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New insights on the specificity of heparin and heparan sulfate lyases from *Flavobacterium heparinum* revealed by the use of synthetic derivatives of K5 polysaccharide from *E. coli* and 2-O-desulfated heparin

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The capsular polysaccharide from *E. Coli,* strain K5 composed of …→4)β-D-GlcA(1→4)α-D-GlcNAc(1→4)β-D-GlcA (1→…, chemically modified K5 polysaccharides, bearing sulfates at C-2 and C-6 of the hexosamine moiety and at the C-2 of the glucuronic acid residues as well as 2-O desulfated heparin were used as substrates to study the specificity of heparitinases I and II and heparinase from *Flavobacterium heparinum.* The natural K5 polysaccharide was susceptible only to heparitinase I forming ΔU-GlcNAc. N-deacetylated, N-sulfated K5 became susceptible to both heparitinases I and II producing ΔU-GlcNS. The K5 polysaccharides containing sulfate at the C-2 and C-6 positions of the hexosamine moiety and C-2 position of the glucuronic acid residues were susceptible only to heparitinase II producing ΔU-GlcNS,6S and ΔU,2S-GlcNS,6S respectively. These combined results led to the conclusion that the sulfate at C-6 position of the glucosamine is impeditive for the action of heparitinase I and that heparitinase II requires at least a C-2 or a C-6 sulfate in the glucosamine residues of the substrate for its activity. Iduronic acid-2-O-desulfated heparin was susceptible only to heparitinase II producing ΔU-GlcNS,6S. All the modified K5 polysaccharides as well as the desulfated heparin were not substrates for heparinase. This led to the conclusion that heparitinase II acts upon linkages containing non-sulfated iduronic acid residues and that heparinase requires C-2 sulfated iduronic acid residues for its activity.

Keywords: **heparan sulfate lyases, specificity; heparinase, specificity; sulfated K5 polysaccharides; heparan sulfate, structure**

Abbreviations: **The abbreviations used were: ΔU,2S-GlcNS,6S, O-(4-deoxy-hex-4-enopyranosyluronic acid 2-sulfate)- (1→4)-2-sulfamino-D-glucose 6-sulfate; ΔU,2S-GlcNS, O-(4-deoxy-hex-4-enopyranosyluronic acid 2-sulfate)- (1→4)-2-sulfamino-D-glucose; ΔU-GlcNS,6S, O-(4-deoxy-hex-4-enopyranosyluronic acid)- (1→4)-2-sulfamino-D-glucose 6-sulfate; ΔU-GlcNS, O-(4-deoxy-hex-4-enopyranosyluronic acid)-(1→4)-2-sulfamino-D-glucose; ΔU-GlcNAc,6S, O-(4-deoxy-hex-4-enopyranosyluronic acid)-(1→4)-2-acetamido-D-glucose 6-sulfate; ΔU-GlcNAc, O-(4-deoxy-hex-4-enopyranosyluronic acid)-(1→4)-2-acetamido-D-glucose; GlcNS,6S, 2-sulfamino-D-glucose 6-sulfate**

Introduction

The heparinase and heparitinases from *Flavobacterium heparinum* have been extremely useful tools for the determination of the structural diversity of heparin and heparan sulfates [1–8]. Nevertheless many details of their structures have been only indirectly inferred due to the absence of model substrates for the proper determination of the enzyme specificities. The isolation of a capsular polysaccharide from a pathogenic *E. Coli,* strain K5 [9], composed of …→4)β-D-glucuronic acid (1→4) α-D-N-acetylglucosamine (1→4) glucuronic acid (1→…, which has the same structure of one of the putative oligosaccharide domains of

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heparan sulfate [2,3,6,7], led to the preparation of several modified polymers bearing sulfates at C-2 and C-6 positions of the hexosamine moiety as well as at the C-2 position of the glucuronic acid residues [10]. These derivatives would theoretically mimic the other proposed oligosaccharide domains of heparan sulfate as well as heparin.

This work reports new information on the specificity of heparitinase I, heparitinase II and heparinase through the use of several modified K-5 polysaccharides as well as a 2-O desulfated heparin as substrates.

Materials and methods

Substrates, enzymes and materials

Heparin from bovine lung and intestinal mucosa and heparan sulfate from bovine pancreas were gifts from Dr. P. Bianchini (Opocrin Research Laboratories, Modena, Italy). The K5 polysaccharide derivatives were prepared as previously described [10]. Heparitinase I (Heparinase III?, see Discussion), heparitinase II (heparinase II, no assigned EC number), heparinase (heparinase I, EC 4.2.2.7), disaccharide sulfoesterase and glycuronidase were prepared from induced *F. heparinum* cells [11,12]. Unsaturated disaccharide standards were prepared from heparan sulfate and heparin by action of the heparitinases and heparinase and characterized as previously described [12,13].

Preparation of 2-O-desulfated heparin

Heparin desulfated at C-2 position of the uronic acid residues was prepared essentially as described by Rej and Perlin [14]. Briefly, 100 mg of bovine lung heparin, with a hexosamine/sulfate ration of 1:2.75 was dissolved in 10 ml of water and the solution stirred in the presence of 10 ml of Dowex 50-X8 for 30 minutes at 5 °C. The solution was then decanted. The resin was washed once with 10 ml of water. The two combined solutions were neutralized with pyridine. One volume of 0.2 N NaOH was added to the solution which was then frozen and lyophilized. The powder was then resuspended in 10 ml of water, dialyzed for 72 hours against water and lyophilized. Sixty to 70 mg were obtained in different preparations. The hexosamine/sulfate ratio of this desulfated heparin was 1:2.1.

Enzymatic degradation

The products formed from the K5 polysaccharides as well as heparan sulfate and heparins by action of heparitinase I, heparitinase II and heparinase were identified as follows: A mixture of 0.1 U of enzymes, 100 μg of subtrates and other additions as indicated, in 0.05 M ethylenediamine-acetate buffer, pH 7.0 in a final volume of 30 μl were incubated for 8 hours at 30 °C. The disaccharide products formed by the action of the enzymes were analyzed by HPLC in a 0.95 × 25 cm SAX ion exchange resin (Dupont) column using a NaCl gradient (0.01–2 M) with a flow of 1 ml/min and monitored at 230 nm. The products were also analysed by paper chromatography in Whatman nr. 1 using isobutyric acid: 1 M NH_3, 5/3, v/v for 48 hours. The unsaturated products formed were detected by short wave UV lamp and silver nitrate staining. They were quantitated by hexosamine, sulfate and uronic acid determinations after elution of the chromatograms. The relative position of the sulfates on the disaccharides formed by action of heparitinase II upon the different substrates were ascertained by their susceptibility to glycuronidase and disaccharide sulfoesterase from *Flavobacterium heparinum* [11,15].

Other methods

Hexosamine was determined after acid hydrolysis (4 M HCl, 100 °C, 6 hours) by the Rondle-Morgan reaction [16]. Uronic acid content was determined by the Dische carbazole reaction [17]. Labile and total sulfate were measured by a method previously described [18] and acetyl residues by gas chromatography [19].

Results

E Coli K5 modified polysaccharides as substrates for heparitinases and heparinase

The major repeating disaccharide sequences of the natural and synthetic polysaccharides and the respective disaccharide products formed by action of heparitinase I, heparitinase II and heparinase are shown in Figure 1. The unmodified K5 (A) is susceptible to heparitinase I forming ΔU-GlcNAc. This disaccharide is also formed from heparan sulfate by the action of the enzyme. Only trace amounts of this disaccharide are formed by action of heparitinase II upon both substrates. Higher amounts of heparitinase II did not increase the amounts of the N-acetylated disaccharide formed either from the natural K5 or heparan sulfate (results not shown).

N-deacetylation followed by N-sulfation of K5 (B) renders it susceptible to both heparitinases whose main product is ΔU-GlcNS. The modified K5 containing sulfate at the C-2 and C-6 position of the hexosamine moiety (C) is susceptible only to heparitinase II producing ΔU-GlcNS,6S. A N-sulfated/N-acetylated and partially C-6 sulfated K5 (D) is susceptible to both heparitinases, forming by action of heparitinase I, ΔU-GlcNAc and ΔU-GlcNS, and by heparitinase II, ΔU-GlcNS and ΔU-GlcNAc,6S. We can thus conclude that the sulfate at C-6 position of the glucosamine is impeditive for the action of heparitinase I and that the natural non sulfated K5 is not a substrate for heparitinase II. These results also suggest that heparitinase II requires at least a C-2 or a C-6 sulfate in the glucosamine residues of the substrate for its activity.

A most interesting result was obtained with the modified K5 with sulfate residues at C-2 and C-6 of the glucosamine

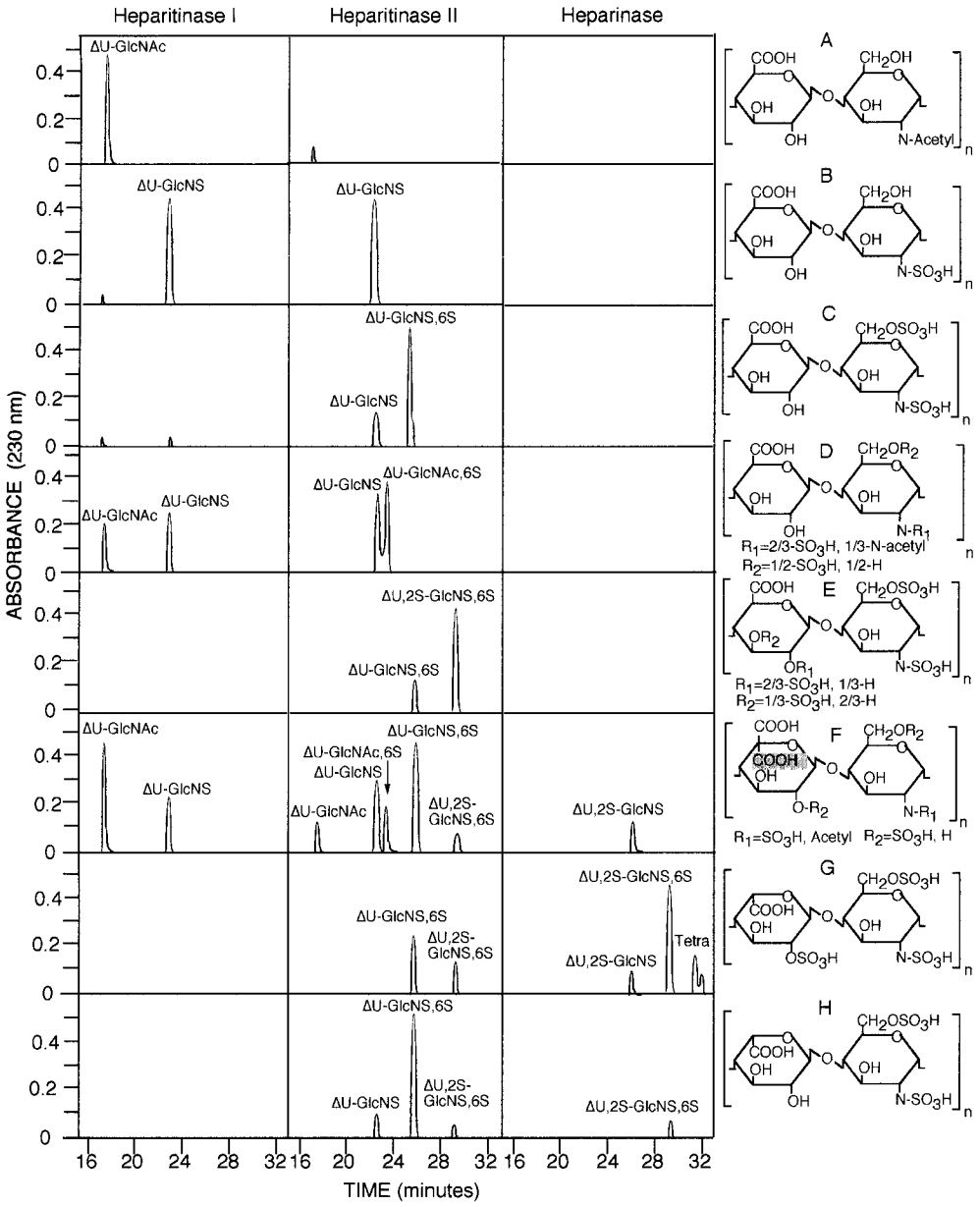

Figure 1. Identification of disaccharide products by HPLC formed by action of heparitinase I, heparitinase II and heparinase upon different substrates. One hundred μg of of the different polysaccharides, whose main disaccharide unit is indicated, were incubated with 0.1 U of heparitinase I or 0.1 unit of heparitinase II or 0.1 unit of heparinase in 1,2-diaminoethane-acetate, pH 7.0 in a final volume of 20 µl for 8 hours. Aliquots were applied to HPLC. The eluted products were monitored at 230 nm. A, unmodified K5 polysaccharide; B, N-deacetylated, N-sulfated K5 polysaccharide; C, N-deacetylated, N,6-disulfated K5 polysaccharide; D, partially N-deacetylated, N,6-disulfated K5 polysaccharide, E, N-deacetylated, N, 6-disulfated, 2,O-sulfated K5 polysaccharide; F, bovine pancreas heparan sulfate; G, bovine mucosa heparin; H, 2,O-desulfated heparin; tetra, heparin tetrasaccharides. The shadow in the carboxyl group of F indicates that part of the uronic acid residues of the disaccharides are iduronic acids.

moiety and C-2 or C-3 of the glucuronic acid residues (E). This compound was susceptible to heparitinase II producing ΔU,2S-GlcNS,6S and smaller amounts of ΔU-GlcNS,6S. Note that the compound as well as all the modified K5 are not substrates for heparinase. The latter enzyme produces the unsaturated trisulfated disaccharide only from heparin (G). This leads to the suggestion that heparinase only acts on substrates containing 2-O sulfated iduronic acid residues as it has been previously proposed [8,12,20–22] and that heparitinase II acts upon substrates containing 2-O-sulfated glucuronic acid residues.

Small amounts of ΔU,2S-GlcNS,6S are also formed from heparin (G) by action of heparitinase II (Fig. 1). This is suggestive that heparin may contain some glucuronic acids sulfated at C-2 position. This is reinforced by the findings that mollusc heparins which, compared to mammalian

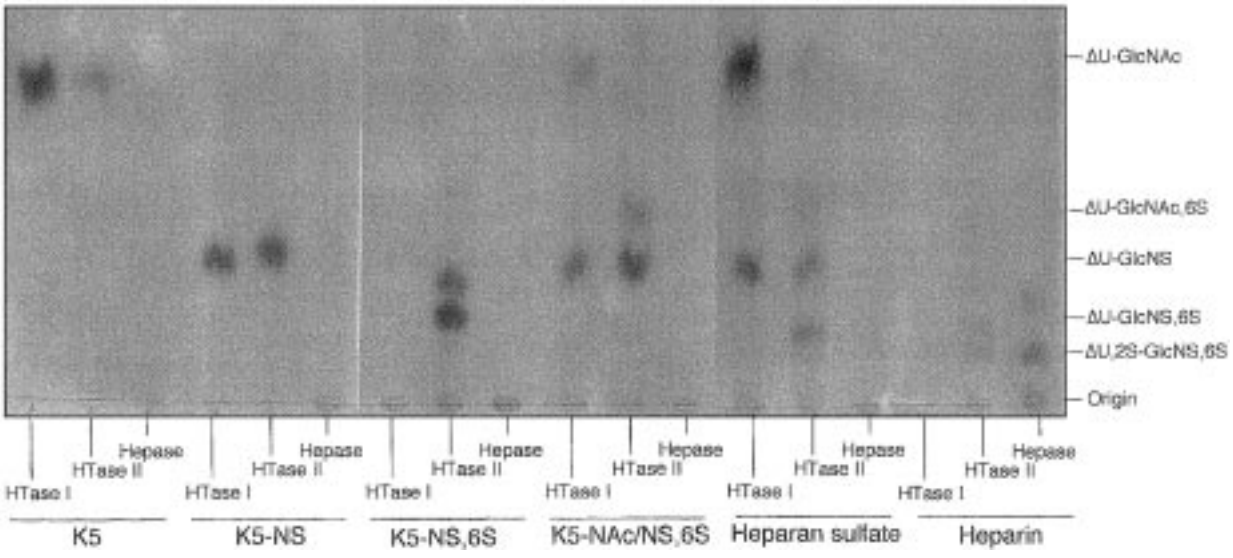

Figure 2. Identification of disaccharide products by paper chromatography formed by action of heparitinase I, heparitinase II and heparinase upon different substrates. The experiment was performed as described in Fig. 1 except that 20μl of the incubation mixtures were applied in Whatman nr.1 paper and subjected to chromatography in isobutyric acid 1 M NH_3 for 48 hours. After drying, the paper was stained with silver nitrate reagent. K5, unmodified K5 polysaccharide; K5-NS, N-deacetylated, N-sulfated K5 polysaccharide; K5-NS,6S, N-deacetylated, N,6-disulfated K5 polysaccharide; K5-NAc/NS,6S, partially N-deacetylated, N,6-disulfated K5 polysaccharide; HTase I, heparitinase I; HTase II, heparitinase II; Hepase, heparinase.

heparin, contain larger amounts of glucuronic acid residues also produce larger amounts of ΔU,2S-GlcNS,6S by action of heparitinase II [23].

Other derivatives prepared from K5, namely: …GlcA,2,3,SO_3H-(1→4)-GlcNSO_3H-3,6,SO_3H… and …GlcA,3SO_3H-(1→4)-GlcNSO_3H-6SO_3H… are not substrates for any of the three enzymes. Thus, the sulfate at C-3 position of glucuronic acid renders the substrate resistant to heparitinase II. This is reinforced by the low yields obtained for the trisulfated disaccharide from the 2- or 3-O-sulfated glucuronic acid K5 polysacharide (E).

Figure 2 shows the paper chromatography of the products formed by action of the enzymes upon some of the modified K5 polysaccharides, heparan sulfate and heparin. As judged by the reducing activity of the compounds the yields obtained for the products of the modified K5 polysaccharides are quite similar to the ones obtained by HPLC (Fig. 1). These products have also the same chromatographic migration as the ones formed from heparan sulfate and heparin by action of the enzymes.

2-O-desulfated heparin as a substrate for heparitinases and heparinase

Heparitinase II has a peculiar behaviour in the degradation of beef lung and mucosal heparins. Whereas the mucosal heparin produces by the action of the enzyme 40% of tri- and di-sulfated disaccharides plus 60% of an oligosaccharide with an average MW of 6 KDa [24], the bovine lung heparin produces, by action of heparitinase II, 10% of disaccharides plus an oligosaccharide of 9 KDa. No further degradation of the oligosaccharides was observed after reincubation with heparitinase II but they were totally degraded by heparinase to di- and tetra-saccharides.

This observation led us to test the 2-O-desulfated bovine lung heparin (H) as a substrate for the heparitinases and heparinase. Figure 1 shows that this modified heparin is no longer a substrate for heparinase but it is an excellent substrate for heparitinase II forming mainly disulfated disaccharide and small amounts of N-sulfated disaccharide. This modified heparin was not a substrate for heparitinase I. These results indicate that heparinase needs 2-O sulfated iduronic acid residues in the molecule for its activity, as previously postulated, whereas heparitinase II acts on non substituted iduronic acid linkages.

These findings together with the ones that the 6 and 9 KDa heparin oligosaccharides are resistant to heparitinase II also suggest that a sequence containing vicinal disaccharides bearing 2-O sulfated iduronic acid residues are impeditive for the heparitinase II action.

The observation that N-sulfated disaccharide is formed from the 2-O-desulfated heparin by heparitinase II but not by the action of heparitinase I is suggestive that the latter does not act on sequences containing iduronic acid residues.

Product identification

The chemical composition of the disaccharides produced by the enzymes upon different substrates shown in Table 1 agree with the assignments made for the compounds in Figures 1 and 2. The disaccharides ΔU-GlcNS,6S and

Table 1. Analyses of the disaccharides produced from modified K5 polysaccharides by action of heparitinases

Disaccharide	ε230 nm ($\times 10^{-3}$)	Uronic acid	Molar proportions to glucosamine		
			Sulfate		Acetyl groups
			Labile	Total	
ΔU-GlcNAc[a]	3.1	1.2	0	0	0.95
ΔU-GlcNS[b]	3.2	0.9	0.9	1.1	0.02
ΔU-GlcNS,6S[c]	3.3	1.1	1.1	1.8	0.01
ΔU-GlcNAc,6S[d]	2.9	1.0	0.2	1.1	1.04
ΔU,2S-GlcNS,6S[e]	3.4	1.0	1.1	2.9	0.02
ΔU,2S-GlcNS[f]	3.0	0.9	1.1	2.0	0.01

Prepared by paper chromatography (see Methods) from the following modified K5 polysaccharides: a, GlcA-GlNAc; b, GlcA-GlcNSO$_3$; c, GlcA-GlcNSO$_3$6SO$_3$; d, GlcA-GlcNSO$_3$ /acetyl,6SO$_3$/H; e, GlcA2SO$_3$-GlcNSO$_3$6SO$_3$ and f, from the 2-O-desulfated heparin.
ε = molar extinction coefficient.

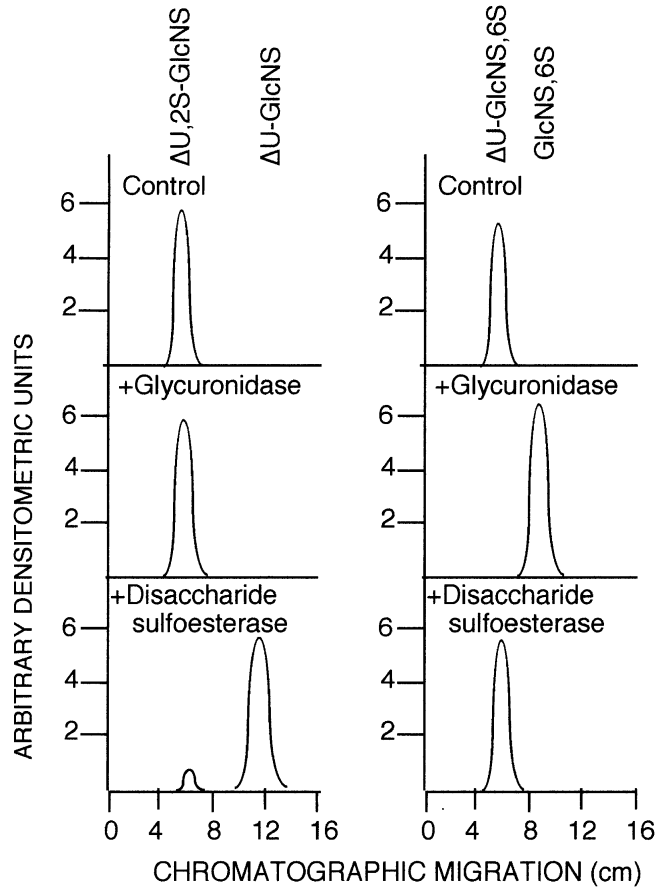

Figure 3. Characterization of the disulfated disaccharides using glycuronidase and disaccharide sulfoesterase from *F. heparinum.* About 20 μg of ΔU,2S-GlcNS and ΔU-GlcNS,6S (Fig. 1) were incubated with 0.1 U of glycuronidade and disaccharide sulfoesterase in 0.1 M ethylenediamine acetate buffer, pH 7.0 for 4 hours in a final volume of 20 µl. The incubation mixtures were applied in Whatman nr.1 paper and subjected to chromatography in isobutyric acid 1 M NH_3 for 24 hours. After drying, the paper was stained with silver nitrate reagent and subjected to densitometry at 450 nm.

ΔU,2S-GlcNS produced by action of heparitinase II upon GlcNS,6S modified K5-polysaccharide and 2-O desulfated heparin, respectively, have the same chemical composition, retention time in HPLC (Fig. 1) and chromatographic migration (Fig. 2). In order to ascertain the relative position of the sulfates in the molecules they were subjected to the action of glycuronidase and disaccharide sulfoesterase from *F. heparinum.* As previously shown [11,15], the glycuronidase does not act on unsaturated disaccharides containing sulfate at the C-2 position of the uronic acid residues and the disaccharide sulfoesterase removes sulfate only from the C-2 position of this residue. Figure 3 shows that ΔU,2S-GlcNS is susceptible only to the disaccharide sulfoesterase forming ΔU-GlcNS whereas ΔU-GlcNS,6S is suceptible only to the glycuronidase producing GlcNS,6S.

Discussion

Figure 4 summarizes the probable mode of action of the three lyases upon a hypothetical glycosaminoglycan. Heparitinase I and heparinase have a selective substrate specificity requiring for their activity β-D-glucuronyl (1→4) glucosamine N-sulfate or N-acetylglucosamine and α-L-iduronyl-2-O sulfate (1→4) glucosamine N-sulfate or glucosamine N,6-disulfate, respectively. Heparitinase II, on the other hand, has a broad specificity acting on most substrates tested, except on the sequences of β-D-glucuronyl (1→4) N-acetylglucosamine and sequences with exclusively α-L-iduronyl-2-O-sulfate (1→4) glucosamine N,6-disulfate. None of the enzymes acted on sequences containing 3-O-sulfated glucuronic acid residues.

The specificity of heparinase, heparitinase I and heparitinase II have also been studied using modified heparins and heparin-derived oligosaccharides [21,22]. These authors have shown that heparitinase I acts on regions containing non sulfated iduronic acid residues [22]. This contrasts with

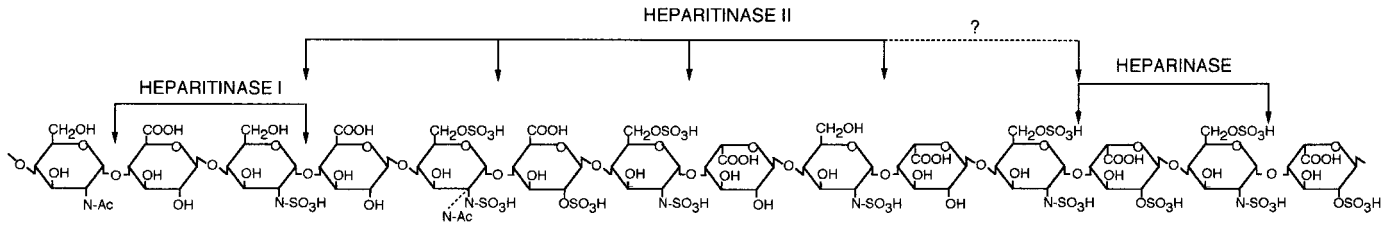

Figure 4. The mode of action of heparin and heparan sulfate lyases over a hypothetical glycosaminoglycan.

the present results where the sulfate at the C-6 position of the hexosamine moiety (linked either to an iduronic or glucuronic acid residue) is impeditive for heparitinase I action. It has been shown earlier that iduronic acid linked to either N-acetylated or N-sulfated glucosamine residues are also not substrates for heparitinase I [4,6].

The modified heparins and heparin oligosaccharides used by the previous authors [21,22] were derived from intestinal mucosa that contains substantial amounts of glucuronic acid residues [25] and were only partially degraded by heparitinase II making it difficult to compare their results with the observations reported here. Alternatively, we may be dealing with two different enzymes. When the heparitinases I and II were first described [26], it was shown that heparitinase I acted only on the glucuronidic linkages of heparan sulfate linked to either N-acetylglucosamine or glucosamine N-sulfate whereas heparitinase II acted on the more sulfated region of the molecule. Nevertheless, the commercially available enzymes sold by Seikagaku Co. (Tokio) and Sigma Chemical Co. (USA) state that the reaction products of heparitinase I include N,6 sulfated disaccharides, in favour of the previously reported specificity [21,22] but contrary to our previous [26] and present observations.

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