PARTIAL PURIFICATION AND CHARACTERIZATION OF A HEPARAN SULFATE SPECIFIC ENDOGLUCURONIDASE

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Summary: A heparan sulfate degrading endoglycosidase was partially purified from human placenta. The analysis of the reducing end groups liberated by the endoglycosidase identified this enzyme as an endoglucuronidase. This endoglucuronidase is most active at pH values below pH 6.5 and appears to be a glycoprotein. The enzyme is specific for heparan sulfate and depolymerizes its substrate to oligosaccharides.

Hyaluronidase-like enzymes are glycosaminoglycan degrading endoglycosidases already known for a longer period (1). These enzymes cleave internal glycosidic bonds exclusively in hyaluronic acid and chondroitin sulfate isomers. Heparan sulfate specific endoglycosidases have only but recently been described to be present in rat liver lysosomes (2) and human platelets (3). These enzymes depolymerize heparan sulfate chains to oligosaccharide fragments. The analysis of the reducing end groups of heparan sulfate fragments stored in fibroblasts of mucopolysaccharidoses suggested that the heparan sulfate degrading endoglycosidases might have the specificities of an endoglucosaminidase and an endoglucuronidase (4,5).

The present paper describes the partial purification of a heparan sulfate specific endoglucuronidase from human placenta and some properties of this enzyme.

Materials and Methods: Isolation of polysaccharides: Bovine aorta thoracica was incubated for 20 h in the presence of 5 µCi $[^{35}S]Na_2SO_4$ (carrier free, Amersham Buchler, Braunschweig)/ml medium and $[^{35}S]$heparan sulfate (6) and a dermatan sulfate rich $[^{35}S]$dermatan sulfate-chondroitin sulfate copolymer (7) were isolated as described. $[^{14}C]$Hyaluronic acid was obtained from bovine arterial tissue after incubation in the presence of $[^{14}C]$glucose (6). $[^{35}S]$Chondroitin-4-sulfate was isolated from bovine nasal cartilage (8). $[^{35}S]$heparin was purchased from Amersham Buchler.

Preparation of placenta homogenate: Fresh human placenta was freed from blood and cut into small pieces. After homogenization in 1.5 volumes of 0.01 M TRIS/HCl, pH 7.5 in 0.15 M NaCl with an Ultra-Turrax (Janke u. Kunkel KG, Staufen), the preparation

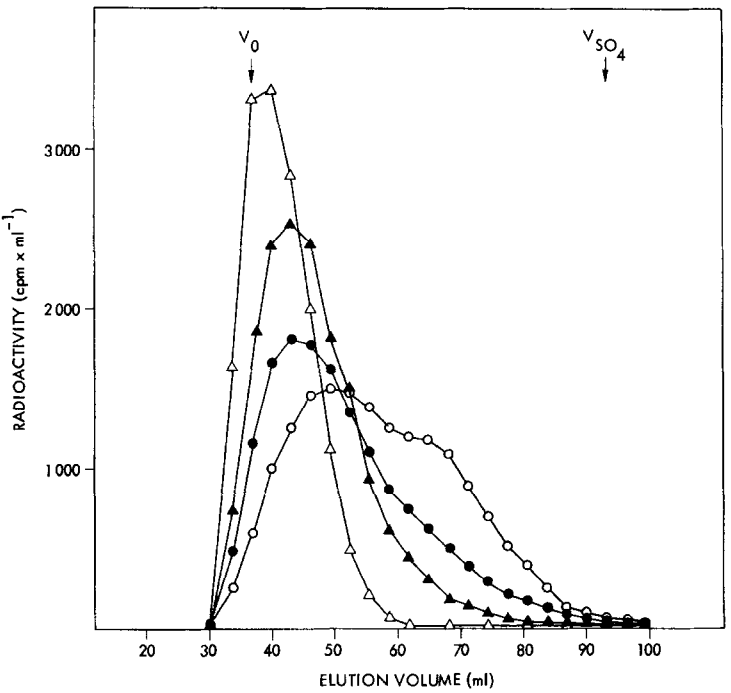

Fig. 1: Chromatography of [^{35}S]heparan sulfate on Sephadex G-100 after incubation with crude placenta homogenate for 8 h (▲—▲), 24 h (●—●) and 72 h (o—o). A control was incubated for 72 h with boiled homogenate (Δ—Δ). V_o and V_{SO_4} are indicated by arrows.

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was centrifuged at 15 000 x g for 60 min. All steps were performed at 2-4 °C. The 15 000 x g supernatant was used for further purification.

Assay for endoglycosidase activity: The standard incubation mixture contained radioactive polysaccharide (30 000 cpm corresponding to 100 µg heparan sulfate or less), enzyme protein (0.1-5 mg), 0.05 M sodium phosphate pH 5.5, 0.15 M NaCl and 0.02 % NaN_3 in a final volume of 60 µl. After incubation 200 µl of 0.1 M NaOH in 0.9 M NaCl were added and the mixture was applied to a 1 x 118 cm Sephadex G-100 column (Pharmacia, Uppsala), equilibrated and eluted with 0.1 M NaOH in 0.9 M NaCl.

For determination of the pH-optimum 0.05 M sodium acetate (pH 4.0-5.0) and 0.05 M sodium phosphate buffers (pH 5.0-7.0) were used.

Identification of reducing end groups liberated by endoglycosidase activity: [^{35}S]heparan sulfate was reduced with $NaBH_4$ (Merck, Darmstadt) (9) and incubated with 80 µg protein of the partially purified endoglycosidase preparation (fraction VIII, Fig. 3 C) under standard conditions. The control contained enzyme boiled for 10 min at 100 °C. After incubation for 72 h at 37 °C the incubation mixtures were applied to a 1.5 x 30 cm Sephadex G-50 column, equilibrated and eluted with 0.2 M pyridine-acetic acid, pH 5.5. The material appearing in the void volume and up to a K_{av} value of 0.17 was pooled and brought to dryness by evaporation.

For analysis of the reducing terminals the incubated [^{35}S]heparan sulfate preparations and the following standards: glucosamine, glucuronic acid (Serva, Heidelberg), iduronic

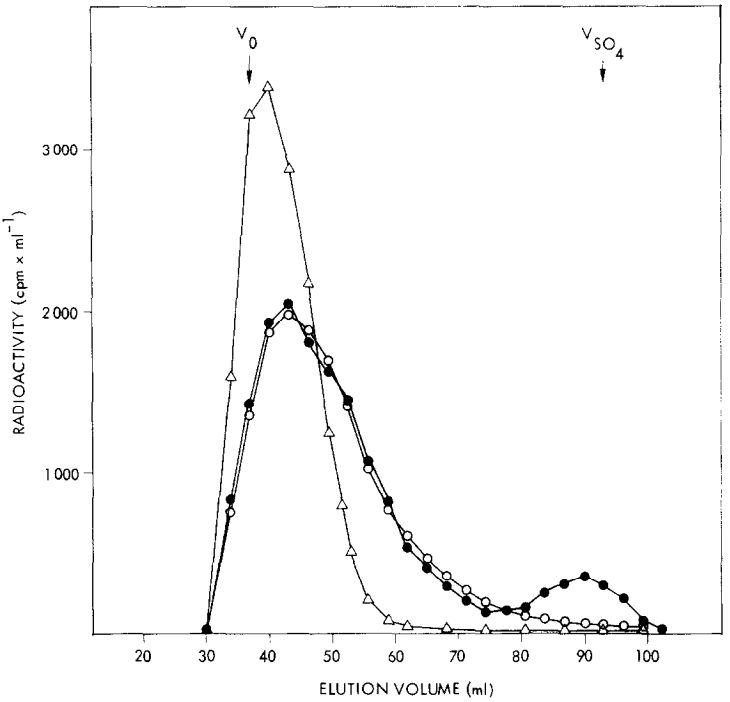

Fig. 2: Chromatography of [^{35}S]heparan sulfate on Sephadex G-100 after incubation with crude placenta homogenate at pH 4.3 (●—●), pH 6.0 (o—o) and pH 7.2 (Δ—Δ). V_o and V_{SO_4} are indicated by arrows.

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acid (prepared from dermatan sulfate, see ref. 10), xylose and galactose (Merck) were reduced with [^{3}H] $NaBH_4$ as described (9).

[^{3}H]Xylitol, [^{3}H]galactitol (11), [^{3}H]glucosaminitol (see preparation of hexosamine fraction in ref. 6) and [^{3}H]aldonic acids (9) were identified as described. The [^{3}H]aldonic acid fraction was isolated, lactonized (12) and further characterized by paper chromatography (9).

Liquid scintillation counting was performed in a 3390 Packard liquid scintillation counter, using Unisolve (Zinsser, Frankfurt) as the scintillation medium.

Results: 1. Demonstration of a [^{35}S]heparan sulfate degrading endoglycosidase in human placenta. Incubation of [^{35}S]heparan sulfate with the 15 000 x g supernatant of a 40 % (w/w) placenta homogenate at pH 6.0 leads to a decrease of the molecular size of the substrate. The depolymerization occurs without a concomitant release of inorganic sulfate (Fig. 1). This indicates that at pH 6.0 [^{35}S]heparan sulfate is degraded by an endoglycosidase acting on internal glycosidic bonds of the polysaccharide chain. The degree of de-

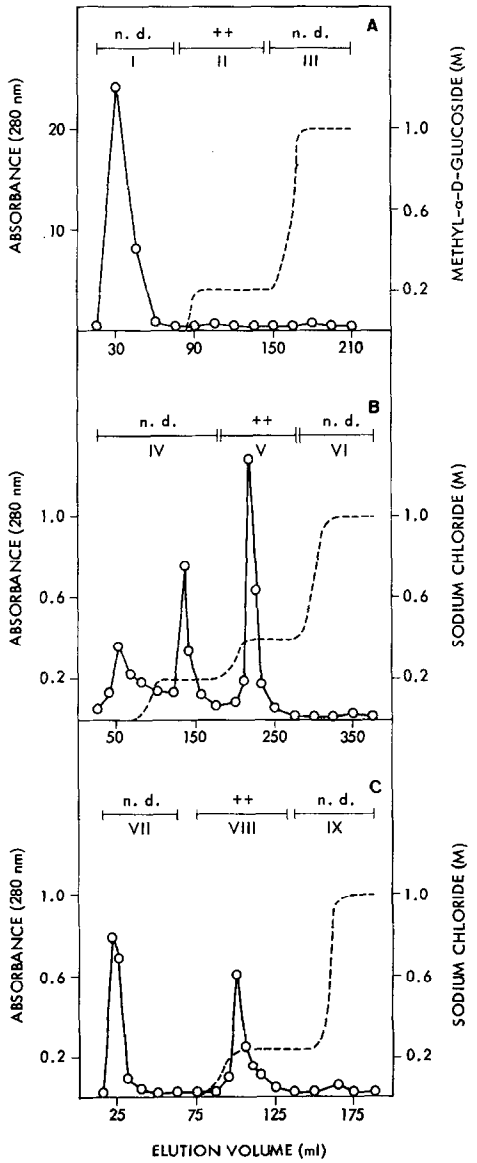

Fig. 3: Purification of [^{35}S]heparan sulfate degrading endoglycosidase activity. 3 A: Elution profile of the endoglycosidase on Concanavalin A-Sepharose 4 B. The 15 000 x g supernatant of the placenta homogenate was made 70 % saturated with ammonium sulfate. The precipitate was suspended in 65 ml 50 mM TRIS/HCl, pH 7.5 and dialyzed against 4 x 5 l of the TRIS/HCl buffer. 20 ml of the dialyzed solution (absorbance 85.0 at 280 nm) were applied on a 1.5 x 20 cm Concanavalin A-Sepharose 4B column (Pharmacia, Uppsala), equilibrated with 50 mM TRIS/HCl, pH 7.5 and eluted stepwise with (I) 50 mM TRIS/HCl, pH 7.5, (II) 0.2 M methyl-α-D-glucoside in 50 mM TRIS/HCl, pH 7.5 and (III) 1.0 M methyl-α-D-glucoside in 50 mM TRIS/HCl, pH 7.5. Absorbance at 280 nm was recorded (o—o) and fractions I-III were pooled as indicated. Fraction II contained the endoglycosidase activity (marked by ++).

polymerization increases with prolonging the incubation period up to 72 hours. Further incubation with fresh enzyme does not affect the elution pattern of [^{35}S]heparan sulfate on Sephadex G-100 (Fig. 1). It seems therefore likely that the number of linkages susceptible to the endoglycosidase is limited. Endoglycosidase activity without concomitant release of inorganic sulfate is observed between pH 5.0 and pH 6.5, with a maximal activity between pH 5.0 and pH 6.0. Above pH 7.0 no endoglycosidase activity is detectable and below pH 4.5 inorganic sulfate is released concomitantly (Fig. 2). From the gel chromatography pattern, however, it seems likely that the endoglycosidase is active down to pH values of pH 4.0.

2. Partial purification of the [^{35}S] heparan sulfate degrading endoglycosidase. The ammonium sulfate precipitation of the 15 000 x g supernatant of the placenta homogenate was applied to a Concanavalin A-Sepharose column, from which the endoglycosidase was eluted with 0.2 M methyl-α-D-glucoside (Fig. 3 A). Further purification was achieved by ion exchange chromatography on carboxymethylcellulose (Fig. 3 B) and diethylaminoethylcellulose (Fig. 3 C). The protein content of the final preparation was 0.35 % of the

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3 B: Elution profile of the endoglycosidase on carboxymethylcellulose. Fraction II obtained after chromatography on Concanavalin A-Sepharose 4B was made 70 % saturated with ammonium sulfate. The precipitate was suspended in 7.5 ml 50 mM sodium acetate, pH 4.5 and dialyzed against 4 x 5 l of the sodium acetate buffer. 14 ml of the dialyzed solution (absorbance 54.8 at 280 nm) were applied after centrifugation at 20 000 x g to a 1.5 x 20 cm carboxymethylcellulose column (Whatman, Maidstone), equilibrated with 50 mM sodium acetate, pH 4.5 and eluted stepwise with increasing sodium chloride concentrations (0.2 M- 1.0 M) in 50 mM sodium acetate, pH 4.5. Absorbance at 280 nm was recorded (o—o) and the fractions IV-VI were pooled as indicated. Fraction V contained the endoglycosidase activity (marked by ++).

3 C: Elution profile of the endoglycosidase on diethylaminoethylcellulose. Fraction V obtained after chromatography on carboxymethylcellulose was made 70 % saturated with ammonium sulfate. The precipitate was suspended in 5 ml 50 mM TRIS/HCl, pH 7.5 and dialyzed against 4 x 5 l of the TRIS/HCl buffer. 6 ml of the dialyzed solution (absorbance 2.04 at 280 nm) were loaded on a 1.5 x 20 cm diethylaminoethylcellulose column (Whatman), equilibrated with 50 mM TRIS/HCl, pH 7.5 and eluted stepwise with increasing sodium chloride concentrations (0.25 M -1.0 M) in 50 mM TRIS/HCl, pH 7.5. Absorbance at 280 nm was recorded (o—o) and fractions VII-IX were pooled as indicated. Fraction VIII contained the endoglycosidase activity and was used for characterization of the enzyme specificity.

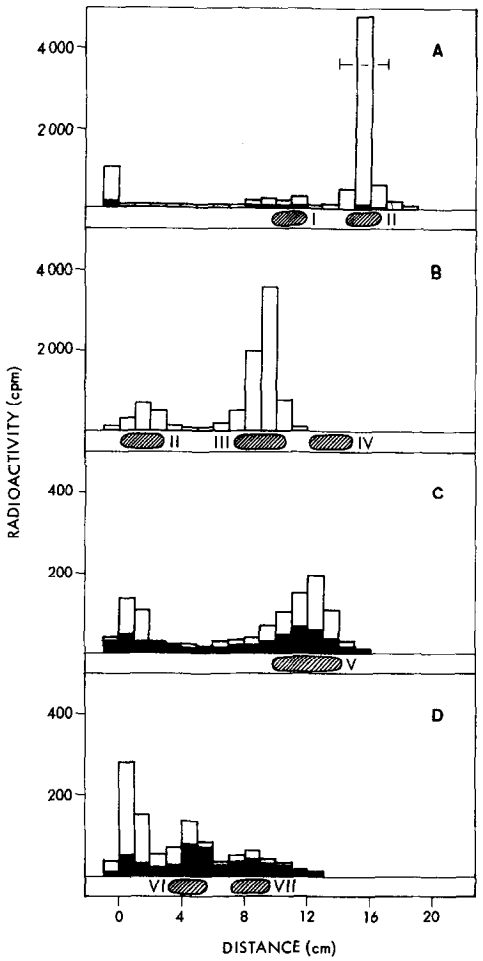

Fig. 4: Identification of the reducing terminals of the [^{35}S] heparan sulfate after incubation with either partially purified endoglycosidase (□) or heat inactivated enzyme (■) (for details see Methods).
4 A: Paper electropherogram of the [^{3}H]aldonic acid fraction. Electrophoresis was performed at 75 V/cm for 45 min in 0.08 M pyridine-acetic acid, pH 5.3 (9).
4 B: Paper chromatogram of the lactonized [^{3}H]aldonic acid fraction obtained after high voltage electrophoresis (Fig. A) in t-amylalcohol-isopropylalcohol-water (4:1:2,v/v/v) (9).
4 C: Electropherogram of the [^{3}H]hexosaminitol fraction. Electrophoresis was performed at 60 V/cm for 165 min in 0.06 M sodium borate, pH 9.5 (14).
4 D: Paperchromatogram of the [^{3}H]neutral sugar fraction in 1-butanol-acetone-water (2:7:1,v/v/v) (11). As references served [^{3}H]uronosylanhydromannitol (I), a mixture of [^{3}H]idonic acid and [^{3}H]gulonic acid (II), [^{3}H]gulonolactone (III), [^{3}H]idonolactone (IV), [^{3}H]glucosaminitol (V), [^{3}H]galactitol (VI) and [^{3}H]xylitol (VII).

starting material. An analytical polyacrylamide gel electrophoresis (system 1 of ref. 13) revealed the presence of at least 6 protein bands in the final preparation.

3. Enzymatic specificity of the partially purified endoglycosidase. The partially purified endoglycosidase was incubated with [^{35}S]heparan sulfate, which had been pretreated with sodium borohydride. A control was incubated with boiled enzyme. The incubation mixtures were subjected to gel chromatography on Sephadex G-50 and the fractions up to a K_{av} value of 0.17 were pooled and treated with [^{3}H]sodium borohydride in order to convert the reducing terminals of the polysaccharide fragments liberated by the endoglycosidase into the corresponding [^{3}H]sugar alcohols.

Analysis of the [^{3}H]sugar alcohols (Fig. 4) revealed that 81 % of the total [^{3}H]activity was present in the fraction of the aldonic acids, which derive from uronic acids present at the reducing terminals of the polysaccharide fragments. 4.5 % of the [^{3}H]activity behaved as glucosaminitol, whereas 3.1 % and 5.2 % cochromatographed with xylitol and galactitol, respectively. A comparison with the [^{3}H]activities detectable in the glucosaminitol, xylitol and galactitol fractions of the control reveals that the activities present in these fractions do not differ significantly in the enzyme assay and the control (Fig. 4 C and 4 D).

The [^{3}H]aldonic acid fraction was eluted from the high voltage electropherogram and identified after lactonization as [^{3}H]L-gulonic acid. L-Gulonic acid is the reduction product of D-glucuronic acid. [^{3}H]L-idonic acid, the reduction product of L-iduronic acid, which is present in heparan sulfate besides D-glucuronic acid, was not detectable (Fig. 4 B).

4. Substrate specificity and properties of the partially purified endoglycosidase. The partially purified endoglycosidase was incubated with the following polysaccharides for 96 h at 37 °C: [^{35}S]chondroitin-4-sulfate, a dermatan sulfate rich [^{35}S]chondroitin sulfate-dermatan sulfate copolymer, [^{35}S]heparin and [^{14}C]hyaluronic acid. None of the polysaccharides was attacked by the endoglycosidase preparation with the exception of [^{35}S]heparin, which showed a minute shift of size distribution towards a lower molecular weight as jugded by chromatography on Sephadex G-50.

The endoglycosidase is completely inactivated by keeping the enzyme preparation for 5 min at 80 °C. Presence of 2 mM dithiothreitol or 2 mM EDTA does not affect the enzyme activity.

Discussion: The demonstration of glucuronic acid as the reducing terminal of polysaccharide fragments liberated from [^{35}S]heparan sulfate by a partially purified endoglycosidase identifies this enzyme as an endoglucuronidase. This endoglucuronidase is specific for heparan sulfate and appears to be a glycoprotein. The pH values, at which the enzyme is active, suggest a lysosomal localization.

The molecular size of the liberated fragments is considerably larger than that of heparan sulfates stored in tissues and fibroblasts from patients with a deficiency of an exoenzyme involved in the degradation of heparan sulfate. Furthermore, the majority of the reducing end groups of stored heparan sulfate fragments are glucosamine residues. These two observations suggest that other endoglycosidases, in addition to the endoglucuronidase described in this paper, participate in the in vivo degradation of heparan sulfate.

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