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Heparinase I Acts on a Synthetic Heparin Pentasaccharide Corresponding to the Antithrombin III Binding Site

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Abstract

A synthetic pentasaccharide, containing an intact antithrombin III (ATIII) binding site that is in clinical studies a specific antifactor Xa agent, serves as a substrate for a heparin lyase (heparinase I, EC 4.2.2.7) from *Flavobacterium heparinum*. Heparinase I, currently being assessed as a heparin reversal agent, also reverses the antifactor Xa activity of this synthetic pentasaccharide by breaking it down to inactive disaccharide and trisaccharide products. © 2000 Elsevier Science Ltd. All rights reserved.

Key Words: Heparinase; Heparin lyase; Pentasaccharide; Neutralization; Reversal

Heparin, widely used as a clinical anticoagulant, has recently begun to be displaced by low molecular weight (LMW) heparins [1]. LMW heparins differ from heparin in that they act primarily as antithrombin III (ATIII)-dependent antifactor Xa agents [2]. A synthetic heparin pentasaccharide, corresponding to the ATIII binding site in heparin, represents the first synthetic, specific, antifactor Xa agent [3].

While LMW heparins offer some important advantages, such as enhanced subcutaneous bioavailability, the therapeutic application of these new drugs also offers some unique challenges. One such challenge, is how to reverse a dose of LMW heparin. Following the administration of a high dosage of heparin, required in procedures such as cardiovascular surgery, the anticoagulant effect of heparin is commonly reversed through the administration of protamine [4]. Protamine, while effective in reversing heparin, has a number of untoward side effects. These range in severity from a modest elevation in blood pressure to severe allergic responses and stroke [4–6]. A heparin reversal system that relies on the enzymatic degradation of heparin using an immobilized flavobacterial heparinase was first proposed by Langer et al. [7]. Soluble heparinase I (heparin lyase 1, EC 4.2.2.7) has recently been evaluated clinically as a heparin reversal agent [8,9]. It has been demonstrated that heparinase I can effectively reverse the anticoagulant activity of various

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LMW heparins, which had been approved for clinical use [10]. The new synthetic heparin pentasaccharide is currently under clinical evaluation in Europe as a specific agent to catalyze the ATIII-based inhibition of factor Xa [3]. We were interested in assessing the utility of heparinase I in reversing the action of this new therapeutic agent.

Studies on the specificities of flavobacterial heparinases on polymeric substrates have demonstrated that heparinase I cleaves the ATIII binding site within the heparin polymer [11]. Similar specificity studies on structurally defined, heparin-derived oligosaccharides demonstrated that while some of these oligosaccharides could serve as substrates, heparinase I often showed poor activity towards particularly small oligosaccharides [12]. There has also been controversy concerning the heparinase I sensitivity of oligosaccharides containing the pentasaccharide sequence corresponding to the ATIII binding site [12–14].

In this study, we examine the heparinase I sensitivity of the synthetic heparin pentasaccharide containing an ATIII binding site. The kinetics of this reaction were studied and the products of the reaction were isolated and characterized.

1. Materials and Methods

1.1. Materials

Synthetic heparin pentasaccharide (SR90107A/ ORG31540) from Sanofi-Synthelabo (Choay, France) exhibited a specific activity of 800 anti-factor Xa U/mg. Heparinase I (Lot No. Hep I G61-64 with an enzyme activity of 201 IU/ml and a specific activity of 123 IU/mg) was provided by IBEX Technologies (Montreal, Canada) and was stored at $-70°C$. Platelet factor 4 was purchased from Stago (Genne-Villiers, France). Protamine sulfate was purchased from Sanofi-Synthelabo (Paris, France). BioGel P2 (45–90 μm particle size) was purchased from BioRad (Hercules, CA). Acrylamide (ultrapure) and Tris (ultrapure) were purchased from Life Technologies (Gaithersburg,

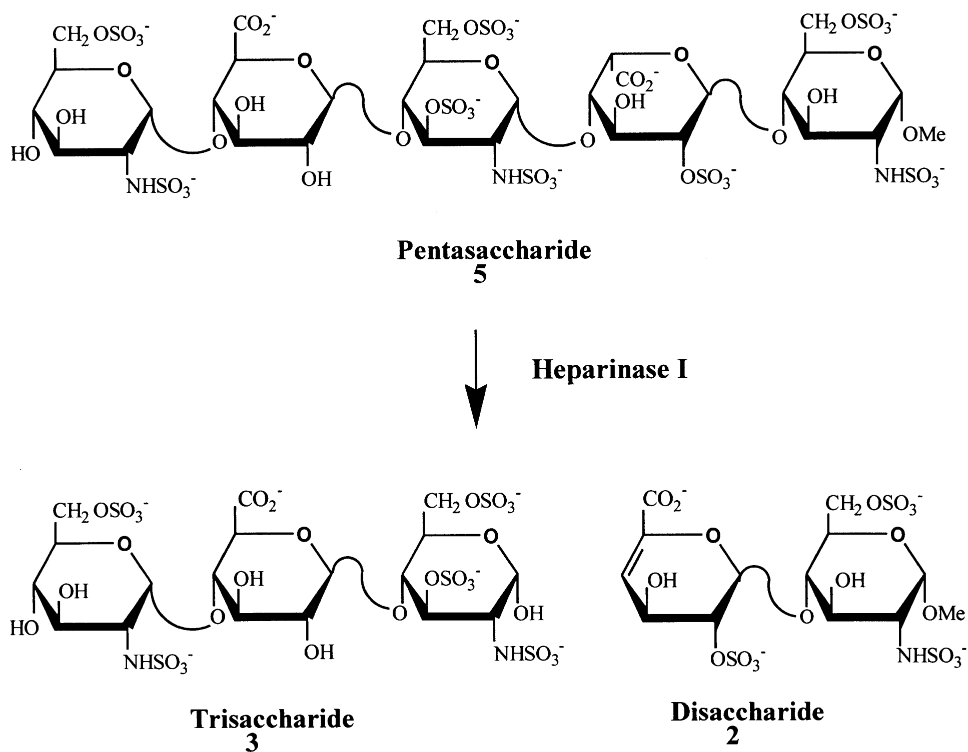

Fig. 1. Synthetic heparin pentasaccharide (5) acted upon by heparinase I affords disaccharide (2) and trisaccharide (3) products.

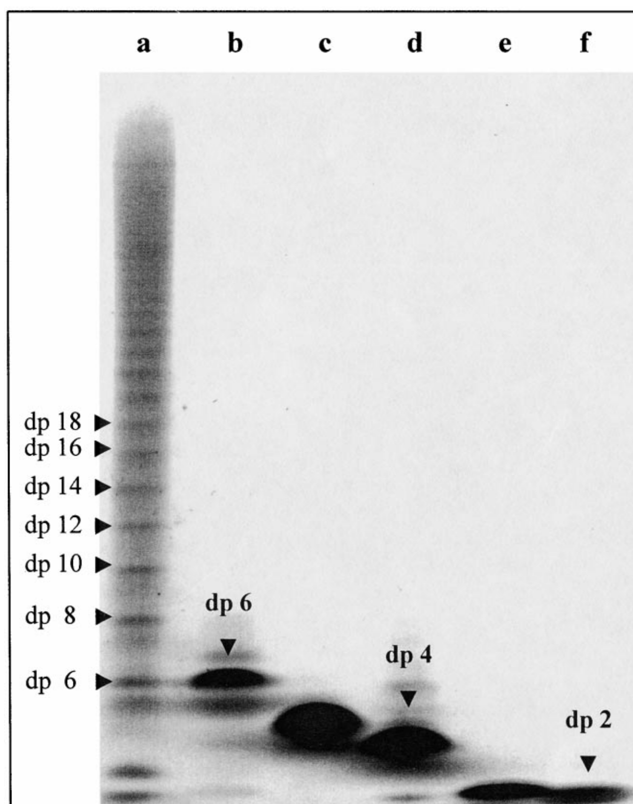

Fig. 2. Gradient PAGE analysis of heparin oligosaccharides. The six lanes shown contain: (a) high molecular weight heparin oligosaccharide standards [16]; (b) heparin hexasaccharide (dp6) standard (MW 1995); (c) synthetic heparin pentasaccharide (MW 1507); (d) heparin tetrasaccharide (dp4) standard (MW 1330); (e) products obtained on exhaustive treatment of synthetic pentasaccharide with heparinase I; (f) heparin disaccharide (dp2) standard (MW 665). The bands are labeled by degree of polymerization (dp).

MD). Alcian blue dye, bromophenol blue dye, and ammonium persulfate were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Glycine, disodium ethylene diaminetetraacetic acid (EDTA), boric acid, sucrose, *N,N*-methylene bisacrylamide, *N,N,N′,N′*-tetramethylenediamine (TEMED) were purchased from Fisher Chemical (Fair Lawn, NJ). All other reagents used were analytical grade.

1.2. Pentasaccharide Depolymerization

Synthetic pentasaccharide (0.5 mg at 1 mg/ml) was depolymerized with 10 U/ml heparinase I in a buffer (200 mM sodium chloride and 5 mM sodium phosphate, pH 7.0) at 30°C for 24 h.

1.3. High-Performance Size Exclusion Chromatography

Synthetic pentasaccharide and its degradation products were analyzed with TSK G3000SW and TSK G 2000SW columns (Tosoh, Japan) and run in series on a Waters Chromatography System (Lexington, MA) using both a refractive index and UV (205 nm) detectors. A sample (20 μl at 10 mg/ml) was injected and separated in 0.3 M sodium sulfate at pH 5 at a flow rate of 0.5 ml/min.

1.4. Separation of Pentasaccharide-derived Oligosaccharides

The pentasaccharide-derived oligosaccharide mixture was desalted on a small BioGel P2

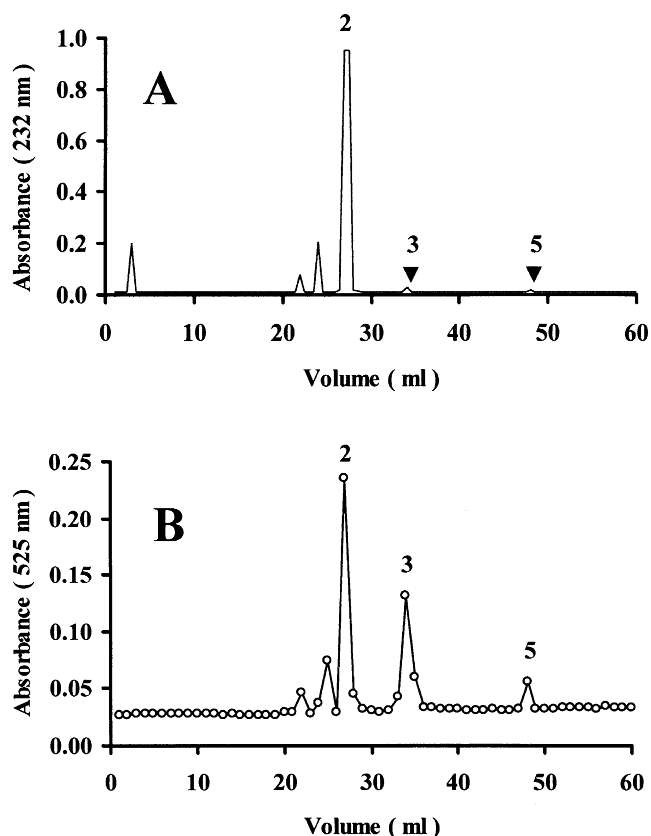

Fig. 3. SAX-HPLC analysis of reaction products afforded by the treatment of synthetic heparin pentasaccharide (5) with heparinase I. Panel A was through direct UV detection at 232 nm. Panel B shows the same chromatogram where fractions were collected and uronic acid content was determined by carbazole assay [15].

Table 1. ESI-MS analysis of oligosaccharides

	Pentasaccharide (5)	Trisaccharide (3)	Disaccharide (2)
Molecular ion mass	$[M\text{-}3H]^{3-}$ 500.7	$[M\text{-}3H]^{3-}$ 304.3	$[M\text{-}H]^{1-}$ 589.7
Parent ion mass[a]	$[M\text{-}10H+8Na]^{2-}$ 840.6	$[M\text{-}4H+Na]^{3-}$ 311.6	$[M\text{-}2H]^{2-}$ 294.4
Molecular mass[b]	1507	916	591

[a] Most intense ion observed in the ESI mass spectrum.

[b] Multiple ions were observed ranging in net change from −1 to −6 and containing multi-sodiated species. These ions could each be used to establish molecular mass. All assignable ions were used to calculate the low-resolution molecular mass of each oligosaccharide.

column (1.0 × 35 cm); fractions containing oligosaccharides were pooled and freeze-dried. The reaction products were separated by strong anion exchange (SAX) high-performance liquid chromatography (HPLC), using a Shimadzu LC10A HPLC system (Columbia, MD). Analyses and small-scale preparation were performed on a 5-μm particle size (4.6 mm × 25 cm) SAX Spherisorb column (Waters, Milford, MA). The sample, dissolved in 200-μl distilled water, was injected into a 250-μl injection loop. The SAX column was equilibrated with 0.2 M sodium chloride at pH 3.5 and the flow rate was 1.0 ml/min. After sample injection, a linear gradient of concentration y [y (in molarity)=(0.0002) x (in seconds) + 0.2] of sodium chloride with a pH 3.5, at 1.0 ml/min was used to elute the oligosaccharides. The separation was monitored by absorbance at 232 nm with 0.2 absorbance units full scale (AUFS). The oligosaccharide content of each fraction was assessed by a carbazole assay for uronic acid [15].

1.5. Gradient Polyacrylamide Gel Electrophoresis (PAGE) Analysis of Pentasaccharide-derived Oligosaccharide

Gradient (12–22%), discontinuous PAGE analysis was performed on a vertical slab (0.1 × 16 × 20 cm) gel system [16]. Heparin oligosaccharides prepared from bovine lung heparin using heparin lyase I were used as the electrophoresis standard ladder [16]. The gel was loaded with ~25 μg of sample and subjected to electrophoresis for 4 h at 400 V. The gel was visualized with Alcian blue (0.5% in 1% acetic acid) staining.

1.6. Electrospray Ionization (ESI) Mass Spectrometry (MS)

Negative-ion ESI-MS was performed on an LCQ spectrometer (Finnigan, Breman, Germany) at the University of Minnesota, MS Service Laboratory by methods previously described [17].

Table 2. Signals of reporter groups observed in the ^1H NMR spectra of oligosaccharides

Sample	Residue				
	1	2	3	4	5
Disaccharide (2)				ΔUA2S	GlcNS6SOMe
H-l ppm				5.48	5.00
OCH_3 ppm					3.39
Trisaccahride (3)	GlcNS6S	GlcA	GlcNS3S6S		
H-1 ppm	5.51	4.59	5.48		
Pentasaccharide (5)	GlcNS6S	GlcA	GlcNS3S6S	IdoA2S	GlcNS6SOMe
H-1 ppm	5.60	4.59	5.52	5.20	5.00
OCH_3 ppm					3.39

1.7. Nuclear Magnetic Resonance (NMR) Spectroscopy

^1H NMR spectroscopy was performed on a Varian 500 MHz spectrometer (Varian, Palo Alto, CA). Samples ($\sim$100 μg) were exchanged three times by freeze-drying from ^2H_2O and transferred to Shigimi tubes for analyses.

1.8. Kinetics Study on Degradation of Pentasaccharide by Heparinase I

Synthetic pentasaccharide (300 μl, at concentrations between 10 and 60 μM in 200 mM sodium acetate and 2.5 mM calcium acetate solution adjusted to pH 7.0) was degraded at 30°C by the addition of 10 mU of heparinase I. The reaction was monitored at 232 nm continuously in the spectrophotometer, Shimadzu (Tokyo, Japan) Model UV-2101PC UV–Vis scanning spectrophotometer, equipped with CPS-260 cell positioner and CPS cell temperature controller. Based on the initial velocities of the reaction with varied concentration of substrate K_m and V_{max} values were calculated.

1.9. Antifactor Xa Assay and Neutralization Studies

Pentasaccharide was supplemented to normal human-pooled platelet-poor plasma (NHP) in a concentration range of 10–0.125 μg/ml. Protamine sulfate at a final concentration of 10 μg/ml was added to one set of pentasaccharide supplemented to NHP. Platelet factor 4 was added to a second set at a final concentration of 10 μg/ml. Heparinase was supplemented with a concentration of 0.1 U/ml to the third set. All samples were analyzed using the amidolytic anti-Xa assay reported in an earlier publication [18].

2. Results

Synthetic heparin pentasaccharide was treated with a preparation of heparinase I to examine its sensitivity to this enzyme (Fig. 1). Initial studies, utilizing high-performance size exclusion chromatography, confirmed that the pentasaccharide had broken down into products of approximately half its molecular weight (data not shown). Gradient PAGE analysis of the product mixture showed that it contained oligosaccharides giving bands between those for the heparin disaccharide and tetrasaccharide standards (Fig. 2, lanes d, e, f). Furthermore, the heparin pentasaccharide starting material gave a band between the bands for the hexasaccharide and tetrasaccharide standard and was extremely pure, containing no smaller contaminating oligosaccharides (Fig. 3, lanes b, c, d). The product mixture, obtained from heparinase I treatment, was desalted for analysis and separated on a SAX-HPLC. SAX-HPLC typically uses UV detection at 232 nm and only detects oligosaccharides containing an unsaturated uroniate residue, afforded through the action of a lyase enzyme such as heparinase I. While injection of the untreated synthetic pentasaccharide showed no peak at 232 nm, it could be detected at a retention volume of 48 ml (data not shown) by collecting fractions and analyzing each by carbazole assay [15]. SAX-HPLC analysis of the product mixture using UV detection at 232 nm, showed a major peak at 27 ml corresponding to a product containing an unsaturated uronate residue (Fig. 3A, Peak 2). Several additional minor peaks were also observed. When fractions were collected and analyzed by carbazole assay (Fig. 3B), two major peaks were observed corresponding to an unsaturated product (Peak 2) and a saturated product

Table 3. Kinetic constants for heparinase I acting on pentasaccharide at 30°C

Kinetic constants[a]		
K_m (μM)	V_{max} (nmol/min)[b]	V_{max}/K_m (nmol/μM min)
18	2.2	0.13

[a] Kinetic constants were determined from computer hyperbolic fit-optimized data. The standard errors were less than 10%.
[b] Determined using a molar absorptivity of 3800 M^{-1}, in 200 mM sodium acetate and 2.5 mM calcium acetate for the disaccharide.

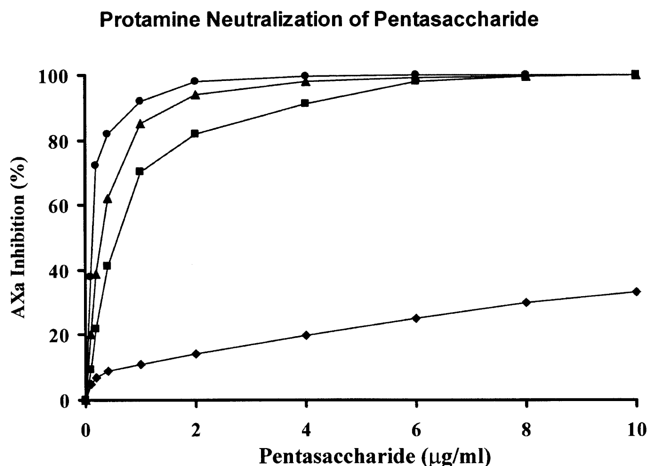

Fig. 4. Neutralization of the ATIII-mediated antifactor Xa activity of the synthetic heparin pentasaccharide. The amidolytic antifactor Xa activity is plotted as a function of pentasaccharide concentration in the (●) absence of neutralizing agent; (■) presence of a molar excess of protamine sulfate; (▲) in the presence of a molar excess of platelet factor 4; and (◆) in the presence of 0.1 U/ml of heparinase I (for 30 min).

(Peak 3). A small peak corresponding to residual synthetic pentasaccharide vide infra (Peak 5) was also detected by carbazole assay.

The collected fractions corresponding to each peak were pooled, desalted, and analyzed by ESI-MS and ^{1}H NMR spectroscopy. Table 1 summarizes of the ESI-MS data for oligosaccharides eluting under Peaks 5, 3, and 2 that corresponds to synthetic pentasaccharide, saturated trisaccharide product, and unsaturated disaccharide product, respectively. Next, the ^{1}H NMR spectra of each were obtained at 500 MHz. The signals assignable to the H-1 (anomeric) protons in each spectra and the methyl ether (OCH_3) in the spectra of the disaccharide and pentasaccharide served as reporter groups (Table 2). These, together with the remaining signals in the ^{1}H NMR spectra, permitted the definitive assignment of the structure of the products formed (Fig. 1).

The kinetics of heparinase I acting on the synthetic pentasaccharide was examined. The reaction afforded Michaelis–Menton kinetics from which a K_m, V_{max}, and V_{max}/K_m were determined (Table 3).

The neutralization of the factor Xa activity of synthetic heparin pentasaccharide was examined. Concentrations of pentasaccharide ranging from 0.125 to 10 μg/ml were analyzed for their ability to catalyze ATIII-mediated inhibition of factor Xa activity using an amidolytic assay [18]. Pentasaccharide samples at the same concentrations were neutralized with a molar excess of protamine sulfate and platelet factor 4 or a catalytic amount of heparinase I (Fig. 4). While both protamine sulfate and platelet factor 4 failed to significantly neutralize the activity of the pentasaccharide, heparinase I showed a high level of efficacy in neutralizing up to 10 μg/ml of pentasaccharide. Similar results were observed using clot-based antifactor Xa assays (data not shown).

3. Discussion

Heparinase I is an eliminase isolated from *Flavobacterium heparinum* that cleaves heparin at the major linkage in the heparin polymer, $\rightarrow$4)α-D-GlcNS6S (1$\rightarrow$4)-α-L-IdoA2S (1$\rightarrow$. Protamine sulfate has been used for many years as a heparin reversal agent [4]. Unfortunately, protamine sulfate exhibits a number of undesirable side effects [4–6] and is not always completely successful in neutralizing the newest anticoagulant agents — the LMW heparins. Heparinase I and platelet factor 4 (a chemokine secreted by platelets) have been under recent investigation as heparin neutralization agents [19]. Both agents have been used successfully in the neutralization of LMW heparins [20,21].

Synthetic heparin pentasaccharide is under intensive investigation as a new anticoagulant/antithrombotic drug. This pentasaccharide, having a strong affinity to human ATIII and only exhibiting antifactor Xa effects, is currently undergoing phase III clinical trials in orthopedic surgery as a prophylaxis of venous thromboembolism. This agent has potent ATIII-mediated antifactor Xa activity and exhibits no anti-factor IIa activities. Furthermore, it exhibits a concentration-dependent, anticoagulant effect in thromboelastographic (TEG) analysis and Heptest assays, demonstrating strong antithrombotic effects in primates and other animal models [3].

Neutralization of anticoagulant/antithrombotic agents is important in cases of overdose or when severe hemorrhagic side effects are

observed. While heparin is routinely neutralized with protamine sulfate, LMW heparins are often incompletely neutralized. Heparinase I has not only demonstrated the ability to neutralize LMW heparins but also their oligosaccharide (dp4–10) components [20,21].

In preliminary studies, synthetic heparin pentasaccharide has been demonstrated to be resistant to neutralization with protamine sulfate as well as platelet factor 4 [20]. Thus, we decided to investigate the heparinase I sensitivity to synthetic heparin pentasaccharide.

There is some controversy in the literature as to the heparinase I sensitivity to the ATIII binding sequence when it occurs in small oligosaccharides [12,13,22]. Detailed studies have demonstrated that while tetrasaccharides containing →4)α-D-GlcNS6S (1→4) IdoA2S(1→ sequences were cleaved by heparinase I, this enzyme demonstrates a reduced value in the catalytic efficiency (V_{max}/K_m) towards such substrates [23].

The current study clearly establishes that the synthetic heparin pentasaccharide is a substrate for heparinase I. The enzyme cleaves the →4)-α-D-GlcNS3S6S (1→4) Ido A2S (1→ linkage, confirming its reported tolerance for a 3-*O*-sulfo group [11,12]. The kinetics observed, indicate that the catalytic efficiency for the pentasaccharide is equivalent to a tetrasaccharide containing a →4)-D-GlcNS6S (1→4) IdoA2S (1→cleavable site [23].

In in vitro studies, the synthetic heparin pentasaccharide produced a concentration-dependent, ATIII-mediated antifactor Xa activity. This activity was poorly reversed by using a molar excess of either protamine sulfate or platelet factor 4. The addition of a catalytic quantity of heparinase I, however, successfully neutralized the activity of the pentasaccharide.

In conclusion, this study has demonstrated that heparinase I degrades the synthetic heparin pentasaccharide to a disaccharide and trisaccharide product. The kinetic analysis of this reaction shows that linkages containing the 3-*O*-sulfo group are sensitive to heparinase I. Finally, heparinase I is capable of neutralizing the ATIII mediated antifactor Xa activity of the pentasaccharide, suggesting the potential utility of heparinase I as a reversal agent for this drug.

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