ISOLATION AND PARTIAL CHARACTERIZATION OF THREE INDUCED ENZYMES FROM FLAVOBACTERIUM HEPARINUM INVOLVED IN THE DEGRADATION OF HEPARIN AND HEPARITIN SULFATES

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SUMMARY: The isolation and partial characterization of two heparitinases and a heparinase from induced extracts from F. heparinum are reported. The heparinase acts upon heparin, heparitin sulfates C and D producing as the main degradation product a trisulfated disaccharide. The heparitinase I degrades only heparitin sulfate A and B producing as main products two N-acetylated disaccharides, one of them O-sulfated. The heparitinase II degrades heparitin sulfate B producing an N-O-sulfated disaccharide as the main product. Based on these results, the mode of action of the three enzymes as well as the structure of heparin and heparitin sulfates are proposed.

INTRODUCTION: A Flavobacterium capable of degrading heparin was isolated from soil by Payza and Korn (1). These authors observed that the bacteria had to grow in a medium containing heparin in order to induce the heparin degrading system. It was later demonstrated that five enzymes acting in concert are necessary to degrade heparin to its basic constituents (2-5) and that at least three of these enzymes are induced when the bacteria are grown either in heparin or heparitin sulfate (6).

Studies on the characterization and chemical composition of commercial heparitin sulfate have shown that this material is composed of at least four distinct mucopolysaccharides (7), two of which were degraded by a purified heparinase isolated from the induced flavobacterium cells (5). The crude induced

extracts on the other hand were able to degrade the four heparitin sulfate fractions (7). These results are indicative that at least another enzyme is present in these extracts which is responsible for the degradation of the two heparitin sulfate fractions resistant to the action of the purified heparinase.

The present paper reports the isolation of two heparitinases and a heparinase able to degrade the four heparitin sulfate fractions. A preliminary communication of these findings has been presented (8).

MATERIALS AND METHODS: Enzymes - F. heparinum (ATCC 13,125) was grown in trypticase without dextrose media (27 g/l) containing heparin and/or heparitin sulfate (150 mg/l). After growth for 36 hours (logarithmic phase) at room temperature the cells were harvested and washed twice with cold 0.1 M ethylenediamine buffer pH 7.0. The cells were then ressuspended in the same buffer, sonicated for 5 minutes in the cold, and centrifuged at 100,000 g for 1 hour. The supernatant was mixed with 10 volumes of acetone at -10°C and the precipitate formed was collected by centrifugation and dried. The dried powder was ressuspended in the same buffer (100 mg/ml) and the insoluble matter was removed by centrifugation. The resulting protein solution was fractionated by large scale electrophoresis in agarose gel as previously described (5).

Materials - Heparin and heparitin sulfate were obtained from the UpJohn Co. through the courtesy of Dr. J.T. Correl. Heparitin sulfates A, B, C, and D were prepared as previously described (7) except that the support used for fractionation of the heparitins was 6% polyacrylamide instead of agarose gel (9).

Other methods - The chromatographic methods and colorimetric determinations of the mucopolysaccharides and their degradation products have already been referred to (2-5). Molecular weight determinations of the mucopolysaccharides were performed as described by Hilborn and Anastassiadis (10).

RESULTS AND DISCUSSION: Chemical composition of the purified heparitin sulfate fractions. Table I shows the results on the analysis of the heparitin sulfates as well as heparin used as substrates in these experiments. Heparitin sulfate A is totally N-acetylated whereas heparitin sulfates C and D are N- and O-sulfated. Heparitin sulfate B contains N-acetyl and N-sulfate

TABLE I

Chemical composition of heparitin sulfate fractions

FRACTION	HEXOSAMINE	ACETYL GROUPS	LABILE SULFATE	TOTAL SULFATE	URONIC ACID	OPTICAL ROTATION $(\alpha)_D^{20}$	AVERAGE M.W.
A	1.0	0.92	0.04	0.45	1.88	+ 92.2	> 170,000
B	1.0	0.60	0.33	0.80	1.80	+ 132.9	25,000
C	1.0	0.07	0.94	2.02	2.28	+ 77.1	9,300
D	1.0	0	1.08	2.62	1.77	+ 53.6	3,800
HEPARIN	1.0	0.09	1.05	2.61	2.00	+ 53.0	12,000

groups within the molecule. These compounds were not degraded by chondroitinase AC and chondroitinase ABC (9).

Fractionation of the heparinase and heparitinases. The enzymatic extracts from the heparin induced cells were fractionated by large scale electrophoresis. Aliquots of each fraction were assayed with each one of the heparitin sulfates and with heparin. The results of this experiment are shown in Fig. 1. Most of the proteins from the extract remain at the origin or migrate towards the positive pole whereas the heparitinases and the heparinase migrate towards the negative pole. Heparin, heparitin sulfate C and heparitin sulfate D are degraded by the same enzyme (fractions 16 - 17). Two other enzymatic activities (fractions 20 - 21 and 12 - 13) degrade heparitin sulfates A and B, producing different types of di- and oligosaccharides. These two activities were called heparitinase I and heparitinase II. The purification obtained for these three enzymes was in the order of 80-fold.

Products formed from the heparitin sulfates by the action of the heparinase and the heparitinases. Fig. 2 shows the

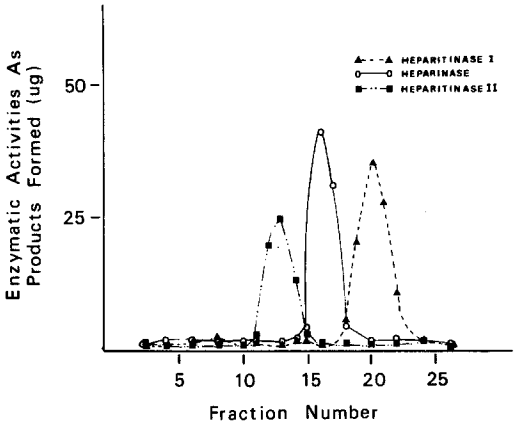

Figure 1. Fractionation of heparitinases and heparinase by large scale agarose gel electrophoresis.

The disaccharide products formed from heparitin sulfates A and B ▲- - -▲ ; ■-··-■ and from heparitin sulfates C and D and heparin O——O were quantified as previously described (5).

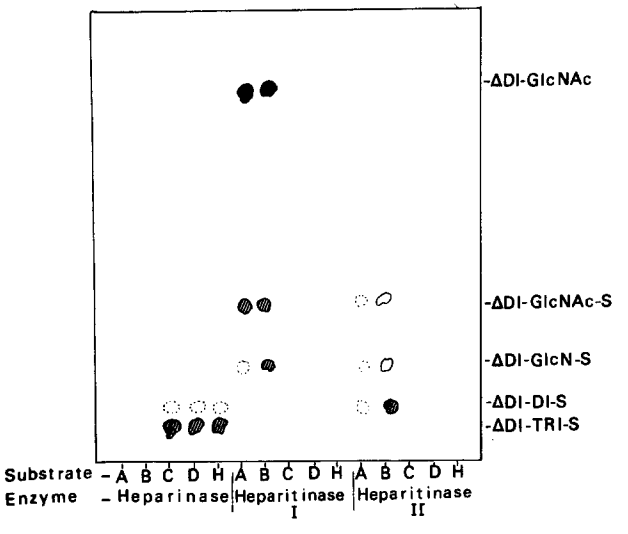

Figure 2. Products formed from heparitin sulfates by the action of the heparinase and heparitinases.

100 µg of heparitin sulfates A, B, C and D and heparin, A, B, C, D, and H respectively, were incubated with about 2 µg of heparinase, heparitinase I and heparitinase II for 16 hours at 30ºC in 0.05 M ethylenediamine acetate pH 7.0 in a final volume of 30 µl. The incubation mixtures were then spotted on Whatman nº 1 paper and chromatographed in isobutyric acid: 1 M NH_3, 5:3, V/V for 48 hours. The products formed were located by the silver nitrate reagent. The shade of the spots indicates the relative amount of the products formed.

relative migration in chromatography of the products formed from the heparitin sulfates and from heparin by the action of the three enzymes. The heparinase acts upon heparitin sulfates C and D and heparin producing an unsaturated trisulfated disaccharide (Δ Di-TRI S) as the major degradation product. A relatively smaller amount of disulfated disaccharide (Δ Di-Di S) and tetrasaccharides is also formed from these substrates. The heparinase does not act upon heparitins A and B, which are in turn degraded by heparitinases I and II. Nevertheless, the products formed by the action of the heparitinases have different chromatographic migration than the ones mentioned above. The major products (68% yield) obtained from heparitins A and B by the action of heparitinase I are an unsaturated acetylated - non sulfated disaccharide (Δ Di-GlcNAc) and an unsaturated N-acetylated O-sulfated disaccharide (Δ Di-GlcNAc-S). A third product of this incubation is an N-sulfated unsaturated disaccharide (Δ Di-GlcN-S). The heparitinase II acts upon one of the substrates, i.e. heparitin sulfate B, producing as a major product (75% yield) an N-O-disulfated disaccharide (Δ Di-Di-S).

The finding that heparitin sulfate B forms different degradation products by the action of heparitinase I or II suggests that this heparitin is a hybrid structure with N-acetylated and N-sulfated regions within the molecule, which is in agreement with the results obtained by chemical analysis (Table I). At least two possible combinations of the N-acetylated and N-sulfated disaccharides within the molecules can be proposed. One of the possibilities is that the structure is composed of alternate N-acetylated and N-sulfated disaccharides. The other is that the molecule is composed of two polymers, one with totally N-acetylated disaccharides and the other with totally

N-sulfated disaccharides. In order to test these possibilities, molecular weight determination of the products formed by the action of heparitinase I was made. The molecular weight of heparitin sulfate B is around 25,000 and the molecular weight of the product formed by the action of heparitinase I which removes the N-acetylated disaccharide residues is in the order of 6,000. This product, when incubated with heparitinase II, produces the sulfated disaccharides shown in Fig. 2. These results indicate that the hybrid heparitin sulfate B is composed of regions made of totally N-sulfated disaccharides and regions made of totally N-acetylated disaccharides. A full account of the analysis of the disaccharide degradation products as well as other properties of the heparitinases will be published elsewhere.

As previously reported, results obtained by the action of the heparinase indicate that large portions of heparin and the heparitin sulfates C and D molecules are composed of trisulfated and disulfated disaccharides repeating units (5). The trisulfated disaccharide repeating units are composed of α L-iduronic acid and glucosamine joined by an α 1-4 linkage (11). The isolation of the trisulfated disaccharide in high yield suggests that the heparinase is specific for N-sulfate glucosamine α 1,4 iduronosyl linkages (Fig. 3). Whether the presence of the sulfate in the iduronic acid moiety is important for the action of the heparinase is now under investigation. The finding that heparitinase I releases mainly N-acetylated products from heparitin sulfates A and B without any action upon N-sulfated portions of the heparitin sulfates C and D or heparin suggests that the enzyme is specific for N-acetyl glucosamine α-1,4 glucuronosyl linkages (Fig. 3). This enzyme has some resemblance to the one previously described by Hovingh and

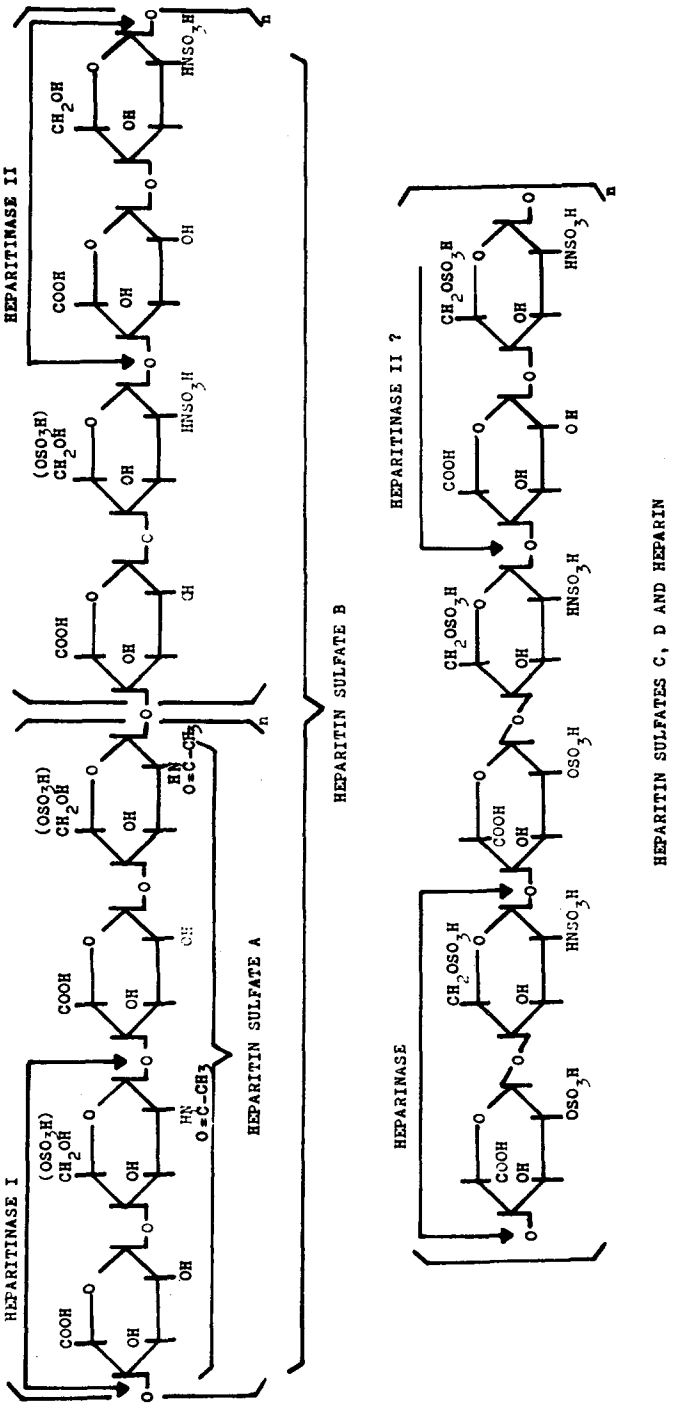

Figure 3. Tentative structure of the heparitin sulfates and heparin. Probable sites of action of the enzymes.

Linker, who isolated a non sulfated N-acetylated disaccharide from crude heparitin sulfate (12). The heparitinase II releases N-O-sulfated disaccharide from heparitin sulfate B, suggesting that N-sulfate glucosamine α 1,4 glucuronosyl linkage is the one susceptible to the action of this enzyme (Fig. 3).

The complete characterization of the heparitin sulfates and heparin degradation products obtained by the action of the above described enzymes will surely bring more information on the structure of these mucopolysaccharides.

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