The Enzymatic Degradation of Heparin and Heparitin Sulfate

III. PURIFICATION OF A HEPARITINASE AND A HEPARINASE FROM FLAVOBACTERIA*

PETER HOVINGH AND ALFRED LINKER

From the Departments of Biological Chemistry and Pathology, University of Utah College of Medicine, and the Veterans Administration Hospital, Salt Lake City, Utah 84113

SUMMARY

Crude enzyme obtained from heparin-induced flavobacteria has been fractionated into a heparitinase acting on heparitin sulfates and related compounds and a heparinase acting mainly on heparin. Purification achieved for each was from 50 to 100 times that of earlier preparations containing a mixture of the two enzymes. In agreement with previous data both enzymes act as eliminases rather than hydrolases yielding products containing \( \Delta 4,5 \)-unsaturated uronic acid. Specificity of the heparitinase appears to require the presence of \( O \)-sulfate and the presence of \( N \)-acetyl or sulfamido groups. Specificity of the heparinase requires the presence of \( O \)-sulfate and sulfamido groups while derivatives containing free amino or \( N \)-acetyl are not substrates. The heparinase degrades heparitin sulfate to some extent acting apparently on the heparin-like portion.

Enzymes from various bacteria are known to degrade mucopolysaccharides by an elimination reaction rather than by the usual hydrolytic pathway. The major end products in most cases are disaccharides containing a \( \alpha, \beta \)-unsaturated uronic acid on the nonreducing end (1–3). Enzymes which degrade heparin and heparitin sulfate have been isolated from flavobacteria grown under conditions of enzyme induction. The crude extracts contain a number of enzymes which degrade the polymers mainly to monosaccharide units (4). Fractionation into an eliminase producing unsaturated di- and oligosaccharides and a glycuronidase degrading these further has been reported by us earlier (5). The purified \( \alpha \)-eliminase still contained traces of \( \beta \)-eliminase, glycuronidase, and sulfatases. The eliminase showed differences in temperature optima and in cationic inhibitions when heparin or heparitin sulfate were used as substrates. This suggested the possibility that two \( \alpha \)-eliminases were present. This paper describes the fractionation of the crude \( \alpha \)-eliminase into a heparitinase and a heparinase by column electrophoresis using a sucrose solution as medium and of phosphocellulose columns similar to those used recently in the purification of the \( \beta \)-eliminases (6).

METHODS

Enzymes—Flavobacteria were grown and extracted as described previously (5).

Analytical Procedures—The following were analyzed by the procedures indicated: reducing sugar by a ferrixyanide method (7); \( N \)-acetyl by the hydroxamic acid reaction after hydrolysis and distillation (8); sulfate by titration (9); free amino by the dinitrofluorobenzene method using \( N \)-desulfated heparin as a standard (10); and proteins by Lowry's method using egg albumin as a standard (11).

Paper Chromatography—Products of enzymatic digestion were chromatographed on Whatman No. 1 paper in butanol-acetic acid-water (50:15:35). The compounds were located by ultraviolet absorption using a short wave length ultraviolet lamp or by spraying with alkali-silver nitrate reagent (12).

Enzyme Assays—Eliminase activity was measured routinely by increase in absorption at 230 mp or by increase in reducing groups where indicated. Sulfatase activity was assayed by measuring the rate of hydrolysis of glucosamine disulfate. Glycuronidase activity was monitored by the rate of disappearance of absorption at 230 mp using as substrate an unsaturated disaccharide obtained from an eliminase digest of heparin (5). Substrate concentrations were 10 mg per ml unless otherwise indicated, and purified enzymes were used in a range of about 3 \( \mu \)g of protein per ml. Assays were carried out routinely in 0.1 \( \mu \) sodium acetate, pH 6.5, for 2 to 3 hours at 25° unless otherwise indicated in the text.

Column Chromatography—Phosphocellulose Whatman P.11 was washed with water by decantation until the material sedimented at a uniform rate and was then washed and equilibrated with solution used for elution of the column. Electrophoresis in a sucrose gradient was accomplished with an LKB 3340C model apparatus (LKB Instruments, Rockville, Maryland). The gradient was prepared with 150 \( \mu \)l of 50% sucrose solution in 0.02 \( \mu \) Tris, pH 7.9, in the mixing chamber and 150 \( \mu \)l of 0.02 w Tris, pH 7.9, in the second chamber. The electrophoresis
TABLE I

Analysis of heparitin sulfate fractions and of heparin derivatives

<table>
<thead>
<tr>
<th></th>
<th>Sulfate</th>
<th>N-acetyl</th>
<th>Free amino</th>
<th>Moles of sulfate per tetrasaccharide</th>
<th>Moles of N-acetyl per tetrasaccharide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin</td>
<td>32</td>
<td>%</td>
<td>%</td>
<td>3</td>
<td>3.6</td>
</tr>
<tr>
<td>N-desulfated heparin</td>
<td>16</td>
<td>%</td>
<td>115</td>
<td>3</td>
<td>1.5</td>
</tr>
<tr>
<td>N-acetylated heparin</td>
<td>16</td>
<td>9.0</td>
<td>3</td>
<td>1.6</td>
<td>2.2</td>
</tr>
<tr>
<td>N, O-desulfated heparin</td>
<td>7</td>
<td>96</td>
<td>0.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-sulfated and O-desulfated heparin</td>
<td>20</td>
<td>%</td>
<td>10</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>N-acetyl-O-desulfated heparin</td>
<td>7</td>
<td>9.0</td>
<td>5</td>
<td>0.6</td>
<td>2.0</td>
</tr>
<tr>
<td>w-heparin (16)</td>
<td>24</td>
<td>2.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heparitin, 1.0</td>
<td>9</td>
<td>4.9</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heparitin, 1.3</td>
<td>15</td>
<td>3.9</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heparitin, 1.5</td>
<td>16</td>
<td>4.0</td>
<td>3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Percentage of total amino groups which are unsubstituted.

** These fractions were eluted from a Dowex 1 column at 1.0 M NaCl, 1.3 M NaCl, and 1.5 M NaCl, respectively.

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** MATERIALS **

Chondroitin sulfate C was obtained from Calbiochem; dermanan sulfate was prepared from pig skin (13). For most experiments a commercial bovine lung heparin was used. The sample had the following analysis: uronic acid (by carbazole), 47%; hexosamine, 30%; sulfate, 32%; nitrogen, 2.2%; [a]_250 = 50°. Anticoagulant activity, 150 units per mg. For some of the experiments a preparation obtained from Nutritional Biochemicals with very similar analysis but of unknown origin was used. Crude enzyme acted on both preparations at the same rate. Heparitin sulfate was purified from a commercial sample as described previously (14), with further fractionation on a Dowex 1 column (15). The fraction used for most experiments had the following analysis: uronic acid (by carbazole), 49%; hexosamine, 28%; sulfate, 15%. Other data are shown in Table I. The following heparin derivatives were prepared by the procedures indicated: N-desulfated heparin (17), N-acetyl-heparin from N-desulfated heparin (18), N,O-desulfated heparin (19). * N-sulfated and O-desulfated heparin (16, 17). Three major fractions eluted with 1.0 M, 1.3 M, and 1.5 M NaCl, respectively, were obtained. Each migrated as a single spot on cellulose acetate electrophoresis; the lowest molarity salt eluate had the slowest migration rate. The [a]_250° of the o-keto acid product does not absorb in this range. Assay of the purified enzymes by increase in reducing sugar is very insensitive due to the nature of the oligosaccharide products.

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** FIG. 1. ** Elution pattern from a phosphocellulose column (5 X 2.1 cm). Each tube represents approximately 0.6 ml. Aliquots (0.3 ml) were assayed in 1.0 ml of 0.1 M sodium acetate, pH 7.0, with 10 mg of substrate. +---+, heparitin 1.0; - - - - , heparin; A---A, chondroitin 6-sulfate; ---*, NaCl gradient.

** TABLE II **

Purification of heparitinase and heparinase

<table>
<thead>
<tr>
<th>Total protein</th>
<th>Heparin substrate</th>
<th>Heparitin substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total activity</td>
<td>Specific activity</td>
<td>Specific activity</td>
</tr>
<tr>
<td>mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>350</td>
<td>15</td>
</tr>
</tbody>
</table>

* Total activity is represented by the change in optical density at 230 mp per hour.

** FIG. 2. ** Elution pattern of heparitinase and heparinase from a sucrose gradient electrophoresis column. Aliquots (0.15 ml) were incubated in 0.1 M sodium acetate, pH 7.0, using 12 mg per ml of substrate. --- , heparin; +---+, heparitin 1.0.
Heparitinase and Heparinase from Flavobacteria

**RESULTS**

**Preparation and Properties of Heparitinase**

Preparation—All purification steps were carried out at 4°. Crude lyophilized extract of flavobacteria (5.0 g) was added to 100 ml of 0.5 M sodium acetate solution, adjusted to a final pH of 6.4, slowly stirred, and centrifuged at 25,000 × g for 30 min. The supernatant solution was treated with protamine sulfate (10 mg per ml of solution), stirred slowly, and centrifuged at 25,000 × g for 30 min. This supernatant solution was diluted to a 0.05 M concentration of acetate and added to a column (4.5 × 2.1 cm) containing 5.0 g of phosphocellulose. The column was washed with 100 ml of 0.05 M sodium acetate and then eluted with a gradient formed by addition of 500 ml of 0.05 M sodium acetate in 0.7 M NaCl to a mixing vessel containing 500 ml of 0.05 M acetate in 0.05 M NaCl. Fractions of 7 ml were collected and checked for activity on heparin, heparitin sulfate, and chondroitin 4-sulfate. The elution pattern is shown in Fig. 1. As can be seen, β-eliminase activity (chondroitinase) is well separated from α-eliminase activity. Material eluted in Peak I from two columns was combined, dialyzed against 0.01 M (NH₄)₂CO₃ (pH 7.9), and lyophilized. This was then dissolved in a small volume of the sucrose gradient solution and placed in the midpoint of the electrophoresis column. Current was applied for 38 hours with an initial voltage of 1100 volts at 28 amp. The column was then eluted in 2.0-ml fractions which were assayed for activity on heparin and heparitin sulfate. The pattern obtained is shown in Fig. 2. Fractions in Peak I and Peak II, respectively, were combined, dialyzed, and lyophilized. Peak I was found to contain enzyme acting on heparin and heparitin sulfate; sulfatase and glycuronidase were also present. Peak II contained α-eliminase acting on heparitin sulfate only. Purification was over 100-fold of that from the Sephadex column. None of the other enzymes could be detected; however, the preparation still showed five bands on gel electrophoresis. Table I gives the pertinent analysis of the heparin and heparitin fractions.
Properties—Enzyme activity with time is shown in Fig. 3. As can be seen, the activity measured by the increase in ultraviolet absorbance parallels that measured by the increase in reducing groups. Figs. 4 and 5 show that activity is proportional to enzyme and substrate concentration. The temperature optimum was 42° (Fig. 6) and the pH optimum occurred at pH 6.8 (Fig. 7). The enzyme was completely inhibited by Cd++, Cu++, Zn++, and Hg++ at 10^{-3} M. However, at concentrations of 10^{-5} M, Cd++, Cu++, and Hg++ were stimulatory while Zn++ still produced 50% inhibition. Very little effect was shown by Fe++, Ba++, Ca++, Mn++, and Mg++ at 10^{-3} M. EDTA and cysteine had no effect. The enzyme was stable for at least 2 months when stored at 40 µg of protein per ml of buffer at 4°.

The substrate specificity of the heparitin eliminase, which has interesting characteristics, is shown in Fig. 8. As can be seen, the enzyme shows fairly good activity with N-acetyl-O-desulfated heparin as a substrate. Paper chromatography of this digest showed nonsulfated di- and tetrasaccharide (21). Though N-acetylheparin and heparin were partially degraded, no disaccharides could be detected as products on paper chromatography. The heparitin sulfate fraction (1.0) with the lowest sulfate content (Table I) was the best substrate. Except for the N-acetylheparin, which inhibited the degradation of heparitin by 60% at a concentration of 10 mg per ml, the other compounds, though not active as substrates, were not inhibitory. Substrate specificity was the same at 22° or at 42°.

Preparation and Properties of Heparinase

Preparation—Since an eliminase acting on heparitin sulfate but not on heparin could be isolated, the question arose whether an enzyme acting only on heparin was also present in the crude extracts. Two peaks had been obtained by column electrophoresis of enzyme obtained from the small phosphocellulose column (Fig. 2). Peak I contained enzyme that acted on both heparin and heparitin. Further fractionation of the crude extract was carried out as follows. Crude extract (6.8 g) was prepared as above and treated with protamine sulfate. The resulting supernatant was diluted to 0.05 M acetate and added to 35 g of phosphocellulose in a column (30 × 2.1 cm). The column was washed with 500 ml of 0.025 M Tris, pH 7.9, in 0.1 M NaCl. A gradient formed by addition of 500 ml of 0.025 M Tris in 0.7 M NaCl, pH 7.9, to a mixing vessel containing 500 ml of 0.025 M Tris in 0.1 M NaCl, pH 7.9, was used for elution. Fractions of approximately 6 ml were collected and assayed for activity with heparin as substrate (Fig. 9). Fractions in the main peak (tubes 53 to 64) were combined, dialyzed against 0.01 M (NH_4)CO_3, pH 7.5, and lyophilized. Table II summarizes the activities of the purified fractions. As can be seen the purification achieved was 50- to 100-fold over

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that obtained by the original Sephadex column procedure (5). No glycuronidase, sulfatase, or β-eliminase (chondroitin 6-sulfate as substrate) could be detected in the final preparation. Polyacrylamide gel electrophoresis, however, still showed the presence of three strong and six weak bands.

Properties—As seen in Table II this enzyme acts on heparitin sulfate as well as on heparin. However, specific activity on heparitin is much lower here than in the heparinase described above. Therefore, substrate specificity was examined. As can be seen in Fig. 10 heparin was the best substrate, w-heparin was degraded to a lesser extent while the heparitin sulfates, N-acetyl-O-desulfated heparin, and N-sulfated and O-desulfated heparin (i.e. heparitin-like compounds) were relatively poor substrates. N-desulfated heparin and N-acetylated heparin were not acted upon. The compounds which were poor substrates or were totally inactive did not inhibit the enzyme at 10 mg per ml. The heparinase did show an atypically slow activity with heparin as substrate in 0.1 M sodium acetate, pH 7.0, at 27°; ——, protein (absorption at 280 mp); ..., NaCl gradient.

Fig. 9. Elution pattern of heparinase from a large phosphocellulose column (30 X 10 cm). Approximately 6 ml were collected in each tube. O-O, heparinase activity with the use of 0.3-ml aliquots with heparin as substrate in 0.1 M sodium acetate, pH 7.0, at 27°; ——, protein (absorption at 280 mp); ..., NaCl gradient.

Fig. 10. Substrate specificity of heparinase. Reaction in 0.1 M sodium acetate, pH 6.5, at 27°. Substrates: O-O, heparin; Δ—Δ, w-heparin; △—△, heparitin 1.6; X—X, heparitin 1.3; ——+, heparitin 1.0; ——●, N-sulfated and O-desulfated heparin.

DISCUSSION

Heparin and heparitin sulfate are mucopolysaccharides closely related in composition and linkage type. They differ in sulfite content which is much higher in heparin, and in the substitution of the amino group of glucosamine. Heparin contains mainly sulfamido, while heparitin contains on the average half N-acetyl and half sulfamido groups. The detailed structure of heparitin sulfate is not known but data available (21, 22) indicate that it contains heparin-like segments and low sulfated N-acetyl glucosamine-containing portions. Each of these appears to be of some size. It should be emphasized that heparitin sulfate is not just a low sulfate heparin but shows considerable structural differences.

Flavobacterium heparinum grown in the presence of heparin was the first source of an enzyme degrading heparin to a significant extent (23). Crude extracts of uninduced organisms contain eliminases acting on chondroitin 4- and 6-sulfates, on dermatan sulfate, and on hyaluronic acid (1, 2, 4, 24) and sulfatases acting on oligosaccharides derived from these polymers (25, 26). When induced to heparin the flavobacteria produce eliminases that degrade heparin and heparitin sulfate, and glycuronidases that act on unsaturated uronides (5, 26). Sulfatases (27, 28) have also been shown to be present. If any of these enzymes are to be used for either identification or structural studies of mucopolysaccharides, fractionation and purification are necessary. In a previous publication (5) the preparation of heparin-induced eliminase, free of glycuronidases, was described. The present work describes the separation of an eliminase, acting mainly on heparitin sulfate, from one that acts on heparin. Good separation between α- and β-eliminases is achieved on a small phosphocellulose column (Fig. 1). A heparitinase tree of the other enzymes is purified by column electrophoresis (Fig. 2, Peak II). The fact that heparitin sulfate and heparin have many structural features in common (21, 22) complicates interpretation of specificity. The heparitinase (Fig. 2, Peak II),
though not pure by criteria of gel electrophoresis, shows none of the other activities present in the crude extracts and has a specific activity 125 times higher than the best previous mixed eliminase preparation (5) (Table II). Substrate specificity is of interest (Fig. 8). The heparitin fraction with the lowest sulfate and the highest N-acetyl content was the best substrate. N-acetyl-O-desulfated heparin, which has structural similarities to heparitin sulfate was degraded fairly well while N-acetylheparin (i.e., O-sulfated) and ω-heparin (having 20% of the amino groups acetylated) were poor substrates. The heparitinase showed no activity on heparin. These data indicate that the specificity of the enzyme requires that the amino group of glucosamine is blocked with either acetyl or sulfamido groups and that O-sulfate be absent.

Fractionation of crude extract on a large phosphocellulose column gave results which differed considerably from those obtained with the much smaller column above. The heparinase obtained (Table II) shows very high specific activity with the enzyme required in preparations described here.

It may be noted that the heparinase and a heparitinase in the present study were obtained from organisms induced to heparin only. Without induction neither enzyme can be detected by the assay methods used here, although eliminases acting on β-linked mucopolysaccharides are present (1, 2, 4).

REFERENCES

The Enzymatic Degradation of Heparin and Heparitin Sulfate: III.
PURIFICATION OF A HEPARITINASE AND A HEPARINASE FROM
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