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Degradation of Heparan Sulfate with Heparin Lyases

Laurie A. LeBrun and Robert J. Linhardt

1. Introduction

Glycosaminoglycan (GAG), heparan sulfate (HS), and heparin are a polydisperse mixture of linear polysaccharides composed of glucosamine residues 1→ 4 linked to uronic acid residues. The major repeating unit in heparin is → 4)-α-D-*N*-sulfoglucosamine-6-sulfate (1→ 4)-α-L-iduronic acid-2-sulfate (1→, corresponds to 75–90% of its sequence ***(1)*** (*see* **Fig. 1A**), whereas heparan sulfate consists of 50–75% → 4)-α-D-*N*-acetylglucosamine (1→ 4)-β-glucuronic acid (1→ and smaller amounts of → 4)-α-D-*N*-acetylglucosamine-6-sulfate (1→ 4)-β-D-glucuronic acid (1→ and → 4)-α-D-*N*-sulfoglucosamine (1→ 4)-β-D-glucuronic acid (1→ (*see* **Fig. 1B**). Heparin, which contains approx 2.7 sulfate groups per disaccharide unit, is more highly sulfated than HS, which contains less than one sulfate per disaccharide unit.

HS proteoglycans (PGs) are localized on the surface of many mammalian cells and in the extracellular matrix. HS proteoglycans are important for several different biological activities such as cell–cell and cell–protein interactions ***(2)***. These biological activities are controlled mainly through the binding of a variety of proteins to the HS chains. Specific sequences in the HS chain are thought to be responsible for the binding of growth factors, protease inhibitors, and adhesion molecules. The use of HS-degrading enzymes can help in separating and identifying biological active oligosaccharides ***(3)***.

HS can be degraded enzymatically by using heparin lyases from bacterial sources. The lyase enzymes degrade GAGs by endolytic cleavage ***(4–6)***. The enzymes cut glucosamine–uronate linkage by elimination (*see* **Fig. 2**), leaving a C4–C5 unsaturated bond containing product that can be easily detected by ultraviolet (UV) absorbance. In contrast, mammalian heparanases cleave this linkage by hydrolysis.

Heparin lyases have been isolated from *Flavobacterium heparinum* ***(7)***, *Bacteriodes* species ***(8)***, *Bacteriodes heparinolyticus* ***(9)***, and *Prevotella heparinolytica* ***(10)***. Heparin lyases from *F. heparinum* have been purified to homogeneity, studied extensively ***(11)***, and are available commercially from Sigma and Seikagaku.

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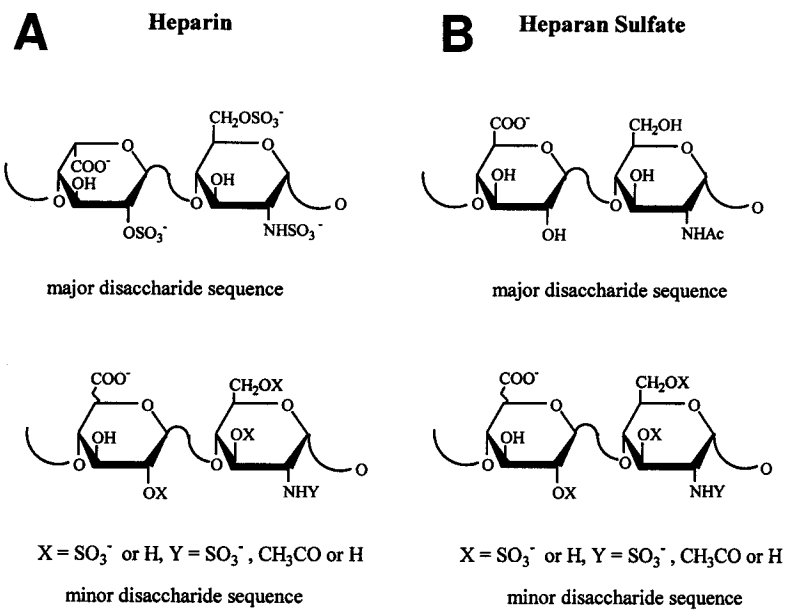

Fig. 1. **(A)** Structure of the major and minor disaccharide sequences of heparin. **(B)** Structure of the major and minor disaccharide sequences of heparan sulfate.

Three types of heparin lyases have been purified from *Flavobacterium*: heparin lyase I, heparin lyase II, and heparin lyase III (*see* **Fig. 2** and **Note 1**). Heparin lyase I acts primarily on heparin, heparin lyase II cleaves both heparin and HS, and heparin lyase III is active only on HS (*see* **Table 1**). The primary linkages cleaved by these enzymes and their relative activities toward heparin and HS are shown in **Table 1** and **Fig. 3**.

From both the DNA and amino acid sequences, there is only 15% alignment between heparin lyase I, II, and III ***(12)***. There are certain conserved sequences such as the heparin-binding sites and the calcium-binding regions in heparin lyase I and III. Recently, chemical modification studies and site-directed mutagenisis have been used to help identify critical residues for enzyme activity ***(13–17)***. Further studies and the crystal structures of the heparin lyases are needed to help us understand better the relationship between function and structure of these enzymes.

This chapter will explain how the heparin lyase enzymes can be used to degrade both heparin- and HS-containing samples and how to assay the activity of the enzyme. The heparin lyase enzymes can be used to identify the presence of HS/heparin in samples or to purify HS oligosaccharides for structural analysis.

2. Materials

2.1. Enzyme Preparation

1. Heparin Lyase I, II, or III. Enzymes can be ordered from Sigma (St. Louis, MO) and Siekagaku America (Falmouth, MA) (*see* **Notes 1** and **2**).

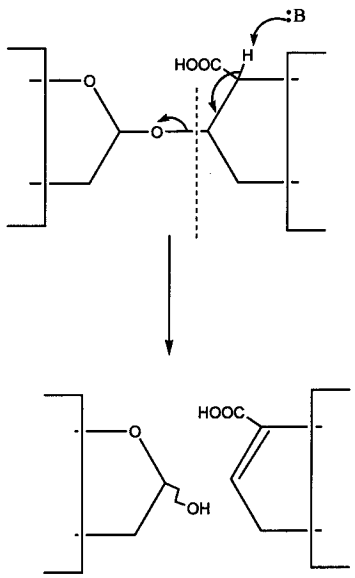

Fig. 2. Eliminative cleavage of GAGs by lyases.

2. Reagents required to make the appropriate buffers:
   a. Dibasic sodium phosphate (EM Science, Gibbstown, NJ).
   b. Phosphoric acid (Fisher Scientific, Fair Lawn, NJ).
   c. Sodium chloride (Fisher Scientific).
3. 500-µL polypropylene microcentrifuge tubes.

2.2. Enzyme Assay

1. HS (bovine kidney HS, sodium salt from Siekagaku America).
2. Heparin lyase solution (*see* **Subheading 3.1.**).
3. Buffer (*see* **Table 2**).
4. UV spectrophotometer.
5. 2 × 1-mL quartz cuvets.

2.3.Sample Digestion

1. HS or heparin samples (*see* **Note 3**) or samples containing radiolabeled HS or heparin.
2. Spectropor dialysis membrane (molecular-weight cutoff [MWCO] 1000) (Spectrum, Los Angeles, CA) or Centricon (YM3, MWCO 3000) centrifugal filter units (Millipore, Bedford, MA).
3. 500-µL polypropylene microcentrifuge tubes.
4. Heparin lyase solution.
5. Water baths at 30°C and 35°C for enzyme digestion and at 100°C to inactivate the enzyme reaction.

2.4. Product Analysis

High-performance liquid chromatography (HPLC), capillary electrophoresis (CE), gel-permeation chromatography, or polyacrylamide gel electrophoresis (PAGE) may be used to purify and analyze oligosaccharides prepared from HS/heparin.

Table 1
Activity of Heparin Lyases

Activity and substrate conversion	Heparin lyase I	Heparin lyase II	Heparin lyase III
Heparin[a]			
Percent activity[b]	100	60	<1
% Conversion[c]	60 (80)[d]	85	6
Heparan sulfate[e]			
Percent activity	10	100	100
Percent conversion	20	40	94

[a]Porcine mucosal heparin.

[b]Percent activity = [initial rate on the substrate examined/initial rate on substrate giving the highest activity] × (100).

[c]Percent conversion = [moles of linkages cleaved/total moles of hexosamine → uronic acid linkages] × (100).

[d]Bovine lung heparin.

[e]Bovine kidney heparan sulfate.

3. Methods

3.1. Preparation of Lyases for Use

1. Preparation of buffers: The following buffers can be stored at room temperature for over 1 mo (*see* **Note 4**).
   a. *For heparin lyase I*, prepare 50 m*M* sodium phosphate buffer containing 100 m*M* sodium chloride at pH 7.1. To prepare 1 L of buffer, dissolve 7.1 g of dibasic sodium phosphate and 5.8 g of sodium chloride into 900 mL of distilled water. Adjust the pH to 7.1 with phosphoric acid and bring the volume up to 1 L with distilled water.
   b. *For heparin lyase II and III*, prepare 50 m*M* sodium phosphate buffer by dissolving 7.1 g of dibasic sodium phosphate in 900 mL of distilled water. For heparin lyase II adjust the pH to 7.1 with concentrated phosphoric acid, and adjust to 7.6 for heparin lyase III. Adjust the final volume to 1 L with distilled water.
2. Aliquot samples:
   a. Dissolve 0.1 U of lyophilized enzyme in 100 µL of the appropriate buffer (*see* **Table 2**).
   b. Store the enzyme in 10-mU aliquots in 500-µL polypropylene tubes at –70°C (*see* **Note 5**).

3.2. Activity Assay for Lyases

1. Add 640'µL of the appropriate buffer (*see* **Table 2**) to a 1-mL quartz cuvet. Warm the cuvet to 30°C in a temperature-controlled UV spectrophotometer (*see* **Note 6**).
2. Thaw 10-µL aliquots of the appropriate enzyme solution (*see* **Table 1**) at room temperature.
3. Remove 90 µL of warm buffer out of the cuvet and transfer the solution into the tube containing the enzyme solution. Immediately transfer the entire 100 µL of buffer and enzyme back into the cuvet, which is incubating at 30°C.
4. Adjust the baseline of the spectrophotometer to zero at 232 nm.
5. Remove the cuvet from the spectrophotometer and add 50 µL of 20 mg/mL of the appropriate substrate HS/heparin (*see* **Table 1**) to the cuvet. Cover the cuvet with Parafilm and

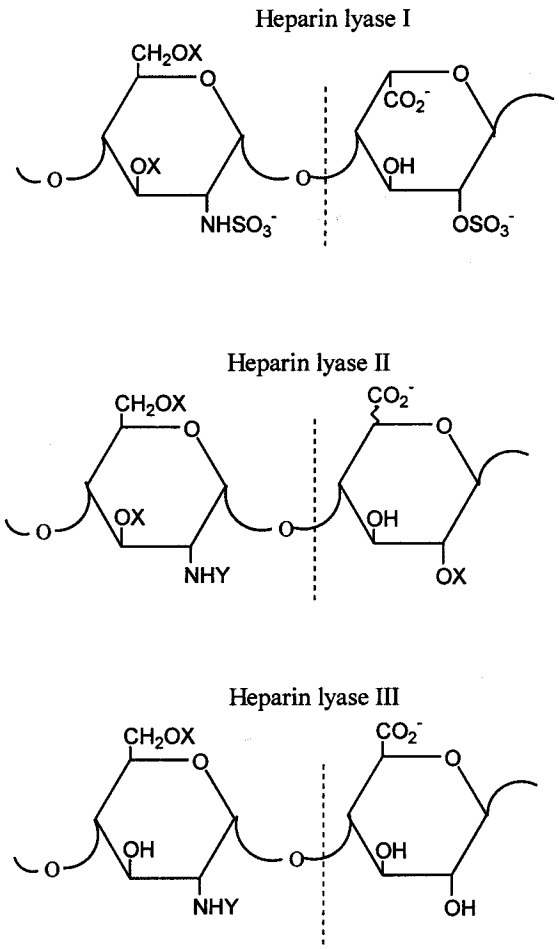

Fig. 3. Primary glycosidic linkages cut by hepain lyases. Abbreviations: X, H or SO_3^-; Y, CH_3CO or SO_3^-. Heparin lyase II cleaves at either glucuronic or iduronic acid residues.

invert two times to mix. Remove the Parafilm and place the cuvet back into the spectrophotometer.

6. Within 30 s after the addition of substrate, begin to measure the absorbance continuously or at 30-s intervals for 2–10 min. Graph absorbance at 232 nm vs time. The initial rate is determined by measuring the slope of the linear portion of absorbance vs time.
7. Calculate the enzyme activity from the initial rate using the extinction coefficient ($\varepsilon = 3800\ M^{-1}$) for the reaction products (*see* **Note 7**). Each product formed has an unsaturated uronic acid residue at its nonreducing terminus that absorbs at 232 nm. The enzyme activity is calculated as

$$\text{Enzyme Activity} = \frac{(\Delta \text{Abs 232 nm/ min})\ (700\ \ \mu\text{L})}{(3800\ \text{M}^{-1})}$$

Table 2
Properties of Heparin Lyases and Reaction Conditions

Enzyme	Substrate	MW (Da)	p*I*	T_{opt} [b] (°C)	Buffer system
Heparin lyase I [a](EC 4.2.2.7)	Heparin	42,800	9.2	30	50 m*M* $NaPO_4$, 100 m*M* NaCl, pH 7.1
Heparin lyase II	Heparin HS	84,100	9.0	35	50 m*M* $NaPO_4$, pH 7.1
Heparin lyase III (EC 4.2.2.8)	HS	70,800	10	35	50 m*M* $NaPO_4$, pH 7.6

[a]EC is the Enzyme Commission number.
[b]T_{opt}, optimum temperature for the enzyme.

3.3.Sample Digestion

3.3.1. Complete Heparin Lyase-Catalyzed Depolymerization of a Sample Containing HS/Heparin

1. Dissolve samples, containing HS/heparin (*see* **Note 8**) in buffer and dialyze using a 1000-MWCO dialysis membrane or a Centricon (YM3, MWCO 3000) centrifugal filter unit (*see* **Note 9**).
2. Thaw 10 µL of the appropriate enzyme solution (*see* **Table 1**) at room temperature (assay if desired as described under **Subheading 3.2.**) and then add 40 µL of the appropriate buffer (*see* **Table 1**) to a 500-µL polypropylene microcentrifuge tube (*see* **Note 10**). Also add 50 µL of buffer to one tube as a blank control.
3. Add 50 µL of the HS/heparin-containing sample to each tube and mix by gently inverting.
4. Incubate for 8–12 h at the appropriate temperature as indicated in **Table 2** (*see* **Note 11**).
5. Terminate the reaction by heating the tubes at 100°C for 2–3 min (*see* **Note 12**).
6. Product formation can be determined by either UV detection, colormetric assay, or HPLC. Pure samples containing >10 µg of HS can be measured by absorbance. The C4–C5 unsaturated bond of the oligosaccharide product is a chromophore that can be measured at a λ_{max} = 232 nm with a molar absorptivity of 5500 M^{-1} in 30 m*M* hydrochloric acid (*see* **Note 13**) ***(18)***. If the sample contains a high concentration of protein, the Azure A metachromatic assay ***(19)*** should be used. For smaller samples or impure samples, gel-permeation chromatography using UV or colorimetric detection ***(20)*** can be used to measure the quantity of product (*see* **Note 14**).

3.3.2. Complete Heparin Lyase-Catalyzed Depolymerization of Radiolabeled HS

1. Dissolve GAG sample containing radiolabeled HS in 50 µL of sodium phosphate buffer. Dialyze sample against sodium phosphate buffer using 1000 MWCO dialysis membrane or a Centricon (YM3, MWCO 3000) centrifugal filter unit (*see* **Note 9**).
2. Thaw 10 µL of heparin lyase III solution at room temperature, immediately prior to use (*see* **Note 5**).

3. Add 30 µL of sodium phosphate buffer to the 500-µL polypropylene microcentrifuge tube containing the enzyme solution.
4. (*Optional*) (*see* **Note 15**). Add 1.7 µL each of 20-mg/mL chondroitin sulfate A, chondroitin sulfate C, and dermatan sulfate substrate solutions (34 µg of each GAG) to the enzyme in buffer.
5. Add 50 µL of radiolabeled heparan sulfate solution and incubate 8–12 h at 30°C.
6. Heat at 100°C for 2 min to inactivate the enzyme.
7. Analyzed depolymerized radioactive sample by gel-permeation chromatography using radioisotope detection methods (*see* **Note 14**).

3.4. Analysis of Product

The heparin and heparan sulfate oligosaccharides can be analyzed by gradient PAGE, capillary electrophoresis (CE), or strong-anion-exchange HPLC ***(21–24)***.

4. Notes

1. Heparin lyase I from *Flavobacterium heparinum* is sold as heparinase I by Sigma and as heparinase by Seikagaku. Heparin lyase II is sold as heparinase II by Sigma and as heparitinase II by Seikagaku. Heparin lyase III from *Flavobacterium heparinum* is sold as heparinase III by Sigma and as heparatinase or heparatinase I by Seikagaku.
2. Often the purchased lyophilized enzyme contains bovine serum albumin (BSA) as a stabilizer. For example, Sigma samples contain 25% BSA.
3. Samples consisting of tissue, biological fluids, PGs, and GAGs that contain microgram quantities of HS can often be analyzed directly using heparin lyases without the use of radioisotopes.
4. Since calcium is an activator for heparin lyase I and III, 20 m*M* sodium acetate buffer in the presence of 2 m*M* calcium acetate can be used with these enzymes. These enzymes are also compatible with a wide range of other biological buffers.
5. Storage: The lyophilized enzyme is stable at –20°C for at least 2 yr. The dissolved enzyme is stable when frozen at –20°C for 1 mo and for over a year at –70°C. The heparin lyases are sensitive to freeze-thawing, especially heparin lyase III. Once heparin lyase III samples are thawed, they should be used immediately.
6. If a temperature-controlled spectrophotometer is not available, activity can be measured at room temperature, or samples can be incubated in a water bath and the absorbance can be measured at fixed time points.
7. One unit is equal to 1 µmol product formed per minute.
8. If the sample is believed to contain HS, heparin lyase III should be used. Samples that contain heparin should be treated with heparin lyase I. In samples where the identity of the GAG is unknown or believed to be a mixture of HS and heparin use either heparin lyase II or an equal unit mixture of heparin lyase I, II, and III to ensure complete depolymerization.
9. The presence of metals, detergents, and denaturants can interfere with the activity of the lyases. Before digesting the samples, detergents should be removed by precipitation with potassium chloride or by using a detergent-removal column such as Biobeads (Bio-Rad). Urea and guanidine should be removed by exhaustive dialysis using controlled-pore dialysis membrane (MWCO 1000).
10. Additional enzyme (10- to 100-fold) may be required to break down small, resistant oligosaccharides (***25–26***).

11. If possible, gently shake the samples during digestion.
12. Following the use of a lyase, residual lyase activity can be destroyed by heating the reaction mixture to 100°C or by adding denaturants or detergents. Most lyases are cationic proteins and can be removed from anionic oligosaccharide products by passing the reaction mixture through a small cation-exchange column, such as SP-Sephadex (Sigma), adjusted to an acidic pH. The oligosaccharide products (void volume) are then recovered, readjusted to neutral pH, and analyzed. This method can also be used to remove BSA, an excipient found in many of the commercial enzymes, from the oligosaccharide products.
13. This assay is to be used with relatively pure GAGs. High concentrations of protein interfere with the measure of oligosaccharide production due to UV absorbance of the protein.
14. In gel-permeation chromotography of HS/heparin, following complete depolymerization using the appropriate heparin lyase, the products should elute close to the column's total volume, corresponding to an apparent molecular weight <1500 daltons (confirming the presence of heparin/HS), while the substrate (control without enzyme) should elute close to the column's void volume, corresponding to a molecular weight of >10,000 daltons.
15. When attempting to use heparin lyases to depolymerize radiolabeled samples that contain very small quantities of heparin or heparan sulfate, it is often useful to add cold substrate as a carrier so that the activity of heparin lyase can be distinguished from that of trace amounts of chondrotin lyases that may be present in heparin lyase preparations *(27)*.

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