Purification and Properties of Steroid Sulfatase from Human Placenta

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Abstract. Steroid sulfatase was purified approximately 170-fold from normal human placental microsomes and properties of the enzyme were investigated. The major steps in the purification procedure included solubilization with Triton X-100, column chromatofocusing, and hydrophobic interaction chromatography on phenylsepharose CL-4B. The purified sulfatase showed a molecular weight of 500-600 kDa on HPLC gel filtration, whereas the enzyme migrated as a molecular mass of 73 kDa on sodium dodecyl sulfate polyacrylamide gel electrophoresis. The isoelectric point of steroid sulfatase was estimated to be 6.7 by isoelectric focusing in polyacrylamide gel in the presence of 2% Triton X-100. The addition of phosphatidylcholine did not enhance the enzyme activity in the placental microsomes obtained from two patients with placental sulfatase deficiency (PSD) after solubilization and chromatofocusing. This result indicates that PSD is the result of a defect in the enzyme rather than a defect in the membrane-enzyme structure. Amino acid analysis revealed that the purified human placental sulfatase did not contain cysteine residue. The Km and Vmax values of the steroid sulfatase for dehydroepiandrosterone sulfate (DHA-S) were 7.8 μM and 0.56 nmol/min, while those for estrone sulfate (E1-S) were 50.6 μM and 0.33 nmol/min, respectively. The results of the kinetic study suggest the substrate specificity of the purified enzyme, but further studies should be done with different substrates and inhibitors.

Key words: Steroid sulfatase, Placenta, Dehydroepiandrosterone sulfate, Estrone sulfate, Placental sulfatase deficiency.

STEROID sulfatase (sterol sulfate sulfohydrolase; EC 3.1.6.2) is an enzyme which hydrolyzes a number of 3β-hydroxysteroid sulfates. This enzyme has been reported to be present in liver, kidney, adrenal, ovary, fibroblast and especially in normal human placenta. Steroid sulfatase and its related physiological effects are important for steroid metabolism in the feto-placental unit. In estrogen synthesis, the sulfo-conjugated steroids are hydrolyzed by steroid sulfatase and this step is essential for further metabolism of sulfated steroid precursors to estrogens.

Steroid sulfatase is a microsomal enzyme, and attempts to purify the enzyme have been reported by several investigators [1–5], with varying results as to molecular mass and properties of the purified enzyme. In this study, the steroid sulfatase from microsomes of normal human placenta was purified and kinetic studies were performed to clarify the enzyme properties. In addition, the same procedure was applied to placentas obtained from patients clinically diagnosed as having placental sulfatase deficiency (PSD) [6].
Materials and Methods

Chemicals

Polybuffer exchanger PBE 94, Phenylsepharose CL-4B, Polybuffer 74, and Ampholine were purchased from Pharmacia LKB Biotechnology. Tris, ethylenediaminetetraacetic acid (EDTA), Triton X-100, dithioerythritol (DTE), phenylmethylsulfonyl fluoride (PMSF), and non-radioactive steroids were obtained from Sigma Chemical. Tritium labeled steroids, dehydroepiandrosterone sulfate (DHA-S) ammonium salt [7-3H(N)] SA 851 GBq/mmol and estrone sulfate (E1-S) ammonium salt [6, 7-3H(N)] SA 1.77 TBq/mmol were purchased from New England Nuclear Corp. For the enzymatic studies, radioactive steroids were purified by thin layer chromatography before use. Other chemicals were reagent grade. Reagents for electrophoresis were of special-reagent grade for electrophoresis or analytical reagent grade as described previously [7].

Materials

Normal and PSD placentas in human were obtained immediately after delivery and after removal of membranes and vascular tissues they were stored at -80°C until studied.

Assay of enzyme activity

Sulfatase activity was measured by desulfation of 3H-DHA-S as reported first by Burstein and Dorfman [8]. The enzyme suspension (20 μl) was mixed with 10 μM DHA-S (containing approximately 3×10⁴ dpm of tritium labeled DHA-S) dissolved in a 50 mM Tris-HCl buffer (pH 7.4), and incubated at 37°C for 20 min. The reaction was terminated by adding of an equal volume of 0.1 N NaOH. The unconjugated products of the reaction were extracted into 1 ml of toluene. The toluene extract (0.5 ml) was then transferred to a scintillation counting vial and added to 10 ml of a Bray scintillation solution. Radioactivity was measured with an Aloka ARC-301B scintillation counter. Enzyme activity is reported in international units (IU).

Protein determination

Protein content was estimated with a commercial dye reagent (Tonein TP kit, Otsuka Pharmaceuticals) according to the procedure of Bradford [9]. Bovine serum albumin was used as a standard protein.

Electrophoresis

Disc electrophoresis was carried out on a 7.5% cylindrical polyacrylamide gel (5 mm I.D.) containing 2% Triton X-100 according to the method of Davis [10]. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was performed on a 10% cylindrical polyacrylamide gel (5 mm I.D.) by the method of Laemmli [11].

Isoelectric focusing was carried on a 4% polyacrylamide gel containing 2% Triton X-100 and 2% Ampholine (pH range 3.5–10.0) as described previously [7]. Gels were stained overnight in 0.05% Coomassie Brilliant Blue R-250 in methanol-acetic acid-water (50:7:43, v/v), and destained in methanol-acetic acid-water (30:7:63, v/v).

HPLC gel filtration

The molecular weight of purified steroid sulfatase was estimated by the LKB HPLC system with a TSK G-3000 SW column (0.8×30 cm) equilibrated with 0.1 M phosphate buffer (pH 7.0). An aliquot of the purified sulfatase was mixed with a small amount of calibration standard proteins and eluted with the same buffer. Enzyme activity was measured for each fraction (0.3 ml/tube) and molecular weight was calculated with thyroglobulin (670 kDa), gamma globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and cyanocobalamin (1.35 kDa) as calibration standard proteins.

Amino acid analysis

Approximately 3 μg of the purified steroid sulfatase was hydrolyzed at 110°C under reduced pressure for 20 h in 6 N hydrochloric acid containing 1% phenol with a Waters PICO-TAG work station. Amino acids were analyzed with a Hitachi L-8500 amino acid analyzer.
**Preparation of microsomes**

Frozen placentas (approximately 1,200 g, wet weight) were thawed and all subsequent steps were carried out at 4°C. The placental tissues were minced and homogenized in an electrical mixer with 3 volumes of buffer I (10 mM Tris-acetate, 0.25 M sucrose and 5 mM EDTA, pH 7.0). Placentas from the patients diagnosed as PSD [6] were also obtained at delivery and prepared by the procedure described above. The resulting homogenate was centrifuged at 10,000 × g for 20 min. The supernatant was finally centrifuged at 105,000 × g for 1 h. The 105,000 × g pellets are referred to as the microsome preparation.

**Solubilization of microsomes**

Microsomal pellets were resuspended in buffer II (10 mM Tris-HCl, 1 mM EDTA, 1 mM DTE, 0.05% PMSF and 0.5% Triton X-100, pH 7.5). The suspension was gently mixed with a magnetic stirrer for 1 h at room temperature for solubilization. The suspension was centrifuged at 105,000 × g for 1 h at 4°C to remove insoluble materials. Under these experimental conditions, approximately 70% of the original activity of steroid sulfatase in the microsomes was released into the supernatant.

**Chromatofocusing**

The resulting supernatant was applied to a PBE 94 column (1 × 38 cm) equilibrated with buffer II (pH 8.0). After thorough washing with 2 bed volumes of the same buffer, sulfatase containing fractions were eluted at 30 ml/h with 500 ml of buffer III (12.5% polybuffer 74, 1 mM EDTA, 1 mM DTE, 0.05% PMSF and 0.5% Triton X-100, pH 4.0) and enzyme containing fractions were collected. An aliquot of each fraction was taken to determine steroid sulfatase activity and protein content.

**Hydrophobic interaction chromatography**

The eluate fractions containing sulfatase activity were pooled and applied to a phenylsepharose column (1 × 5 cm) equilibrated with the buffer II. The column was washed with 10 bed volumes of buffer II, then eluted with the same buffer containing 1.25% Triton X-100. Active fractions were collected and used for all experiments as purified steroid sulfatase preparations.

**Results**

**Purification of steroid sulfatase**

Homogenates, microsomes and solubilized microsomal fractions were prepared from eight normal term placentas. The solubilization followed by chromatofocusing and hydrophobic interaction chromatography of normal placental microsomes resulted in a 167-fold increase of its specific activity (Table 1). Solubilized microsomes were applied to a PBE 94 column equilibrated to pH 8.0 with buffer II. The column was then washed with the same buffer and 5 ml-fractions were collected. Sulfatase activity, protein content and the pH of each fraction were recorded. Figure 1 shows the elution pattern obtained in chromatofocusing of solubilized microsomes. The active fractions in normal placentas were eluted from pH 7.0 to 6.6 (Fig. 1a). They were pooled and were applied to a

<table>
<thead>
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<th>Table 1. Purification of steroid sulfatase from human placenta</th>
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<tr>
<td>Intact microsomes</td>
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<tr>
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Fig. 1. Elution pattern from a chromatofocusing column of solubilized microsomes. Solubilized microsomes were applied to a PBE 94 column (1×38 cm), and 5 ml fractions were collected for determination of pH, sulfatase activity and protein content. In the left panel, microsomes from normal placentas (1a). In the right panel, microsomes from PSD placentas (1b).

Fig. 2. Elution pattern from a phenylsepharose column of chromatofocusing pool. The pooled chromatofocusing fractions were applied to a column (1×5 cm) of phenylsepharose CL-4B equilibrated with buffer II containing 0.5% Triton X-100. 2 ml fractions were collected. Sulfatase was eluted with 1.25% Triton X-100 in the same buffer. The sulfatase activity of each fraction was assayed. Column fractions from 1 to 20 were collected and pooled as fraction A; 21–26, B; 27–37, C. Sulfatase activity was present in fraction C.

phenylsepharose CL-4B column equilibrated with buffer II. The column was then washed with 40 ml of buffer II and eluted with the same buffer containing 1.25% Triton X-100. Fractions containing steroid sulfatase activity (fraction numbers 27–37 in Fig. 2) were pooled and used as purified enzyme preparations. With this experimental procedure, 1.7 mg of steroid sulfatase was purified from 1,764 mg of normal human placental microsomes with approximately a 15.8% yield (Table 1).

**PSD microsomes**

The same purification steps were performed on
Fig. 3. Polyacrylamide gel electrophoresis. A, fraction numbers 1–20; B, fraction numbers 21–26; and C, fraction numbers 27–37 collected by phenylsepharose column. Enzyme activity of C gel was measured with DHA-S as substrate on 2 mm gel slices.

Fig. 4. SDS polyacrylamide gel electrophoresis. A, fraction numbers 1–20; B, fraction numbers 21–26; and C, fraction numbers 27–37 collected by phenylsepharose column. Marker proteins were α2-macroglobulin (170 kDa), phosphorylase b (97.4 kDa), glutamate dehydrogenase (55.4 kDa), lactate dehydrogenase (36.5 kDa) and trypsin inhibitor (20.1 kDa).

Fig. 5. Molecular weight determination of steroid sulfatase by HPLC. By means of a LKB HPLC system with a TSK G-3000 SW column, the purified sulfatase was mixed with a small amount of calibration standard proteins and eluted with 0.1 M phosphate buffer (pH 7.0). Fractions of 0.3 ml were collected and the enzyme activity of each fraction was measured. Marker proteins were 1, thyroglobulin (670 kDa); 2, gamma globulin (158 kDa); 3, ovalbumin (44 kDa); 4, myoglobin (17 kDa); and 5, cyanocobalamin (1.35 kDa).
2 PSD placentas. No sulfatase activity was found in microsomes, solubilized microsomes or after chromatofocusing of solubilized microsomes. Figure 1b shows the elution pattern from chromatofocusing of PSD placental solubilized microsomes. The addition of phosphatidylcholine to the incubation buffer following the method of McNaught and France [12] did not yield any sulfatase activity.

Electrophoretic analysis

The purified enzyme preparation obtained by phenylsepharose CL-4B column (fraction C) was analyzed by disc electrophoresis on a 7.5% polyacrylamide gel in the presence of 2% Triton X-100. When steroid sulfatase activity was measured after electrophoresis, the activity corresponded well with the major protein band (Fig. 3). On the other hand, the sulfatase migrated as a single band according to a molecular mass of 73 kDa on SDS polyacrylamide gel (Fig. 4).

Estimation of isoelectric point

The isoelectric point of the steroid sulfatase was estimated to be 6.7 on isoelectric focusing in polyacrylamide gel in the presence of 2% Triton X-100.

Estimation of molecular weight

The apparent molecular mass of the purified steroid sulfatase was calculated to be 500–600 kDa on HPLC gel filtration (Fig. 5).

Amino acid analysis

The amino acid composition was examined with approximately 3 μg of the purified sulfatase preparation. The results are given in Table 2. This protein contained relatively large amounts of glycine and leucine residues, and small amounts of glycine and leucine residues.

<table>
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<th>Amino acid</th>
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<tr>
<td>Arg</td>
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Fig. 6. Lineweaver-Burk graphs illustrating the effect of the DHA-S concentration on purified steroid sulfatase.

Table 2. Amino acid analysis of purified steroid sulfatase
methionine, tyrosine and histidine residues. It is of interest that there was no cysteine.

**Kinetic experiments**

Kinetic experiments were carried out with the purified steroid sulfatase and DHA-S and E1-S as substrates. 10 μl of enzyme preparation (3 μg/ml protein concentration) was incubated at 37°C for 20 min in a metabolic incubator in a room air atmosphere. Km and Vmax values for DHA-S were 7.8 μM and 0.56 nmol/min (Fig. 6), while those for E1-S were 50.6 μM and 0.33 nmol/min, respectively (Fig. 7).

**Discussion**

The placental steroid sulfatase has been purified approximately 170-fold by solubilization of the microsome with Triton X-100, chromatofocusing column and phenylsepharose column chromatography. The purified enzyme revealed a single band on both disc and SDS polyacrylamide gel electrophoresis indicating apparent homogeneity. In previous studies reported by other laboratories [1-5, 13], 3 to 5 chromatographic separations were used to obtain homogeneity on SDS polyacrylamide gel electrophoresis. In the present study we have used only a 2 step procedure.

![Fig. 7. Lineweaver-Burk graphs illustrating the effect of the E1-S concentration on purified steroid sulfatase.](image)

The molecular weight of steroid sulfatase was assessed to be 500–600 kDa by HPLC gel filtration on a TSK G-3000 SW column. The molecular mass of steroid sulfatase found by means of the gel filtration technique was reported as 166–440 kDa previously with DHA-S as the substrate [1–5]. Our data are in agreement with the report of McNaught and France [12] who found two peaks of sulfatase activity after sepharose 4B chromatography with molecular weights of 1,000 kDa and 600 kDa. When the purified enzyme was electrophoresed on SDS polyacrylamide gel, the sulfatase migrated as a single band with a molecular mass of 73 kDa. Similar values have been reported by Dibbelt and Kuss (64 kDa) [1], Vaccaro et al. (78 kDa) [2], Noel et al. (62 kDa) [3], Burns (74 kDa) [4], Loos et al. (63.7 kDa) [5] and Moriyasu et al. (72 kDa) [13]. The different results with respect to the molecular weight may be due to the various methods used for enzyme preparation. The different results may also be due to the structure of the enzyme studied. Previous reports indicate that the steroid sulfatase is composed of identical subunits [1, 2, 4, 5].

McNaught and France [12] reported that there was no steroid sulfatase activity in untreated PSD microsomes. However, the addition of phosphatidylcholine enhanced the sulfatase activity of the solubilized preparation. They proposed the hypothesis that the biochemical basis of PSD may
involves a defect in the membrane-enzyme structure which blocks normal function of the enzyme. In the present study, following solubilization of the microsomes and chromatofocusing, no sulfatase activity was detected although phosphatidylcholine was added to each preparation.

The amino acid composition of the steroid sulfatase was calculated for the first time from the sample of purified enzyme preparation, and it was found that cysteine was not contained in this enzyme. This suggests that there is no disulfide bond in the conformation of steroid sulfatase. Further study is required to clarify the amino acid sequence and higher-order structure of this enzyme. A kinetic study of the purified steroid sulfatase was performed with DHA-S and E1-S as substrates. In previous reports, the Km of microsomal and solubilized steroid sulfatase were reported to be 100 µM and 25 µM [12], and 1.4 µM [14, 15], with DHA-S as the substrate. The properties of purified arylsulfatase C (ASC) have been reported by Vaccaro et al. with a Km for DHA-S of 50 µM [2]. In our study, Km values for DHA-S and E1-S were 7.8 µM and 50.6 µM with purified steroid sulfatase as the enzyme preparation, and Vmax values for these substrates were 0.56 nmol/min and 0.33 nmol/min, respectively. Noel et al. [3] have also reported the Km for DHA-S of purified steroid sulfatase to be 14 µM. Evidence has been presented that arylsulfatase C and steroid sulfatase is ranked among the microsomal type I arylsulfatases. However, it is not yet clear whether this group really consists of several different enzyme species or of only one sulfatase of low substrate specificity [16]. Further study is required to solve this problem by determining the activity of the purified enzyme with sulfate esters such as DHA-S, E1-S, and p-nitrophenylsulfate as substrates and inhibitors.

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References

