0.2 ml of gel buffer containing 10% each of SDS and mercaptoethanol [13] and then boiled for 2 min. The sample was applied to the top of 5 mm dia cylindrical gels (3% acrylamide stacking gel, 1.5 cm in length and 7.5% acrylamide separating gel, 5.0 cm in length) for electrophoresis. The gels were fixed and stained by 0.25% coomassie brilliant blue, 45% MeOH and 10% acetic acid in water for 2 h and destained in 15% MeOH and 10% acetic acid in water overnight.

Enzyme assays

Assay for ESFT activity were performed by the procedure described previously [9]. The radioactive steroids: [^{3}H]steroids: estrone (E_1), E_2, estriol (E_3), dehydroepiandrosterone (D) and [^{14}C]E_1 and D were purchased from New England Nuclear (NEN) and crystaline steroids from Steraloids or Sigma. 0.2 ml assay solution contained the enzyme solution (4 mg protein/ml), 0.1–10 μM [^{3}H]steroids (sp. act. of the radioactivity was adjusted to 700–2,000 dpm/pmol), 0.16 mM PAPS and 15 mM $MgCl_2$ in buffer B without PMSF. The reaction was initiated by addition of ^{3}H-steroids to the mixture of PAPS and the enzyme solution, prewarmed to 37°C and terminated by extracting the free steroids from the reaction mixture with ethylacetate. The activities were estimated from the rate of sulfurylation at 5 and 10 min incubation periods (blank value subtracted). The rate of sulfurylation remained constant up to 30 min under assay condition. The products, [^{3}H] steroid sulfate: estron sulfate (E_1S), estradiol-3-sulfate (E_2S), estriol-3-sulfate (E_3S) and dehydroepiandrosterone sulfate (DS) in aqueous solution were further purified on thin layer chromatography [9]. [^{14}C] Steroid sulfates: E_1S, E_2S, DS and [^{125}I]E_3S was separately added to the corresponding reaction mixture at the end of incubation as the recovery marker. [^{14}C]E_1S and DS were separately synthesized from [^{14}C]E_1 and D and purified on thin layer chromatography [14] and [^{14}C]E_2S was synthesized from [^{14}C]E_1S [9]. [^{125}I]E_3S was synthesized from [^{125}I]E_3 (Amersham) using partially purified bovine adrenal ESFT. 1×10^6 cpm [^{125}I]E_3 was reacted with 0.1 ml (1 mg protein/ml) adrenal ESFT solution and 0.16 mM PAPS at 37°C for 2 h. The product [^{125}I]E_3S was separated from the precursor E_3 by extracting the E_3 with ethylacetate and further purified on paper chromatography [2]. estrogen sulfatase activity was measured by the method previously described [9]. Protein content was determined by Bio-Rad method [15]. Radioactivities were measured in a liquid scintillation counter, Beckman Model LS 9000.

RESULTS

Purification of human placental ESFT

The specific activities of ESFT at various stages of purification are shown in Table 1. The enzyme activity in the supernatant of 100,000 *g* homogenate can be detected only when high specific radioactive E_2 (sp. act. 52 Ci/mol) was used as the substrate. The specific activity increases readily through the $(NH_4)_2SO_4$ precipitation and the agarose gel filtration mainly due to the removal of estrogen sulfatase, Figs 1 and 2. The enzyme activity after agarose column were remained up to 15 days at −80°C. However, 70% of the enzyme activity was lost after 7 days at 4°C in buffer B. Further purification accelerates the loss of the activity indicating the unstable nature of the enzyme.

The enzyme activity was abolished by isoelectrofocusing, but retained by chromatofocusing, Fig. 3. The value of pI of human placental ESFT is 5.8, equivalent to the values for ESFT in bovine adrenal glands (Fig. 4) determined by isoelectrofocusing.

When 5 mg of the effluent from the 2nd agarose column was incubated with 0.5 ml of E_2HDODS resin. After extensive washing, the resin was incubated with [^{3}H]E_2 and PAPS at 37°C for 30 min to

Table 1. Purification of human placental ESFT

Preparation	Total protein (mg)	Specific activity pmol/h·mg protein	Purification factor
1. 100,000 *g* supernatant	10,000	~0.01	
2. $(NH_4)_2SO_4$ ppt 35–50%	1800	5	1
3. 1st agarose gel filtration	260	35	7
4. 2nd agarose gel filtration	30	560	117
5. E_2HDODS affinity chromatography	<0.6	450	—

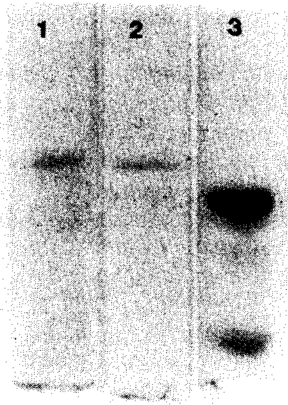

Fig. 5. SDS–PAGE of ESFT extracted from E_2HDODS affinity resin, No. 1: extracted with [^{3}H]E_2 and PAPS at 37°C. No. 2: extracted with [^{3}H]E_2 at 4°C. No. 3: marker proteins, ovalbumin (43,000) and horse heart cytochrome *c* (12,400).

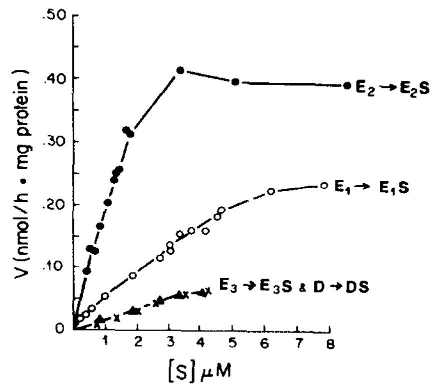

Fig. 6. Substrate specificity of human placental ESFT. The assay condition and separation of the product from the substrate were described in the text. Data are presented as the mean value of duplicate determination of the initial rate of conversion of 5 and 10 min (blank substracted) (▲) and (×) indicate the rate of sulfurylation of E_3 and D, respectively.

extract the enzyme bound to resin. Twenty-two percent of the original enzyme activity was recovered indicating that the enzyme protein was extracted off from the resin. When [^{3}H]E_2 alone was used to extract the resin at 4°C overnight, less than 0.1 mg protein was extracted from the resin with no increase of the specific activity of the enzyme. Analysis of the extracts on SDS–PAGE showed multiple protein bands for the extract of E_2 + PAPS and one major protein band for the extract of E_2 alone (Fig. 5, tube no. 1 and 2, respectively). These results indicate that the proteins are selectively extracted by the combination of E_2 + PAPS or E_2 alone. However, the enzyme activity is rapidly lost during the processes of affinity chromatography and extraction. The molecular weight estimated from SDS–PAGE is 67,500.

Steroid specificity of isolated human placental ESFT

The kinetic study was carried out using the effluent of the second agarose gel filtration, Fig. 2, fraction no. 21–24. Figure 6 shows the rate of conversion of 4 steroids, E_2, E_1, E_3 and D, to the corresponding sulfate. The K_m values of E_2 and E_1, estimated from the reciprocal plot of V vs S are 4 and 20 μM, respectively. The conversion rates of E_3 to E_3S and D to DS are similar but they are much less than that of E_2 to E_2S. The relative rate of sulfurylation of these 4 steroids at 4 μM substrate concentration for E_2, E_1, E_3 and D are 1, 0.3, 0.08 and 0.08, respectively.

DISCUSSION

The present studies demonstrate the presence of ESFT in human placenta. Although this enzyme is not detectable in tissue homogenate and in cytosol, it is able to convert estrogen to estrogen sulfate in intact cotyledon [11] suggesting that this enzyme is active *in vivo*. Steroid sulfotransferase, in general, is unstable [1–5]. The present studies indicate that human placental ESFT is extremely unstable. DEAE cellulose chromatography, conventionally used for the isolation of bovine adrenal and placental ESFT [1–5], isoelectrofocusing and affinity chromatography were found to be unsuitable for the human placental ESFT. The Bio-Gel agarose chromatography was able to separate the ESFT from estrogen sulfatase and simultaneously remove the $(NH_4)_2SO_4$ in a relatively short period. It is necessary to find stabilizing agents for further study. Chromatofocusing in large scale may be useful for the further purification of ESFT in human placenta.

The estimated molecular weight of human placental ESFT is lower than that of bovine adrenal and placental ESFT, 74,000 [3] but similar to the molecular weight of human adrenal dehydroepiandrosterone sulfotransferase [16]. The isoelectric point of ESFT (5.8) in human placenta and in bovine adrenal glands are same as found in the chorion and uterus of guinea pig [10], but different from the porcine endometrial ESFT (pI = 6.1) [17].

The kinetic studies indicate that E_2 is the preferred substrate for human placental ESFT, Fig. 6, and for human endometrium [18] but no preference among E_1, E_2 and E_3 for bovine adrenal and placental ESFT [3]. These findings reveal that the substrate binding site of the enzyme may be modified in different species. Further studies are necessary to evaluate the similarity and discrepancy of this enzyme among species.

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