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IDENTIFICATION AND PARTIAL CHARACTERIZATION OF TWO ENZYME FORMS OF IDURONATE SULFATASE FROM HUMAN PLACENTA

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Iduronate sulfatase of human placenta separates on DEAE Bio-Gel A chromatography into two components, a less acidic form A and a more acidic form B. The two forms have different mobilities on gel electrophoresis and different isoelectric points, pH 5.0 for form A and pH 4.5 for form B. They show the same pH optima in sodium acetate buffer and similar K_m values for [^{3}H]disulfated disaccharide substrate. Iduronate sulfatase A is more heat labile than iduronate sulfatase B. Different molecular weights were found by gel filtration while similar values were estimated by sucrose gradient centrifugation. Neuraminidase treatment of the two forms gives evidence that these enzymes contain sialic acid residues.

Introduction

Hunter syndrome is a lysosomal storage disease characterized by the accumulation of heparan sulfate and dermatan sulfate [1]. It is caused by an X-linked mutation that results in a deficiency of the enzyme iduronate sulfatase [2]. Quantitative determination of the activity in human tissues and body fluids can be made on normal and pathological samples [3]. The enzyme has been partially purified from normal urine [4]. In the present paper we describe our examination of iduronate sulfatase in human placenta and show that two distinct but presumably related forms are present.

Materials and Methods

Chemicals. Bovine serum albumin, catalase, aldolase, ovalbumin, cytochrome *c*, methyl-α-D-glucopyranoside, neuraminidase from *Clostridium perfringens* (type VI) were obtained from Sigma. Concanavalin A-Sepharose, Sephadex G-200, dextran blue were from Pharmacia, DEAE Bio-Gel A, ECTEOLA (Cellex E) were from Bio-Rad Laboratories. All electrophoretic reagents were from British Drug Houses. Carrier ampholytes (pH 7–5 and pH 5–3.5) were obtained from LKB Produkter. Ultrapure sucrose was from Schwarz Mann. Scintillation fluid, Lumagel, was obtained from Supelchem. The substrate, *O*-(α-L-idopyranosyluronic acid 2-sulfate) (1 → 4)-2,5-anhydro-D-[^{3}H]mannitol 6-sulfate was prepared as described [5].

Iduronate sulfatase assay. The iduronate sulfatase has been measured as previously described [3] with minor modifications. Briefly, 30 μl of a substrate solution (radioactive disulfated disaccharide, 0.02 mM, $1 \cdot 10^6$ cpm/ml in 0.27 M sodium acetate buffer, pH 4.0/13 mM NaN_3) were mixed with suitable enzyme aliquots. The enzyme samples were diluted with 1 mg/ml solution of fatty acid-free bovine albumin, extensively dialyzed vs. 10 mM Tris-HCl, pH 7.4, to eliminate low molecular weight inhibitors of enzyme. The volume of reaction mixture was adjusted to 80 μl with the same bovine serum albumin solution. NaCl concentration, in the reaction mixture, was never above 10 mM. Incubation time was for 1 h at 37°C; in these conditions, the reaction is linear up to 30% hydrolysis of the substrate. Reaction was stop-

ped with 1 ml of 1 mM Na_2HPO_4 and the radioactive, monosulfated product was separated from the disulfated substrate on 0.4 ml ECTEOLA columns. A unit of iduronate sulfatase activity is defined as the amount of enzyme required to catalyse the hydrolysis of 1% of the substrate/h. Protein was determined by the method of Lowry et al. [6] using as standard, bovine serum albumin.

Preparation of crude extract. In a typical preparation one placenta, processed immediately after delivery or after freezing for months at −20°C, was thawed in a plastic bag in tap water. Amnion and umbilical cord were removed. The tissue was weighed, cut in pieces and water was added (300 ml H_2O/100 g placenta). The slices were homogenized in a Waring Blendor at low, medium and high speed each for 1 min, the homogenate was filtered through cheesecloth and then centrifuged at 9000 rev./min in a Sorvall GS 3 rotor for 15 min. The supernatant thus obtained was designated placental crude extract. These and the following procedures were carried out at 4°C.

Partial purification of iduronate sulfatase. Placental crude extract was brought to pH 5 with 2 N acetic acid and $(NH_4)_2SO_4$ was added to bring the sample to 70% saturation. The mixture was stirred overnight at 4°C and then centrifuged at 8000 rev./min in a Sorvall GS 3 rotor for 30 min. The resulting precipitate was weighed and 1.3-fold volume of H_2O was added. The solution was brought to pH 5.5–6.0 by addition of 0.5 M Na_2HPO_4, then centrifuged at 15000 rev./min in a Sorvall SS 34 rotor for 10 min and the supernatant dialyzed vs. two changes, 6 l each, of 25 mM sodium phosphate buffer, pH 6.0/0.15 M NaCl for 16–24 h. After centrifugation, the material was applied at 4°C on a concanavalin A-Sepharose column (2.6 × 18 cm) equilibrated with the same buffer, at a flow rate of 30 ml/h. The column was washed at a flow rate of 60 ml/h with 15 ml of the above buffer, then with 45 ml of phosphate buffer/0.5 M NaCl.

Subsequently chromatography was performed at room temperature while the effluent was collected at 4°C. A 90 ml wash with phosphate buffer/1 M NaCl was applied followed by elution with 0.5 M methyl-α-D-glucopyranoside in 25 mM phosphate buffer/1 M NaCl. An aliquot of iduronate sulfatase from the Con A-Sepharose column was dialyzed vs. 10 mM Tris-HCl buffer, pH 7.4, and then applied on DEAE Bio-Gel A column (2.2 × 5.5 cm). Chromatography was started with 10 mM Tris-HCl, pH 7.4 at about 20 ml/h, then the buffer was changed to 10 mM sodium acetate, pH 5.0, later the elution buffer was replaced by 10 mM sodium acetate, pH 5.0/0.15 M NaCl.

Ampholyte displacement chromatography of form A and form B on DEAE Bio-gel A. Ampholyte displacement chromatography was carried out according to a published procedure [7]. The two enzyme species were applied individually to the columns (1.2 × 5 cm) at a flow rate of 10 ml/h in 10 mM Tris-HCl buffer, pH 7.4; for form A the column was washed with 2 vol. distilled water to remove the buffer, then two ampholyte solutions, 15-ml each, diluted 1 : 15 were applied, pH 7.0–5.0 first and subsequently pH 5.0–3.5. For form B the column was washed with 10 mM sodium acetate pH 5, then with 2 vol. distilled water followed by 15 ml ampholyte solution diluted 1 : 15, pH 5.0–3.5.

Neuraminidase treatment of forms A and B. Neuramidase treatment was carried out by incubating 0.5 ml form A (0.94 mg) and 0.5 ml form B (1.5 mg) with 15 μl of a 10 units/ml solution of *Cl. perfringens* neuraminidase in 10 mM sodium acetate buffer, pH 5.0, for 6 h at 37°C. Additional 15 μl batches of neuraminidase were added after the first 3 h incubation. Control experiments in the absence of neuramidase were also performed. The samples were frozen until used. The neuraminidase preparation was free of acid phosphatase, glucosaminidase and mannosidase activities as checked by methods described [3].

Polyacrylamide-gel electrophoresis. Buffer system 1954, 3. VI operative at pH 6.8, 0°C was used [8]. Electrophoresis was performed in 5 × 130 mm tubes at 4°C in 8% gels at a constant current of 1 mA/tube, approx. 50–150 μg protein were layered onto each gel. Following electrophoresis the gels were sliced into 2 or 3-mm-wide segments that were incubated overnight at 4°C in 0.25 ml solution of 0.5 mg/ml bovine serum albumin (fatty acid-free, extensively dialyzed vs. 75 mM NaCl). Enzyme activity was determined on suitable aliquots.

Sucrose gradient centrifugation. 0.5 ml enzyme samples were individually layered on a linear 5–20% sucrose gradient in 10 mM Tris-HCl, pH 7.4/10 mM NaCl. Concurrently, 5 mg aldolase and 5 mg bovine serum albumin were used as marker proteins. Centri-

fugation was performed in the swinging bucket rotor SW 41 of a Beckman L 5.75 centrifuge. Centrifugation was at 39 000 rev./min for 22 h at 4°C. Fractions were collected from the bottom with a glass capillary micropipette connected to a varioperpex pump. The molecular weight was estimated according to the formula proposed by Martin and Ames [9].

Results

A summary of partial purification procedure of placental iduronate sulfatase is presented in Table I. First purification steps include an $(NH_4)_2SO_4$ precipitation and a Con A-Sepharose chromatography (Fig. 1).

Glycoprotein eluted from Con A-Sepharose, when applied on DEAE Bio-Gel A, resulted in two enzyme components, form A (15% of the total activity) that is not adsorbed at pH 5 and form B (85% of total activity) that is adsorbed at pH 5 and eluted with 0.15 M NaCl (Fig. 2). Upon rechromatography using the same column the two enzyme forms were eluted at the same conditions. Ampholyte displacement chromatography on DEAE Bio-Gel A provided evidence of different isoelectric points. Form A was eluted from the column at its isoelectric point of 5.0, while form B was eluted at an isoelectric point of 4.5 (Fig. 3). Electrophoretic examination at pH 6.8 reveals different mobilities of the two enzyme forms: R_F for A = 0.22 and R_F for B = 0.36 (Fig. 4). By treating the two forms with neuraminidase the values were changed to 0.29 and 0.23, respectively. The treatment did not change the elution conditions on

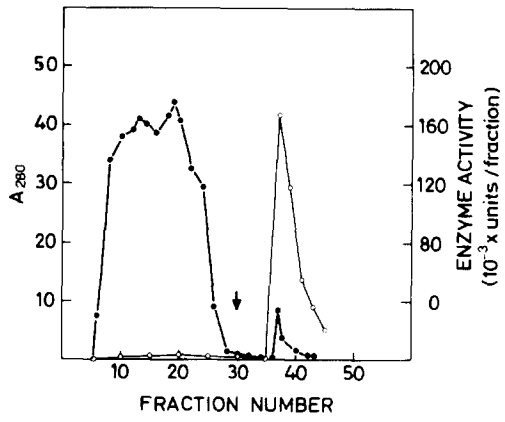

Fig. 1. Chromatography of iduronate sulfatase on Con A-Sepharose. The sample, 292 mg, was applied at 4°C to a column (2.6 × 18 cm) in 25 mM sodium phosphate, pH 6/0.15 M NaCl. The arrow denotes the beginning of the elution. Fractions of 15 ml were collected and absorption at 280 nm (●———●) and enzyme activity (○———○) were measured.

DEAE Bio-gel A chromatography for the form A, while 25% of the B form behaved as the A form following the treatment.

Both forms showed maximum activity at pH 4.0 in 0.1 M sodium acetate buffer. Michaelis-Menten constants, K_m, obtained by plotting reciprocals of initial velocity of enzymatic reaction vs. substrate concentration were very similar: 13 μM for form A (r = 0.92) and 15 μM for form B (r = 0.87) (data not shown). The catalytic activity of the two forms is not influenced by treatment with dithiothreitol (0.1–10 mM) while total inhibition is observed in the presence of phosphate and sulfate (1 mM, as sodium salts). Also

TABLE I

PARTIAL PURIFICATION OF IDURONATE SULFATASE FROM HUMAN PLACENTA

Step	Total protein (mg)	Total activity (units)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude Extract	14 000	1 500 000	107	–	–
1. $(NH_4)_2SO_4$	7 287	1 025 550	140	1.3	68
2. Con A-Sepharose	292	605 900	2 075	19	40
3. DEAE Bio-Gel A [a]					
Pool A	49	35 100	716	6.7	2.3
Pool B	79	318 120	4 026	37.6	21.2

[a] An aliquot of enzyme from Step 2 (172 mg and 356 900 units) was applied on DEAE Bio-Gel A column.

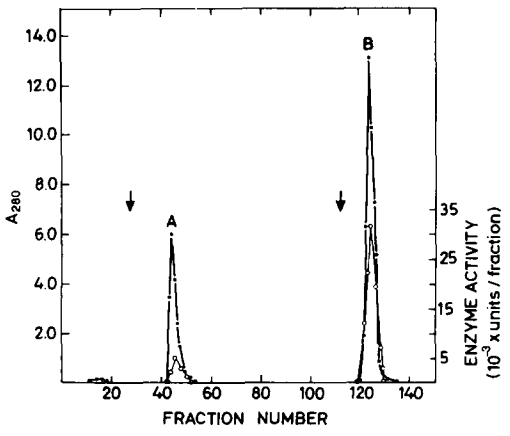

Fig. 2. Separation of forms A and By by DEAE Bio-Gel A Chromatography. Enzyme from step 2 (172 mg) was applied on a column (2.2 X 5.5 cm) in 10 mM Tris-HCl, pH 7.4. The elution buffer was changed to 10 mM sodium acetate, pH 5 (first arrow) and later replaced by the same buffer containing 0.15 M NaCl (second arrow). Absorbance at 280 nm (●———●) and activity (○———○) were measured in each 3 ml fraction.

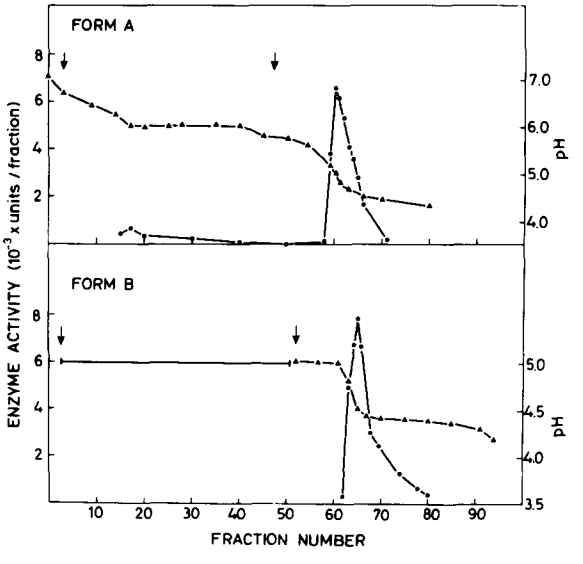

Fig. 3. Ampholyte displacement chromatography of form A and form B on DEAE Bio-Gel A. Iduronate sulfatase A and B were applied individually to a column of DEAE Bio-Gel A (1.2 X 5 cm). To elute iduronate sulfatase A two ampholyte solutions were used: pH 7.0–5.0 (first arrow) and pH 5.0–3.5 (second arrow). For form B the column was washed with 10 mM sodium acetate buffer, pH 5 (first arrow), followed by an ampholyte solution pH 5.0–3.5 (second arrow). Enzyme activity (●———●) and pH (▲———▲) were measured in each 0.5 ml fractions.

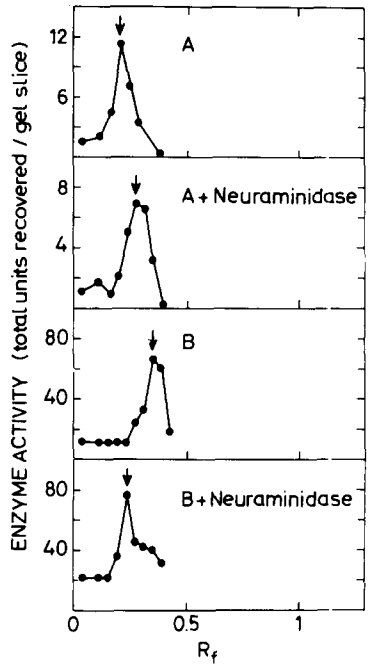

Fig. 4. Polyacrylamide gel electrophoresis of form A and B before and after neuraminidase treatment. Arrows refer to migrated distance used for determination of R_F values.

NaCl inhibited enzyme activities; at 60 mM NaCl, 50% of activity was observed, and at 150 mM NaCl the residual activity was 20%.

Thermal stability of the two enzymes was studied by incubating enzyme aliquots for 5 min at different

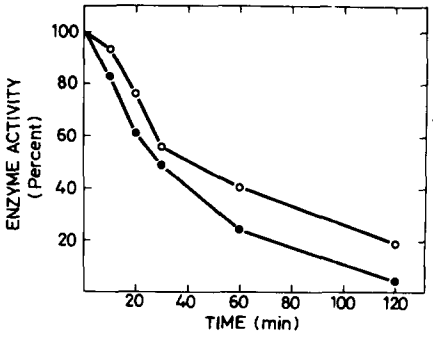

Fig. 5. Thermal stability of forms A (●———●) and B (○———○). Samples of enzymes in 10 mM Tris-HCl, pH 7.4, were heated at 55°C between 0–120 min in the presence of 1 mg/ml bovine serum albumin. Enzyme aliquots were taken at various times, placed in ice and then assayed for iduronate sulfatase. The activity is expressed as percent of that of non-incubated enzyme.

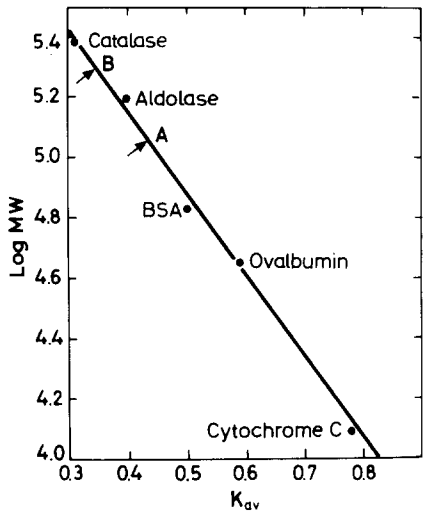

Fig. 6. Gel filtration on Sephadex G-200 of enzymes A and B. A Sephadex G-200 column (2 × 91 cm), running in 10 mM Tris-HCl buffer (pH 7.4)/10 mM NaCl was calibrated with marker proteins. Fractions of 2.3 ml were collected at a flow-rate of 10 ml/h.

temperatures from 25 to 80°C. Both forms were stable for 5 min up to 50°C. At 55°C the enzyme activity started to decrease and 50% of activity was lost at 64°C. In a second series of experiments, the two enzyme forms were incubated at 55°C from 0 to 120 min. The A form lost about 50% of enzyme activity following 30 min of incubation, whereas a slower inactivation was observed with the B form losing 50% of its activity within 45 min of incubation (Fig. 5). After 120 min incubation a nearly total denaturation was observed for form A, while form B retained 20% residual activity.

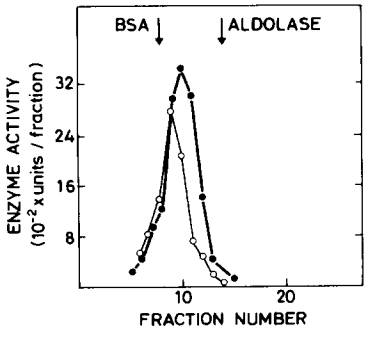

Fig. 7. Sucrose gradient analysis of forms A and B. Enzyme activity of form A (●———●) and form B (○———○).

The molecular weights of iduronate sulfatase A and B were determined by gel filtration and by sucrose gradient centrifugation. Chromatography on Sephadex G-200 (Fig. 6) resulted in an apparent molecular weight of 80 000–115 000 for form A and 170 000–190 000 for form B. Centrifugation on a 5–20% sucrose gradient (Fig. 7) resulted in an apparent molecular weight of 83 000–94 000 for form A and 81 000–83 000 for form B.

Discussion

This report presents evidence that iduronate sulfatase from human placenta is a glycoprotein existing in two distinct forms, a less acidic form A and a more acidic form B. Forms A and B comprise 10–20% and 90–80% of the total activity, respectively. The separation of these two forms is obtained on a DEAE-Bio Gel A, where the component A is eluted at pH 5.0 without salts and the component B is eluted at pH 5.0 with 0.15 M NaCl. Consistent with this behavior form A has a p*I* of 5.0 and form B has a p*I* of 4.5. Difference in the charge of these two forms was further indicated by different electrophoretic mobilities. At pH 6.8 form B migrated faster towards the anode than form A. The possibility that these two components were a consequence of freezing the tissue, was ruled out since the same results were obtained in fresh placenta, processed immediately after delivery. No evidence was obtained of interconversion of forms A and B after their individual rechromatography on DEAE or re-electrophoresis; both enzymes migrated in the original positions.

Partial characterization of these enzymes indicated that the two forms show the same pH optimum, similar K_m for the substrate, similar PO_4^{2-}, SO_4^{2-} and NaCl inhibition, but different isoelectric points and different thermal stability at pH 7.4, the B enzyme being more heat stable than the A enzyme. Neuraminidase treatment of each enzyme caused a change in their charge. Following treatment the electrophoretic mobility of form A was increased, but its elution profile on DEAE-Bio Gel A was unaffected, eluting without salt before and after treatment. The unexpected increase of the electrophoretic mobility of form A after removal of sialic acid can be attributed to either a conformational change of the enzyme molecule or to a possible subunit dissociation

or to interaction with other proteins. After removal of sialic acid the electrophoretic mobility of form B decreased and also its elution profile on DEAE-Bio Gel A was influenced, giving two peaks of activity, one eluting without and the other with salt.

The apparent molecular weights as determined by sucrose gradient centrifugation were in the range of 80 000–100 000 for both enzymes. Similar range was calculated for the urinary enzyme [4]. The discrepancy between the apparent molecular weight of form B obtained on gel chromatography (170 000–190 000) and on centrifugation (81 000–83 000) can be explained either by a different state of the enzyme molecule due to different amount of protein used in the two systems or by a high carbohydrate content [10].

Our results suggest that these two enzyme forms are related. They show similar kinetic properties, while they differ in charge, probably due to carbohydrate content.

In further studies we wish to clarify the relationship of the two enzyme forms and their physiological significance.

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