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A SINGLE STEP PROCEDURE FOR PURIFICATION OF ESTRADIOL 17 β-DEHYDROGENASE FROM HUMAN PLACENTA

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Summary: A new and simple procedure for purification to homogeneity of the soluble estradiol 17 β-dehydrogenase [E C.1.1.1.62] from human placenta has been developed. The purification is achieved in a single step by affinity-absorption of Cibachrome Blue F3G-A coupled to Sepharose and selective elution with $NADP^+$. Homogeneous estradiol 17 β-dehydrogenase has a specific activity of 7.2 units/mg. and has been purified 2400-fold with a 93 % recovery.

Estradiol 17 β-dehydrogenase [E C.1.1.1.62] from human term placenta is the only enzyme involved in the metabolic transformations of steroid hormones that has been purified from human tissues. This enzyme was first isolated by Langer and Engel (1) and catalyzes the reversible interconversion of estradiol 17-β and estrone utilizing either NAD^+ or $NADP^+$ as coenzyme. This dehydrogenase also catalyzes the apparent irreversible reduction of 16 α-hydroxyestrone to estriol (2), and recently we reported that dehydroepiandrosterone, the principal androgen from fetal adrenals, is a substrate of the enzyme (3). The wide range of steroids accepted as substrates and the easy measurement of its activity have made of this enzyme an important tool for the study of steroid-protein interactions.

Abbreviations used: estradiol 17β-dehydrogenase,-oestradiol-17β:NAD^+ 17-oxidoreductase [E C.1.1.1.62]; estradiol 17-β, estra-1,3,5,(10)-triene-3,17β-diol; estrone, 3-hydroxy 1,2,5,(10)-estratriene-17-one; 16α-hydroxyestrone, 1,3,5(10) estratrien-3, 16α-diol-17one; estriol, 1,3,5(10)-estratrien-3,16α, 17β-triol; dehydroepiandrosterone, 5-androsten-3β-ol-17 one; Blue-Sepharose, Cibachrome Blue FG3-A Sepharose. SDS, sodium dodecyl sulfate.

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In this paper we report a simple method for purification of estradiol 17 β-dehydrogenase from human placenta. The purification is achieved in a single affinity chromatographic step, based on the interaction between the enzyme with Cibachrome Blue F3G-A coupled to Sepharose and its elution by $NADP^+$. Estradiol 17 β-dehydrogenase appeared homogeneous by means of different criteria of purity and recovery was the highest so far reported.

MATERIALS AND METHODS

Reagents. Estradiol 17-β was purchased from Sigma Chemical Co. (St. Louis, Mo., USA) and repurified by thin layer chromatography before use. NAD^+, $NADP^+$, Coomassie Blue R250, Coomassie Blue G, N,N' methylenebis-(acrylamide), ammonium persulfate and sodium dodecyl sulfate were also obtained from Sigma. Acrylamide, N,N,N',N'-tetramethylenediamine and organic solvents were obtained from Merck (Darmstadt, Germany); Sepharose 6 B and molecular weight standards were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Cibachrome Blue FG3-A was a gift from Ciba-Geigy Mexicana, S.A. de C.V. Diaflo P M 10 membranes from Amicon (Lexington, USA), glycerol, inorganic acids, and most common chemicals were analytical reagent grade and purchased from Merck.

Assay Method. All enzyme assays were done by spectrophotometric measurement of the reduction of NAD^+. Reaction velocities were calculated from the initial linear increase in absorbance at 340 nm. in a single beam automatic recording spectrophotometer (Gilford model 252). Assay system contained 0.1 μmol of NAD^+ in 5 mM potassium phosfate buffer containing 20% glycerol, in a final volume of 0.5 ml, pH 7. Reactions were started by the addition of the enzyme and conducted at 25° C.

One unit of enzyme is defined as the amount of enzyme that catalyzes the conversion of 1 μmol of NAD^+ to NADH per minute in a cuvette of 1.0 cm light path at 25°C.

Protein concentrations were determined by the Coomssie Blue G-binding method of Bradford (4) with chicken egg albumin as reference. This protein was standarized spectrophotometrically by means of its absortivity at 280 nm. (5).

Blue Sepharose was prepared coupling Cibachrome Blue F3G-A to Sepharose-6 B by the method of Böhme (6). After the remotion of the non coupled dye with distilled water, a column with 200 ml of Blue Sepharose-6B gel was packed (internal diameter 26 mm) and equilibrated with 5mM potassium phosphate buffer containing 1 mM EDTA, 0.2g/l NaN_3 and 20% glycerol (Medium A).

Gel Electrophoresis. Electrophoresis under non-denaturing conditions were performed in 80 x 80 x 2.7 mm polyacrylamide gel slabs with a linear pore gradient obtained by varying the monomers concentrations from 4 to 30 g/dl., with a constant cross linkage of 4%. Samples of 4 μg. and protein standards were run at 4°C in 90 mM tris-base/80 mM boric acid buffer (pH 8.35) at 125 V for 16 h. Gels were stained for protein by standard methods as well as Karavolas et. al. for estradiol 17 β-dehydrogenase activity (7). Electrophoresis under denaturing conditions were made after treating the samples with 1 g/dl sodium

dodecyl sulfate and 0.15 M mercaptoethanol at 92° C for 5 min. Samples with 4 μg of protein were run in 180 x 140 x 0.7 mm polyacrylamide gel slabs using the discontinuous buffer of Laemmli (8), and stained by standard methods.

RESULTS AND DISCUSSION

Purification Procedure. All operations were carried out at 4° C. with the exception of the affinity chromatography, which was done at room temperature. Term placentas were obtained immediately after delivery and transported to the laboratory on ice. The villous tissue was homogenized in Medium A with a weight/volume ratio of 1/2. Between 266-365 g of villous tissue were obtained per placenta after dissection away from fetal membranes and conective tissue. A single normal term placenta contains between 7 to 10 mg of enzyme. The homogenization was carried out in a 3-liters Waring blendor container with three 20 sec burts at full speed. The homogenate was centrifuged at 12,000 x g for 1 h to sediment debris and mitochondria. The supernatant solution was applied to Blue Sepharose column, then Blue Sepharose was washed with 10 bed volumes of Medium A containing 0.1 M of KCl and then with 5 bed volumes of the same buffer without KCl. (the reduction of the ionic strength is used to stop the elution of proteins that ocurrs at this concentration of KCl). No dehydrogenase activity was found in the column washings. Elution of estradiol 17 β-dehydrogenase was performed with a linear $NADP^+$ gradient in the 0 to 30 μM range. Chromatographic peak from the affinity matrix is symmetrical, with constant specific activity of 7.2 units/mg. This activity agrees with that reported by Chin et. al. for the crystallized enzyme (9). Estradiol 17 β-dehydrogenase also could be eluted by an increase of the concentration of KCl, phosphate or with NAD^+ but in each case there was elution of other proteins. The fractions containing the enzyme were pooled, concentrated by ultrafiltration on a Diaflo concentrating device using a P M -10 membrane and dialyzed against the same buffer but containing 50% glycerol. The purified enzyme then

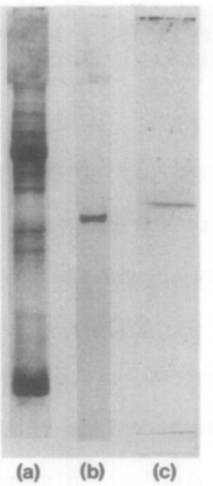

Fig. 1.- Polyacrylamide gel electrophoresis.- (a) Denatured supernatant after homogenization of human term placenta. (b) Denatured sample after Blue-Sepharose chromatography. (c) Pore-gradient gel electrophoresis of a sample after Blue-Sepharose chromatography stained for estradiol 17β-dehydrogenase activity. The electrophoresis and staining were conducted as described under Materials and Methods.

may be stored at 4° C for at least five months with little loss of activity. The enzyme preparation migrated as a single band on SDS polyacrylamide electrophoresis and acrylamide pore-gradient electrophoresis. With either NAD^+ or $NADP^+$ as cofactor, the single protein band developed by acrylamide pore gradient electrophoresis stained for estradiol 17 β-dehydrogenase activity. Fig. 1 shows some representative gels, corresponding to different techniques.

A molecular weight of 33,600 ± 300 was obtained for the protein subunit in SDS polyacrylamide gel electrophoresis; for the native enzyme a molecular weight of 68,000 ± 1500 was estimated using polyacrylamide pore-gradient gel electrophoresis. These values correspond to those reported for estradiol 17 β-dehydrogenase (10). A summary of the purification procedure is given in table I.

Compared with the initial extract, the enzyme was purified 2400-fold with a 93% yield. The over-all recovery is higher than the obtained by longer procedures based on ion exchange chromatography (7,11-13) or on estrogen-bound affinity chromatography (14,15).

TABLE I

PURIFICATION PROCEDURE FOR ESTRADIOL 17β-DEHYDROGENASE FROM HUMAN PLACENTA

Stage	Protein (mg/ml)	Volume (ml)	Total activity (units)	Specific activity (units/mg Protein)	Yield (%)	Purification Factor
12,000 x g Supernatant	31	626	58	0.00303	100%	I
Cibachrome-Blue F3G-A-Sepharose	.21	36.2	55	7.2	93%	2400

The purification of estradiol 17 β-dehydrogenase from human placenta in one relatively easy step, with high recovery, will permit to obtain sufficient quantities of enzyme to allow more detailled studies on substrate specificity, enzyme structure, physiological role and steroid-protein interactions.

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