

Hydrolysis of Heparinase I to Synthetic Heparin-Pentasaccharide and Effect on its Anti-factor Xa Activity*

Yu Guangli (于广利)¹ Guan Huashi (管华诗)¹ Robert J. Linhardt² Jawed Fareed³

(1 *Institute of marine Drugs and Foods, Ocean University of Qingdao,
Qingdao 266003, China*)

(2 *Division of Medicinal and Natural*

Products Chemistry, University of Iowa, IA 52242, USA)

(3 *Department of Pathology and Pharmacology,*

Loyola University Medical Center, IL 60153, USA)

Abstract The hydrolysis of Heparinase I (from *Flavobacterium heparinase*, EC 4.2.2.7) to synthetic heparin-pentasaccharide (SHP) which contained an antithrombin III binding site was examined. The kinetics of this reaction was studied and the products were separated by strong anion exchange high performance liquid chromatography (SAX-HPLC). The structure of disaccharide and trisaccharide derived from SHP were confirmed by NMR and MS techniques. The experiment results showed that, heparinase I, which currently is used as a heparin reversal agent, can also reverse this synthetic pentasaccharide and make it losing its anti-factor Xa activity.

Key words heparinase I; synthetic heparin-pentasaccharide (SHP); anti-factor Xa activity

Heparin had recently begun to be displaced by low molecular weight heparin (LMWH) where they act primarily as antithrombin III (ATIII) and anti-factor Xa agents^[1]. A synthetic heparin-pentasaccharide (SHP), corresponding to the ATIII binding site in heparin, represents the ultimate, specific activity of anti-factor Xa^[2]. LMWH had some important advantages, such as enhanced subcutaneous bioavailability. The therapeutic application of these new drugs also offered some unique challenges, for example, how to reverse a dose of LMWH. A high dosage of heparin was required in procedures of cardiovascular surgery and the anticoagulant effect of heparin is commonly reversed by the administration of protamine. Although protamine is effective in reversing heparin's activity, it has a number of untoward side effects, such as modest elevation in blood pressure to severe allergic responses and

* This work was sponsored by NIH (GM 38060), RJL (HL52622) and finished in Dr. Linhardt's Lab, MNPC, College of pharmacy, the University of Iowa.

Received: 2000-09-22; Revised: 2001-02-21

stroke^[3-5]. A heparin reversal system relies on the enzymatic degradation of heparin by the use of an immobilized heparinase was first proposed by Langer and his coworkers^[6]. Soluble heparinase I had recently been evaluated clinically as a heparin reversal agent^[7,8]. We carried out extensive studies on the specificities of Flavobacterial heparinases on polymeric substrates, which demonstrated that heparinase I cleaved the ATIII binding site found within the heparin polymer. Similar studies on structurally defined oligosaccharide demonstrated some of these oligosaccharides could serve as substrates^[9].

In this study, the hydrolysis of heparinase I to SHP was examined. The kinetics of this reaction was studied and the products of the reaction were isolated and structure was confirmed.

1 Materials and Methods

1.1 Materials SHP (anti-factor Xa activity 800U/mg) and protamine sulfate were from Sanofi-Synthelabo (Choay, France). Heparinase I was from IBEX Technologies of Canada, and Platelet factor 4 was from Genne-Villiers of France. Biogel P2 (45~90 μm) was from BioRad Company (USA). Acrylamide (ultra-pure) and Tris (ultra-pure) were purchased from Life Technologies Inc. (Gaithersburg, MD). Alcian blue dye, bromophenol blue dye and ammonium persulfate were from Biochemicals. Glycine, Na₂EDTA, boric acid, sucrose, N,N-methylene bisacrylamide, TEMED were from Fisher Chemical Company. All other reagents used were analytical grade.

1.2 Depolymerization of SHP SHP (0.5 mg at 1mg/mL) was depolymerized with 10 units/mL heparinase I in a buffer (200mmol/L sodium chloride and 5mmol/L sodium phosphate, pH 7.0) at 30°C for 24 h.

1.3 Separation of SHP Derived Oligosaccharides^[10] The SHP derived oligosaccharides was desalted on Biogel P2 column (1.0 \times 35cm), and then separated by SAX-HPLC column (Spherisorb, 5 μm , 4.6mm \times 25cm, Waters Corporation). The column was pre-equilibrated with 0.2mol/L NaCl (pH3.5) at 1.0mL/min, and then in a linear gradient of NaCl from 0.2mol/L to 2.0mol/L in 150 minutes. The separation was monitored at 232 nm and the content of each fraction was tested by carbozole assay.^[11]

1.4 PAGE Analysis of Pentasaccharide-Derived Oligosaccharide^[12] Gradient (12%~22%) discontinuous Polyacrylamide gel electrophoresis (PAGE) analysis was performed on a vertical slab (0.1cm \times 16cm \times 20 cm) gel system. Bovine lung heparin oligosaccharides used as standards. Each sample was loaded with 25 μg and subjected to electrophoresis for 4 h at 400 V. The gel was visualized with Alcian blue (0.5% in 1% acetic acid) staining.

1.5 Electrospray Ionization Mass Spectrometry (ESIMS)^[13] Negative-ion ESI-MS was performed on an LCQ spectrometer (Finnegan, Germany) at the University of Minnesota, MS Service Laboratory by methods previous described in our laboratory.

1.6 Nuclear Magnetic Resonance (NMR) Spectroscopy ¹H-NMR spectroscopy was per-

formed on a Varian 500 MHz spectrometer (Varian, Palo Alto, CA). All samples (100 μ g) were exchanged three times by freeze-drying from D₂O before analysis.

1.7 Kinetics Study on Degradation of SHP by Heparinase I The SHP (10~60 μ mol/L in 200mmol/L NaAC and 2.5mmol/L Ca(AC)₂ at pH 7.0) was degraded at 30°C by addition of 10 mU of heparinase I and monitored at 232 nm continuously in the spectrophotometer (Shimadzu UV-2101PC UV-Vis spectrophotometer, equipped with 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.

1.8 Anti-factor Xa Assay and Neutralization Studies^[14] Activity of Xa the anti-factor of SHP and the neutralization of protamine sulfate were studied by using the method described before.

2 Results and Discussion

2.1 The Separation of SHP SHP was treated with heparinase I to examine its sensitivity. SHP had broken down into lower molecular weight products were confirmed (data not shown) by using HPGPC (TSK G3000SW and TSK G2000SW columns run in series on a Waters Chromatography System, RI and UV detectors were used at the same time) technique. Gradient PAGE analysis also showed the same results (Figure 1, lane d, e, f). Furthermore, the SHP starting material migrated between the hexasaccharide (dp6) and tetrasaccharide (dp4) standard (Figure 1, lanes b, c, d).

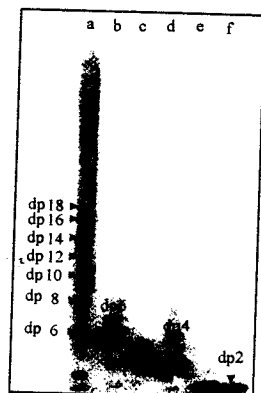


Fig. 1 The gradient PAGE analysis of SHP (c) and its hydrolyzed products (e).

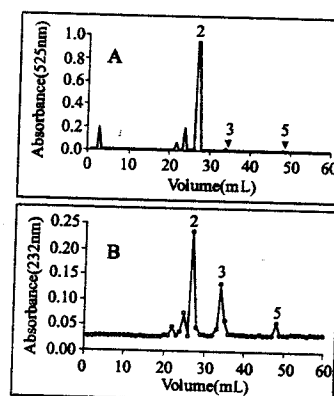


Fig. 2 SAX-HPLC analysis (panel A) and carbazole analysis (Panel B) of SHP and its hydrolyzed products.

The heparinase I treatment mixture was desalted for analysis and separated by SAX-HPLC (Figure 2A). The untreated SHP showed no peak at 232 nm but could be detected by collecting fractions and analyzing each fraction by carbazole assay (Figure 2B). SAX-HPLC analysis showed a major peak at 27 mL corresponding to a product containing an unsaturated uronate residue (Figure 2A, peak 2). Two major peaks (Figure 2B) were observed corre-

sponding to an unsaturated product (peak 2) and a saturated product (peak 3). A small peak corresponding to residual SHP (peak 5) was also detected by carbazole assay.

2.2 The structure of SHP and its hydrolyzed products The collected fractions corresponding to each peak were pooled, desalted and analyzed by ESI-MS and $^1\text{H-NMR}$ spectroscopy. Table 1 shows the summary of the ESI-MS data for oligosaccharides eluting under peaks 5, 3 and 2 correspond to SHP, saturated trisaccharide and unsaturated disaccharide product, respectively (Table 1). The $^1\text{H-NMR}$ spectra of each compounds was obtained from Varian 500 MHz spectrometer. The signals assignable to the H-1 (anomeric) protons in each spectrum and the methyl ether (OCH_3) in the spectra of the disaccharide and pentasaccharide were listed in Table 2. These, together with the remaining signals in the $^1\text{H-NMR}$ spectra, permitted the definitive assignment of the structure of the products formed by SHP (Figure 3).

Table 1 ESI-MS analysis of oligosaccharides

	Pentasaccharide (5)	Trisaccharide (3)	Disaccharide (2)
Molecular Ion Mass	$[\text{M}-3\text{H}]^{2-}$ 500.7	$[\text{M}-3\text{H}]^{3-}$ 304.3	$[\text{M}-\text{H}]^{-}$ 589.7
Parent Ion Mass ^a	$[\text{M}-10\text{H}+8\text{Na}]^{2-}$ 840.6	$[\text{M}-4\text{H}+\text{Na}]^{3-}$ 311.6	$[\text{M}-2\text{H}]^{2-}$ 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 charge from -1 to -6 and containing multi-sodiated species.

Table 2 Signals of groups observed in the $^1\text{H-NMR}$ spectra of oligosaccharides

Sample	Residue				
	1	2	3	4	5
Disaccharide (2)				$\Delta\text{UA}2\text{S}$	GlcNS6SOMe
H-1, ppm				5.48	5.00
OCH_3 , ppm	3.39				
Trisaccharide (3)	GlcNS6S	GlcA	GlcNS3S6S		
H-1, ppm	5.51	4.59	5.48		
SHP (5)	GlcNS6S	GlcA	GlcNS3S6S	$\text{IdoA}2\text{S}$	GlcNS6SOMe
H-1, ppm	5.60	4.59	5.52	5.20	5.00
OCH_3 , ppm					3.39

Table 3 Kinetic constant of heparinase I acting on SHP at 30 C

Kinetic Constants ^a		
K_m (μM)	V_{max} (nmol/min) ^b	V_{max}/K_m (nmol/(M · min))
18	2.2	0.13

^a Kinetic constants were determined from computer hyperbolic fit-optimized data.

^b Determined using a molar absorbance of $3,800\text{M}^{-1}$ in 200 mmol/L NaAC and 2.5 mmol/L calcium acetate for the disaccharide.

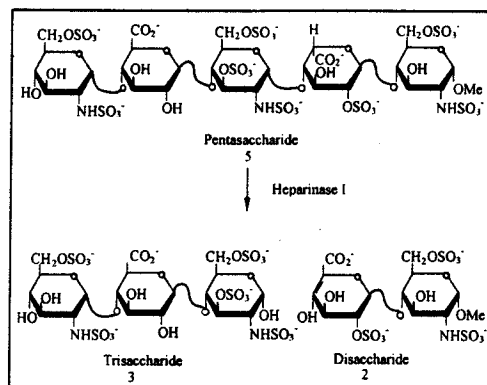


Fig. 3 Structure of pentasaccharide (5) and its hydrolyzed Products of disaccharide (2) and trisaccharide (3)

2.3 The Kinetics study and anti-factor Xa activity of SHP The kinetics of heparinase I acting on SHP was examined. The reaction afforded Michaelis-Menton kinetics. From the results of table 3, the value of K_m , V_{max} and V_{max}/K_m were 18, 2.2 and 0.13, respectively.

Concentration of SHP ranging from 0 to $10\mu\text{g/mL}$ were analyzed for their ability to catalyze ATIII mediated inhibition of factor Xa activity using an amidolytic assay. Pentasaccharide samples at the same concentrations were then neutralized

with a molar excess of protamine sulfate and platelet factor 4 or a catalytic amount of heparinase I. From the results of figure 4 we knew that both protamine sulfate and platelet factor 4 failed to significantly neutralize the activity of the SHP, but heparinase I showed a high level of efficacy in neutralizing up to $10\mu\text{g/mL}$ of pentasaccharide.

Protamine sulfate had been used for many years as a heparin reversal agent. Unfortunately, protamine sulfate exhibited a number of undesirable side effects and was not always completely successful in neutralizing LMWH. Heparinase I and platelet factor 4 (a chemokine secreted by platelets) had been recently investigated as heparin neutralization agent^[15].

SHP having a strong affinity to human ATIII and only exhibit anti-factor Xa activity was currently undergoing phase III clinical trials in the prophylaxis of post-orthopedic surgical thrombosis. In preliminary studies, SHP had been demonstrated resist to be neutralized by protamine sulfate as well as platelet factor 4^[16]. Thus, we decided to investigate the heparinase I sensitivity on SHP. Detailed studies in our laboratory had demonstrated that hep-

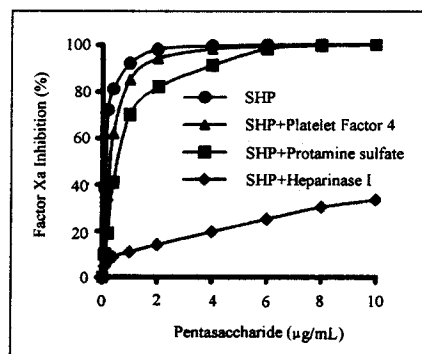


Fig. 4 Anti-factor Xa inhibition of SHP

arin tetrasaccharide could be cleaved by heparinase I^[17].

The current studies clearly showed that SHP was a substrate of heparinase I and cleaved the $\rightarrow 4$ - α -D-GlcNS3S6S (1 \rightarrow 4) Ido A2S (1 \rightarrow linkage. The kinetics indicated that the catalytic efficiency for the SHP was equivalent to a heparin-derived tetrasaccharide containing $\alpha\rightarrow 4$ -D-GlcNS6S (1 \rightarrow 4) IdoA2S (1 \rightarrow cleavable site^[18, 19].

3 Conclusion

The study had demonstrated that heparinase I degraded the synthetic heparin pentasaccharide to a disaccharide and trisaccharide product. The kinetic analysis of this reaction showed that linkages containing the 3-O-sulfo groups were sensitive to heparinase I. Finally, heparinase I was capable of neutralizing the ATIII mediated anti-factor Xa activity of the pentasaccharide, suggesting the potential utility of Neutralase as a reversal agent for this drug.

References

- 1 Fareed J, Jeske W, Hoppensteadt D, et al. Low-molecular-weight heparins: pharmacological profile and product difference. [J] *Am J Cardiol*, 1998, 82:3~10
- 2 Walenga J M, Jeske W P, Bara L, et al. Biochemical and pharmacological rationale for the development of a synthetic pentasaccharide. [J] *Thromb Res*, 1997, 86:31~36
- 3 Carr J A, Silverman N. The heparin-protamine interaction. [J] *J Cardiovasc Surg*, 1999, 40:659~66
- 4 Porsche R, Brenner Z R. Allergy to protamine sulfate. [J] *Heart & Lung*, 1999, 28:418~428
- 5 Shenoy S, Harris R B, Sobel M. Development of heparin antagonists with focused biological activity. [J] *Curr Pharm Design*, 1999, 5:965~986
- 6 Langer R, Linhardt R J, Hoffberg S. et al. An enzymatic system for removing heparin in extra corporeal therapy. [J] *Science*, 1982, 217:261~263
- 7 Kilgore K S, Tanheco E J, Naylor K B, et al. Ex vivo reversal of heparinase after ischemia and reperfusion. [J] *J Pharm & Exp Ther*, 1999, 290:1031~1037
- 8 Michelsen L G, Kikura M, Levy J H, et al. Heparinase I (neutralase) reversal of systemic anticoagulation. [J] *Anesthesia*, 1996, 85:339~346
- 9 Desai U R, Wang H M, Linhardt R J. Substrate specificity of the heparin lyases from *Flavobacterium heparinum*. [J] *Arch Biochem Biophys*, 1993, 306:461~468
- 10 Yamada S, Sugahar K. Structure of oligosaccharides isolated from heparan sulfate/heparin and substrate specificities of the degrading enzymes of bacterial origin. [J] *Trends Glycosci Glycotechnol*, 1998, 10:95~125
- 11 Bitter T, Muir H M. A modified uronic acid carbazole reaction. [J] *Anal Biochem*, 1962, 4:3330~3334
- 12 Edens R E, Al-Hakim A, Weiler J M. Gradient polyacrylamide gel electrophoresis for determination of molecular weights of heparin preparations and low-molecular-weight heparin derivatives. [J] *J Pharm Sci*, 1992, 81:823~827
- 13 Kim Y S, Ahn M Y, Wu S J, et al. Determination of the structure of oligosaccharides prepared from acharan sulfate. [J] *Glycobiology*, 1998, 8:869~877
- 14 Walenga J M, Bara L, Samama M M, et al. Amidolytic anti-factor Xa in the laboratory evaluation of heparin and low molecular weight fractions. [J] *Semin Thromb Hemost*, 1985, 11:100~107
- 15 Levy J H, Cormack J G, Morales A. Heparin neutralization by recombinant platelet factor 4 and protamine. [J] *Anesthesia & Analgesia*, 1995, 81:35~37
- 16 Iqbal O, Silver P, Walenga J M, et al. Heparinase is capable of neutralizing anticoagulant and anti-Xa effects of

- synthetic pentasaccharide: potential clinical application. [J] Blood, 1999, 94: 234
- 17 Linhardt R J, Turnbull J E, Wang H M, et al. Examination of the substrate specificity of heparin and heparan sulfate lyases. [J] Biochemistry, 1990, 29: 2611~2617
- 18 Rice K G, Linhardt R J. Study of structurally defined oligosaccharide substrates of heparin and heparan monosulfate lyases. [J] Carbohydr Res, 1989, 190: 219~233
- 19 Wang Qun, Yu Guangli. Combinatorial Synthesis of Carbohydrates. [J] Journal of Ocean University of Qingdao, 2001, 31(1): 45~52

肝素酶 I 对合成的肝素五糖的酶解作用及其抗 Xa 因子活性的影响

于广利¹ 管华诗¹ Robert J. Linhardt² Jawed Fareed³

(1 青岛海洋大学海洋药物与食品研究所, 青岛, 266003)

(2 Division of Medicinal and Natural Products Chemistry, University of Iowa, IA 52242, USA)

(3 Department of Pathology and Pharmacology, Loyola University Medical Center, IL 60153, USA)

摘要 研究肝素酶 I (来源于 *Flavobacterium heparinase*, EC4. 2. 2. 7) 对人工合成的且含有与抗凝血酶 III 特定结合位点的肝素五糖 (SHP) 的酶解作用, 并对酶解作用的动力学进行研究。利用强阴离子高效液相色谱 (SAX-HPLC) 对酶解混合物进行分离, 利用质谱 (ESIMS) 和核磁共振波谱 (¹H-NMR) 技术对得到的二糖和三糖的结构进行确证。研究结果表明, 这种被作为肝素反向试剂的肝素酶 I 可水解人工所合成的肝素五糖, 从而使之丧失抗 Xa 因子活性。

关键词 肝素酶 I (Heparinase I); 合成肝素五糖 (SHP); 抗 Xa 因子活性

中图分类号 R282.77

文章编号 1001-1862(2001)04-529-07

海洋人物

朗格, K. (Kaare Langlo, 1913-10-07~) 挪威气象学家。生于挪威的卑尔根。历任特罗姆塞气象台助研 (1940~1943)、研究员 (1943~)、世界气象组织干事 (1952)、副总干事长 (1971~1975)。嗣后在南极考察委员会、国际极地气象委员会、气象研究所等机构任职 (1976~)。主要从事大气臭氧层研究。主著《论大气臭氧层的含量及其气象条件的关系》(1952)、《世界气象观测》(1966)、《31 亿人——唯一的生命层》(1971)。

(刘安国)

• 美国国立健康研究学会 NIH(GM38060) 基金资助, 在美国 Iowa 大学 Robert J. Linhardt 实验室完成
 收稿日期: 2000-09-22; 修订日期: 2001-02-21
 于广利, 男, 1964 年 12 月出生, 硕士, 副教授。