Purification and Substrate Specificity of Heparitinase I and Heparitinase II from *Flavobacterium heparinum*

ANALYSES OF THE HEPARIN AND HEPARAN SULFATE DEGRADATION PRODUCTS BY \(^{13}\)C NMR SPECTROSCOPY*

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The purification of two heparitinases and a heparinase, in high yields from *Flavobacterium heparinum* was achieved by a combination of molecular sieving and cation-exchange chromatography. Heparinase acts upon N-sulfated glucosaminido-L-iduronic acid linkages of heparin. Substitution of N-sulfate by N-acetyl groups renders the heparin molecule resistant to degradation by the enzyme. Heparitinase I acts on N-acetylated or N-sulfated glucosaminido-glucuronic acid linkages of the heparan sulfate. Sulfate groups at the 6-position of the glucosamine moiety of the heparan sulfate chains seem to be imperative for heparitinase I action. Heparitinase II acts upon heparan sulfate producing desulfated, N-sulfated and N-acetylated-6-sulfated disaccharides, and small amounts of N-acetylated disaccharide. These and other results suggest that heparitinase I acts preferentially upon N,6-sulfated glucosaminido-L-iduronic acid linkages of heparan sulfate. The total degradation of heparan sulfate is only achieved by the combined action of both heparitinases. The \(^{13}\)C NMR spectra of the disaccharides formed from heparan sulfate and a heparin oligosaccharide formed by the action of the heparitinases are in accordance to the proposed mode of action of the enzymes. Comparative studies of the enzymes with the commercially available heparinase and heparitinase are described.

*Flavobacterium heparinum* produces a number of constitutional and induced enzymes which are able to degrade glycosaminoglycans to their basic disaccharide units. Among the constitutional enzymes a chondroitinase AC (1) and a chondroitinase C (2) able to degrade chondroitin sulfates have been identified. When the bacteria are grown in the presence of chondroitin sulfate or dermatan sulfate two new chondroitinas, namely, chondroitinases ABC and chondroitinase ABC are induced in the bacteria (3, 4). Also, a heparitinase and two heparitinases are induced when the bacteria are exposed to heparin, heparan sulfate or their di- and oligosaccharides (5, 6).

The presence of these different activities in the bacteria, which act upon closely related substrates, namely heparin and heparan sulfate, poses difficult problems regarding not only their purification but also in obtaining precise information on their mode of action.

Whereas the substrate specificity of the heparinase is reasonably well established (6-13) a clear definition of the specificity of the heparitinases as well as their purification from each other and from the heparinase and the constitutional enzymes is still not completely settled.

The purpose of this paper is the description of a new method for the purification of the enzymes as well as an attempt to define their specificities using natural and modified heparins and heparan sulfates.

**EXPERIMENTAL PROCEDURES**

Substrates and Materials—Heparin from bovine lung tissue was a kind gift from L. L. Coleman (Upjohn Co., Kalamazoo, MI). Heparin from bovine intestinal mucosa and heparan sulfate from bovine pancreas were gifts from Dr. P. Bianchini (Opocrin Research Laboratories, Modena, Italy). Heparan sulfate from bovine lung (formerly designated heparan sulfate B) was purified as previously described (14). Chondroitin 4- and 6-sulfates were purchased from Miles Laboratories (Elkhart, IN). Di- and tetrasaccharides from heparin and heparan sulfates were prepared in large scale by methods previously described (6, 15). Bio-Gel A-0.5 m, DEAE-cellulose, and carboxymethylcellulose were purchased from Bio-Rad. Ethylenediamine (1,2-diaminoethane) was purchased from Aldrich. Commercial heparinase and heparitinase were purchased from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan).

Purification of the Enzymes—F. heparinum (ATCC 13125) was grown in trypticase soy broth minus dextrose in the presence of 150 mg of heparin essentially as previously described (16). The bacteria were harvested in the mid-log phase of growth, suspended in 20 ml of 0.1 M ethylenediamine-acetate buffer, pH 7.0 (prepared by adding acetic acid to 0.1 M 1,2-diaminoethane up to pH 7.0) and sonicated for 5 min in pulses of 30 s/min in the cold. After sonication, the mixture was centrifuged at 100,000 × g for 60 min. 15 ml of the supernatant (20 mg/ml of protein) were applied to a DEAE-cellulose column (2 × 20 cm) previously equilibrated with 0.1 M ethylenediamine-acetate buffer, pH 7.0. Ten-ml fractions were collected with a flow of 20 ml/h. Aliquots of the fractions were assayed with the different substrates (see below). The fractions containing the enzymatic activities were pooled, concentrated by pressure dialysis under nitrogen, diluted to 2 ml and applied to a column of Bio-Gel A-0.5 m (4 × 120 cm) previously equilibrated with the same buffer. 10-ml fractions were collected and aliquots assayed with the different substrates. The fractions containing the heparitinase II, heparinase, and heparitinase I activities were concentrated by pressure dialysis, suspended in 2 ml of 0.01 M ethylenediamine-acetate buffer, pH 7.0, and applied to columns of carboxymethylcellulose (1 × 10 cm). The enzymes were eluted from the columns with different molarities (0.01-0.1 M) of ethylenediamine-acetate buffer, pH 7.0.

**Assay of the Enzymes**—A typical incubation mixture contained 10 μl of enzyme, 100 μg of substrate, and other additions as indicated in 0.05 M ethylenediamine-acetate buffer, pH 7.0, in a final volume of 20 μl. The incubation mixtures were spotted in Whatman no. 1 paper and subjected to chromatography in isobutyric acid, 1 M NH₃ (5/3, 16807
FIG. 1. Fractionation of crude *F. heparinum* extracts by DEAE-cellulose. Supernatant from 100,000 x g (15 ml) prepared from induced cells of *F. heparinum* were applied to a DEAE-cellulose column (2 x 20 cm) previously equilibrated with 0.1 M ethylenediamine-acetate buffer, pH 7.0. The proteins (absorbance at 280 nm) were eluted (10-ml fractions) with 0.1 and 0.5 M of buffer as indicated. Aliquots (10 

FIG. 2. Fractionation of enzymes by Bio-Gel chromatography. Aliquots (10 

FIG. 3. Further purification of the enzymes by cation-exchange chromatography. Pools of the fractions from the Bio-Gel column were concentrated by pressure dialysis, resuspended in 0.01 M ethylenediamine-acetate buffer, pH 7.0, and applied to a column of carboxymethylcellulose (1 x 10 cm). The column was then eluted with ethylenediamine-acetate buffer at the molarities indicated. The resulting fractions (10 ml) were assayed for the enzymatic activities as described in Fig. 2. Pools of the Bio-Gel fractions (Fig. 2) applied to the carboxymethyl column: 90-100 (A); 115-120 (B); 128-140 (C).

| Table I | Purification of heparinase, heparitinase II, and heparitinase I |
|---------|-------------------------------------------------------------|
| Step    | Total units \( \text{mg} \) | Protein \( \text{units/mg} \) | Protein protein | Purification \( \text{fold} \) |
| Crude   | 361 | 0.18 | 1 |
| Heparinase | 125 | 0.53 | 2.9 |
| Heparitinase II | 91.0 | 0.53 | 171.7 |
| Heparitinase I | 77.0 | 0.23 | 378 |
| DEAE-cellulose | 66.5 | 0.18 | 16.2 |
| Heparinase | 114.1 | 2.5 | 45.6 |
| Heparitinase II | 89.6 | 1.3 | 68.9 |
| Heparitinase I | 77.0 | 0.23 | 304 |

\( ^{a} \text{One unit} = 1 \mu \text{mol of unsaturated products formed/h, at pH 7.0, 30} \text{C.} \) 

\( ^{b} \text{Due to the presence of glycuronidas, the total activity of the enzymes present in the crude extract (supernatant of 100,000 x g) and in the DEAE-cellulose fractions was calculated by the total activity of each enzyme present in the Bio-Gel fractions. It was assumed that no loss of enzyme activity occurred during the DEAE and Bio-Gel fractionation.} \)
mixtures by spectrophotometry at 230 nm in 0.1 M KCl-HCl buffer, pH 2.0. For the individual quantitation of the products, the incubation mixtures were subjected to paper chromatography and the unsaturated di- and tetrasaccharides located by UV lamp, eluted with water, and measured at 230 nm in 0.1 M KCl-KCl buffer or by the carbazole reaction (17).

13C NMR Spectroscopy.—The 13C spectra of the heparan sulfate disaccharides were recorded at 35 °C with a Varian CT-20 Fourier transform spectrometer (at 20 MHz) equipped with a 5-mm probe. The unsaturated disaccharides derived from heparan sulfate (50–80 mg) were dissolved in 0.4 ml of H2O (99.7% 2H) and measured at 20 °C with a Varian XL-300 spectrometer at 75 MHz.

Other Methods.—Protein was determined by the Lowry method (18). N-Desulfated, N-acetylated heparin, and heparan sulfate were prepared by methods previously described (19). Hexosamine was determined after acid hydrolysis (4 M HCl, 100 °C; 6 h) by the Rondle-Morgan reaction (20). Labile and total sulfate were measured by a method previously described (21) and acetyl residues by gas chromatography (14). The orcinol/carbazol ratios of the different heparins according to Fransson et al. (22). The quantitation of iduronic and glucuronic acid in the different glycosaminoglycans was performed essentially as described by Kosakai and Yosizawa (23).

RESULTS

Purification of the Enzymes.—The fractionation of the 100,000 × g supernatant of F. heparinum extracts by DEAE-cellulose column chromatography is shown in Fig. 1. This step does not fractionate the enzymes acting upon heparin and heparan sulfate but removes most of the contaminating proteins together with possible proteolytic enzyme(s) which render the glycosaminoglycanases completely stable for at least 1 year at 5 °C. The fractions containing the enzymatic activities were pooled, concentrated, and fractionated on Bio-Gel A-0.5 m. The individual fractions were assayed with heparin and heparan sulfate (Fig. 2). Three enzymatic activities acting upon heparin and heparan sulfate are completely resolved from each other. The activity eluted between fractions 130–140 acts only upon heparan sulfate. This enzyme was earlier defined as heparitinase I (7). The activity present in fractions 113–122 acts only upon heparin from lung and intestinal mucosa and was defined as the heparinase (6). Finally, the activity present in fractions 90–100 acts upon heparan sulfate and partially upon heparin from bovine intestinal mucosa. Fig. 2 shows that when heparin from bovine lung is used for the assay no significant degradation of this substrate is observed. This enzyme was previously described as heparitinase II (7).

These fractions were also tested with other substrates (Fig. 2). The trisulfated disaccharide (ΔU,2S-GlcNS,GS) is degraded to disulfated disaccharide (ΔU-GlcNS,GS) by the fractions containing the heparitinase II which indicates the presence of sulfoiduronate sulfatase previously named disaccharide sulfosterase (6). Also the disulfated disaccharide (ΔU-GlcNS,GS) is degraded by GlcNS,GS by the fractions containing the heparinase indicating the presence of a glycuronidase. In order to separate these enzymes, the fractions 90–100 (Fig. 2) were combined and fractionated on carboxymethylcellulose. The disaccharide sulfosterase is eluted at 0.03 M, and the heparitinase II is eluted at 0.05 M concentration of ethylenediamine-acetate buffer (Fig. 3). Likewise, the heparinase is eluted together with the glycuronidase, and the heparitinase I overlaps with the chondroitinase AC in the Bio-Gel column (Fig. 2). The fractions containing these enzymatic activities were pooled, applied to a carboxymethylcellulose column, and eluted with increasing concentrations of ethylenediamine-acetate buffer. The heparinase is eluted at 0.05 M whereas the glycuronidase is not retained by the column. The heparitinase I is eluted at 0.03 M and the chondroitinase AC between 0.05 and 0.07 M ethylenediamine-acetate buffer (Fig. 3).

Table I shows the recovery and degree of purification of the enzymes by these combined procedures. Assuming no loss of activity by the DEAE and Bio-Gel fractionation the heparinase, heparitinase II, and heparitinase I were purified 378-, 950-, and 1855-fold, respectively, by these combined procedures.

The purified enzymes were incubated with all the sulfated disaccharides listed in Table III as well as 4- and 6-sulfated disaccharides prepared from chondroitin sulfates. None of the

Heparitinases from F. heparinum

Chemical analyses of natural and modified heparins and heparan sulfates used as substrates for the enzymes

| Substrate | Uronic acid | Sulfate | N-acetyl groups | Iduronic acid | Glucuronic acid |
|-----------|-------------|---------|----------------|--------------|----------------|
| Heparin   |             |         |                |              |                |
| Lung      | 1.40        | 1.02    | 2.80           | 0.02         | 80 (>90)<sup>a</sup> |
| Mucosa    | 1.60        | 0.85    | 2.60           | 0.10         | 65 (73)        | 20 (35) |
| Heparan sulfate |         |         |                |              |                |
| Pancreas  | 1.55        | 0.56    | 1.20           | 0.45         | 6 (<5)<sup>b</sup> |
| Lung      | 1.60        | 0.51    | 1.05           | 0.52         | >2             | <2    |
| N-Desulfated, N-acetylated heparin | 1.45 | 0.15 | 1.80 | 0.81 |
| N-Desulfated heparan sulfate | 1.55 | 0.16 | 0.65 | 0.76 |

<sup>a</sup> NMR determination (Ref. 24).
<sup>b</sup> NMR determination (25).
FIG. 4. Unsaturated products formed from heparin and heparan sulfate by action of the different enzymes in function of time. 100 µg each of lung heparin, intestinal heparin, pancreas heparan sulfate, N-acetylated heparin, and N-acetylated heparan sulfate were incubated with 0.05 unit of heparinase, heparitinase I, heparitinase II, and heparitinases I + heparitinase II, as indicated, in 0.05 M ethylenediamine-acetate buffer, pH 7.0, at 30 °C in a final volume of 20 µl for different times as indicated in the figure. The heparinase was incubated with the different substrates in the presence of 0.02 M MnCl₂. The UV absorbing products were determined as described in Fig. 2. HS, heparan sulfate; HEP, heparin; N-ACETYL HS, N-desulfated, N-acetylated heparan sulfate; N-ACETYL HEP, N-desulfated, N-acetylated heparin.

disaccharides were desulfated or hydrolyzed by the enzymes indicating that they were essentially free from sulfatases and glycuronidases (results not shown).

Chemical Data of the Substrates—The chemical analyses of the substrates used for the studies of the specificities of the enzymes are shown in Table II. The heparin from lung tissue contains 80–90% of the uronic acid residues as iduronic acid and the intestinal mucosal heparin between 65–75% of this uronic acid (24). The estimation of the iduronic acid content of the two heparins (chemical analyses and nuclear magnetic resonance) gave similar results. Conversely, the heparan sulfates used in these experiments contain at least 94% of glucuronic acid, measured by those methods (25).

Substrate Specificity of Heparinase—Bovine lung and intestinal heparins are extensively degraded by heparinase (90 and 60% yields, respectively) as shown in Fig. 4 and Table III. Since heparin contains 65–80% of iduronic acid residues and is extensively degraded it is reasonable to assume that heparin acts upon glucosaminido-iduronic acid linkages. Conversely, pancreas and lung heparan sulfate, which differs from heparin by the almost exclusive presence of glucuronic acid residues in the molecule (Table II), is a very poor substrate for the enzyme (Fig. 4).

The N-sulfate residues in the heparin molecule seem to be essential for the heparinase action since substitution of this sulfate by acetyl groups renders the heparin molecule resistant to the enzyme (Fig. 4). The C-6 sulfate substitution of the hexosamine moiety is not an impediment for the enzyme action since ΔU-2S-GlcNS is formed from heparin by the heparinase (Table III). Regarding the C-2 sulfate substitution of the uronic acid moiety, no definite information could be obtained with the substrates used. According to other authors (12, 13) the C-2 substitution of the uronic acid moiety of heparin is essential for the heparinase action. The tetrasaccharides also released from heparin by action of heparinase in high yields (Fig. 5, Table III) contain a non-sulfated internal uronic acid residue (6). This could suggest that the hepa

![FIG. 5. Chromatography of the products formed from heparin by action of the heparinase and heparitinases. 100 µg of intestinal and lung heparins were incubated with heparitinase II (I), heparinase (2), and heparitinase I (3) for 8 h, as described in Fig. 4. The incubation mixtures were chromatographed in Whatman no. 1 paper using isobutyric acid, 1.25 M NH₃ (5/3.6, v/v) during 48 h. The compounds were visualized by toluidine blue staining. ΔTetra, mixture of unsaturated sulfated tetrasaccharides.](http://www.jbc.org/)

![Table III](http://www.jbc.org/)

| Enzyme          | Products formed (µg/100 µg substrate) |
|-----------------|---------------------------------------|
|                 | Heparin | Heparan sulfate |
| Heparinase      |         |                |
| ΔTetra          | 45.6    | 37.5           |
| ΔU,2S-GlcNS,6S  | 45.2    | 36.1           |
| ΔU,2S-GlcNS     | 1.5     | 2.1            |
| ΔU-GlcNS        | ND      | 1.2            |
| GlcNS,6S        | 6.5     | 3.0            |
| Heparitinase II |         |                |
| ΔU,2S-GlcNS,6S  | 2.3     | 7.4            |
| ΔU-GlcNS,6S     | 2.0     | 12.5           |
| ΔU-GlcNS        | 0.7     | 3.2            |
| ΔU-GlcNAc,6S    | ND      | 20.5           |
| ΔU-GlcNAc       | ND      | 4.1            |
| GlcNS,6S        | ND      | 5.2            |
| Heparitinase I  |         |                |
| ΔU-GlcNS        | ND      | 14.2           |
| ΔU-GlcNAc,6S    | ND      | 19.1           |
| ΔU-GlcNAc       | ND      | 24.5           |

ND, not detected.

ΔU-GlcNS and ΔU,2S-GlcNS have the same chromatographic migration (Fig. 5). They were distinguished from each other by their differential susceptibility to glycuronidase and disaccharide sulfatase. ΔU-GlcNS,6S is a substrate for glycuronidase producing upon degradation GlcNS,6S. Conversely, ΔU,2S-GlcNS is desulfated by the sulfoesterase producing ΔU-GlcNS. For further details see Ref. 27.
FIG. 6. Disaccharide products formed from heparan sulfate and N-acetylated heparan sulfate by action of enzymes. Heparan sulfate and N-desulfated, N-acetylated heparan sulfate were incubated with heparitinase I (1), heparitinase II (2), heparitinase I + heparitinase II (3), and heparinase (4) in the conditions described in Fig. 4. The products were analyzed as described in Fig. 5 except that the chromatography was run for 24 h.

Fig. 7. $^{13}$C NMR spectra of heparan sulfate disaccharides. U, uronic acid; H, hexosamine; HO-CH$_3$, internal standard of methanol.

Heparitinase does not act upon non-sulfated uronic acid-glucosaminido linkages. Nevertheless, recent analysis of the tetrasaccharides has shown that the uronic acid is indeed glucuronic acid as previously suggested (6).

A monosaccharide, GlcNS,6S, is also formed from the heparins by action of heparinase in about 3–6% yield. Since no saturated disaccharides were detected as degradation products and the enzyme is free of sulfatases and glycuronidase, it is reasonable to suppose that this monosaccharide is released from the non-reducing end of the molecules by the action of the enzyme.

Specificity of Heparitinase I—Heparitinase I degrades extensively unmodified and N-acetylated heparan sulfate with formation of UV absorbing products. No activity could be detected upon heparin or N-acetylated heparin (Fig. 4). The type of disaccharide products formed by action of the enzyme upon the two heparan sulfates is shown in Fig. 6 and Table III. AU-GlcNS and AU-GlcNAc are the main products formed by action of this enzyme upon heparan sulfate. A decrease of AU-GlcNS with a correspondingly increase of AU-GlcNAc is observed when N-acetylated heparan sulfate is incubated with heparitinase I (Fig. 6). Note that no or negligible amounts of AU-GlcNAc,6S and AU-GlcNS,6S are formed by action of heparitinase I upon heparan sulfate or N-acetylated heparan sulfate. Since the heparan sulfate used contains more than 90% of its uronic acid residues as glucuronic acid and that the N-acetylated heparin (which contains mostly iduronic acid) is not a substrate for the enzyme it is suggestive that heparitinase I acts upon glucosaminido-glucuronic acid linkages. The findings that no or negligible amounts of AU-GlcNS,6S and AU-GlcNAc,6S are formed by action of heparitinase I from both natural and modified heparan sulfates suggest that the sulfate at the C-6 position of the hexosamine moiety is impeditive for the enzyme action.

Specificity of Heparitinase II—Except for lung heparin all the other substrates used in this study were degraded by heparitinase II (Fig. 4). Nevertheless, the extent of degradation and the yield of the disaccharide products formed from the different substrates were quite variable. Thus, intestinal heparin yields 23% of disaccharides. The remaining oligosaccharide was only degraded by heparinase yielding the characteristic products (Fig. 5, Table III). Heparitinase II acts upon heparan sulfate producing the four main types of disaccharides present in these molecules. Nevertheless, the yield of AU-GlcNac is much lower than that obtained by action of
5 and 6 except that the commercial heparinase (0.1 unit) was incubated with lung heparin (upper panel) and commercial heparitinase with pancreas heparan sulfate (lower panel) in 3 mM calcium acetate, pH 7.0, for different periods of time as indicated. St. 1, ATetra; St. 2, AU,2S-GlcNS,6S; St., mixture of AU-GlcNS,6S and GlcNS.

heparitinase I (Table III). Addition of excess of heparitinase II or longer incubation periods did not increase the yield of this disaccharide. Only the combination of the two heparitinases degrades completely the heparan sulfate (Figs. 4 and 6). The heparitinase II also acts upon the tetrasaccharides formed by the action of either heparitinase I or heparinase. These combined data suggest that heparitinase II removes preferentially glucuronic acid-containing disaccharides from the heparan molecule.

FIG. 9. Products formed from lung heparin and pancreas heparan sulfate by the action of commercial heparinase and heparitinase. The experiments were performed as described in Figs. 5 and 6 except that the commercial heparinase (0.1 unit) was incubated with lung heparin (upper panel) and commercial heparitinase with pancreas heparan sulfate (lower panel) in 3 mM calcium acetate, pH 7.0, for different periods of time as indicated. St. 1, ATetra; St. 2, AU,2S-GlcNS,6S; St., mixture of AU-GlcNS,6S and GlcNS.

Product Identification—All these products have been extensively analyzed (6, 10, 14) and their proposed structures have been confirmed by 1H and 13C nuclear magnetic resonance (26, 27).

The 13C spectra of the anomeric carbons of bovine intestinal heparin and its oligosaccharide produced by action of heparitinase II is shown in Fig. 8. The signal at 103 ppm which corresponds to the C-1 of the glucuronic acid is significantly decreased in the oligosaccharide when compared with the intact heparin. This is in agreement with the suggestion that heparitinase II removes preferentially glucuronic acid-containing disaccharides from the heparin molecule.

Substrate Specificity of the Commercial Heparinase and Heparitinase—The products formed by action of the commercially available heparinase and heparitinase upon heparin and heparan sulfate are shown in Fig. 9. At short incubation periods, the heparinase produces tetrasaccharides, tri- and disulfated disaccharides from bovine lung heparin. At longer incubation periods there is a decrease of trisulfated disaccharide with a corresponding increase of disulfated disaccharide and glucosamine 2,6-disulfate (GlcNS,6S). This indicates that the heparinase is contaminated with the disaccharide sulfosterase (an enzyme that removes a sulfate from the C-2 position of the uronic acid moiety) and with glycuronidase (27).

The commercially available heparinase produces upon heparan sulfate-disulfated disaccharide, N-sulfated disaccharide, and glucosamine 2,6-sulfate. It resembles the heparitinase II described in this paper but with low activity and contaminated with glycuronidase. Also both enzymes show a weak chondroitinase activity (results not shown).

DISCUSSION

Functionally purified heparinase, heparitinase I, and heparitinase II free of sulfatases, glycuronidases, and chondroitin sulfate lyases were prepared in high yields by the combination of molecular sieving and ion-exchange chromatography. This methodology also allows the preparation of disaccharide sulfosterase, glycuronidase, and chondroitinase AC free of the other mucopolysaccharidases. Higher yields of enzymes than the ones previously described (6) could be obtained by the present method.

The mode of action of the heparinase upon the different glycosaminoglycans is still compatible with the earlier propositions that the enzyme is an eliminase acting upon glucosaminido α,1,4-L-iduronic acid linkages of the heparin molecule (6, 8, 9, 12). Thus, bovine lung heparin which contains a large proportion of iduronic acid is more extensively degraded than the intestinal one which contains relatively higher amounts of glucuronic acid residues. The present results also show that replacement of the sulfate radicals from the amino groups of the hexosamines by acetyl groups renders the heparin mole-
cule completely resistant to degradation by the heparinase.

No evidence for the presence of disaccharides containing sulfate at the C-5 position of the hexosamine, that could theoretically be formed by the action of heparinase or heparitinase II, were obtained. These disaccharides represent less than 0.3% of the heparin molecule (29) and could not be detected by the present methodology.

The lung and pancreas heparan sulfates used in the present study are poor substrates for the heparinase. The small amounts of UV absorbing materials after heparinase upon the heparan sulfate prepared from pancreas could be an indication that a few iduronic acid residues are present in this heparan which is mostly constituted of glucuronic acid residues as revealed by chemical analyses and NMR studies. Both heparan sulfates are extensively degraded by each one of the heparitinasea. N Acetylated and N sulfated disaccharides are the exclusive products of the heparitinase I whereas disulfated, N-sulfated, and N-acetylated 6-sulfated disaccharides are the major products of heparitinase II. Small amounts of N-acetylated disaccharide are also formed from the heparan sulfate by heparitinase II. This suggests that this enzyme is somewhat nonspecific having a lower affinity for the N-acetylated regions. The total degradation of the heparan sulfate is achieved only by the combined action of the two heparitinases (Fig. 5, Table III). The heparitinase II also acts extensively upon the intestinal heparin releasing considerable amounts of tri- and disulfated disaccharides and an oligosaccharide which accounts for 75% of the original heparin molecule. This oligosaccharide is in turn degraded by heparinase producing Δtetra and trisulfated disaccharide. It thus seems that heparin contains two distinct regions, one of them composed of mostly iduronic acid-containing disaccharides and another composed of glucuronic acid-containing disaccharides. This was also observed by 13C NMR spectra of the oligosaccharide obtained from heparin after heparitinase II degradation which shows a significant decrease of the anomic carbons of glucuronic acid. This glucuronic acid region seems to be more extensive in the intestinal heparin than in the lung heparin as judged by the amount of products formed from the two heparins by action of heparitinase II (Table III) and NMR spectroscopy where glucuronic acid is a minor component of this type of heparin (24).

These combined results suggest that heparitinase II acts preferentially upon N-6-sulfated and/or N-acetylated, 6-sulfated glucosaminido-α-1,4-glucuronic acid linkages. If this is the case the trisulfated disaccharide obtained from heparin by action of this enzyme was derived from the heparin region containing sulfated glucuronic acid residues. This glucuronic acid-containing trisulfated disaccharide has been recently reported by Fedarco and Conrad (30) in a peculiar intracellular heparan sulfate from nuclei of hepatocytes. In favor of this hypothesis are the findings that the sulfated tetrasaccharides (which contains an internal glucuronic acid residue) formed from heparin by the action of heparinase are excellent substrates for the heparitinase II (6). An alternative possibility would be that heparitinase II is again somewhat nonspecific acting preferentially upon the less sulfated regions of the intestinal heparin. Its lack of activity upon the lung heparin and the heparinase-sensitive region of the intestinal heparin would be related to the special conformation of these more sulfated regions. It is clear that more model compounds and a better understanding of the structure of the substrates are needed to define the specificity of heparitinase II.

Except for a few details, the specificity of heparinase and heparitinase II described in this paper agrees with the specificities reported by other authors (8–13). Also, except for the contamination with glycuronidase, sulfatase, and chondroitin sulfate lyase, the commercially available heparinase and heparitinase, correspond in our hands, to the heparinase and heparitinase II described in this paper.

The other commercially available enzymes, namely, heparinase II and heparitinase III were not tested with the present methodology. Nevertheless, as judged by the data presented by other authors (11, 13) these two enzymes seem to differ from the heparitinase I and II described in this paper.

Finally, the present results confirm the previous studies that commercial bovine lung heparin has a quite homogeneous structure where more than 90% of its structure is composed of octasaccharide-repeating units (6). This homogeneity has also been stressed by Gatti et al. (24) through 1H and 13C NMR studies of this type of heparin.

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REFERENCES

1. Linker, A., Hoffman, P., Meyer, K., Sampson, P., and Korn, E. D. (1969) J. Biol. Chem. 235, 3061–3065
2. Michelacci, Y. M., and Dietrich, C. P. (1976) J. Biol. Chem. 251, 1154–1158
3. Morbelacci, V. M., and Dietrich, C. P. (1975) Biochem. J. 151, 121–129
4. Michelacci, Y. M., Horton, S. P. Q., and Poblacion, C. A. (1987) Biochem. Biophys. Acta 923, 291–301
5. Dietrich, C. P. (1989) Biochemistry 8, 3342–3347
6. Silva, M. E., and Dietrich, C. P. (1975) J. Biol. Chem. 250, 6941–6946
7. Silva, M. E., Dietrich, C. P., and Nader, H. B. (1976) Biochim. Biophys. Acta 437, 129–141
8. Linker, A., and Hovingh, P. (1977) Fed. Proc. 36, 43–46
9. Otonami, N., Kikuchi, M., and Yosizawa, Z. (1981) Carbohydr. Res. 88, 291–303
10. Linhardt, R. J., Grant, A., Cooney, C. L., and Langer, R. (1982) J. Biol. Chem. 257, 7316–7321
11. McLean, M. W., Long, W. F., Moffat, C. F., and Williamson, F. B. (1987) Proceedings of the 30th International Symposium of Glycoconjugates, p. 341, September 25–29, Houston, TX
12. Rice, K. G., and Linschau, R. J. (1989) Carbohydr. Res. 190, 219–233
13. Turnbull, J. E., and Gallaguer, J. T. (1990), Biochem. J. 265, 715–724
14. Dietrich, C. P., and Nader, H. B. (1974) Biochim. Biophys. Acta 343, 34–40
15. Dietrich, U. P., Nader, H. B., and Straus, A. H. (1983) Biochim. Biophys. Res. Commun. 111, 865–871
16. Dietrich, C. P. (1986) Biochem. J. 108, 647–654
17. Töster, Z. (1947) J. Biol. Chem. 167, 189–186
18. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
19. Silva, M. E., and Dietrich, C. P. (1973) Biochimie 55, 1101–1106
20. Rondle, C. J. M., and Morgan, W. T. J. (1956) Biochem. J. 61, 566–568
21. Nader, H. B., and Dietrich, C. P. (1977) Anal. Biochem. 78, 112–118
22. Fransson, L. A., Sjöberg, L., and Havsmark, B. (1980) Eur. J. Biochem 106, 69–69
23. Rosalki, M., and Yosizawa, Z. (1970) Anal. Biochem. 89, 419–427
24. Gatti, G., Casu, B., Hamer, G. K., and Perlin, A. S. (1979) Macromolecules 12, 1001–1007
25. Nader, H. B., Perrein, T. M. P., Chavante, S. P., Toma, L., Dietrich, C. P., Casu, B., and Torri, G. (1988) Carbohydr. Res. 184, 292–300
26. Perlin, A. S., Mackie, D. M., and Dietrich, C. P. (1971) Carbohydr. Res. 15, 193–191
27. Dietrich, C. P., Michelacci, V. M., and Nader, H. B. (1980) in Mechanisms of Carbohydrate Polymerisation and Depolymerisation (Marshall, J. J., ed) pp. 317–329, Academic Press Inc., New York
28. Dietrich, C. P., Nader, H. B., Moras, T. C., Porcionatto, M. A., Casu, B., and Torri, G. (1990) XIII International Carbohydrate Symposium, p. 163, Cornell University, Ithaca, NY
29. Lindahl, U., Feingold, D. S., and Roden, L. (1986) Trends Biochem. Sci. 11, 221–225
30. Pedarco, N. S., and Conrad, H. E. (1986) J. Cell Biol. 102, 587–599
Purification and substrate specificity of heparitinase I and heparitinase II from Flavobacterium heparinum. Analyses of the heparin and heparan sulfate degradation products by 13C NMR spectroscopy.
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