Analysis of Glycosaminoglycans with Polysaccharide Lyases

Polysaccharide lyases are a class of enzymes useful for analysis of glycosaminoglycans (GAGs) and the glycosaminoglycan component of proteoglycans (PGs). These enzymes cleave specific glycosidic linkages present in acidic polysaccharides and result in depolymerization (Linhardt et al., 1986). These enzymes act through an eliminase mechanism resulting in unsaturated oligosaccharide products that have UV absorbance at 232 nm. The lyases are derived from a wide variety of pathogenic and nonpathogenic bacteria and fungi (Linhardt et al., 1986). This class of enzymes includes heparin lyases (heparinases), heparan sulfate lyases (heparanases or heparitinases), chondroitin lyases (chondroitinases), and hyaluronate lyases (hyaluronidases), all of which are described in this unit.

Polysaccharide lyases can be used, alone or in combinations, to confirm the presence of GAGs in a sample as well as to distinguish between different GAGs (see Table 17.13B.1 and Commentary). The protocols given for heparin lyase I are general and, with minor modifications (described for each lyase and summarized in Table 17.13B.2), can be used for any of the polysaccharide lyases.

The basic protocol describes depolymerization of GAGs in samples containing 1 µg to 1 mg of GAGs. The alternate protocol describes depolymerization of GAGs in samples containing <1 µg of radiolabeled GAG. Two support protocols describe assays to confirm and quantitate the activity of heparin and chondroitin ABC lyases. It is recommended that enzyme activity be assayed before the enzyme is used in an experiment to be sure it is active and has been stored properly.

The standard definition of a unit (U), 1 µmol product formed/min, is used throughout this article. Some lyases are sold in nonstandard units (e.g., 0.1 µmol/hr, ΔA_{232}/min), and these should either be converted to standard units or the activity should be determined using the appropriate support protocol.

Table 17.13B.1 Polysaccharide Lyases Used to Identify Glycosaminoglycans

Glycosaminoglycan	Enzyme
Heparin, heparan sulfate, chondroitin sulfate, dermatan sulfate, hyaluronate	Heparin lyases I, II, III, and chondroitin ABC, AC lyases
Heparin, heparan sulfate, chondroitin sulfate, dermatan sulfate	Heparin lyases I, II, III, and chondroitin ABC lyase
Heparin, heparan sulfate	Heparin lyases I, II, III
Chondroitin sulfate, dermatan sulfate, hyaluronate	Chondroitin ABC, AC lyases
Chondroitin sulfate, dermatan sulfate	Chondroitin ABC lyase
Heparin	(Heparin lyase I–heparin lyase III)[a]
Heparan sulfate	Heparin lyase III
Chondroitin sulfate	Chondroitin AC lyase (or chondroitin ABC lyase–chondroitin B lyase)[a]
Dermatan sulfate	Chondroitin B lyase (or chondroitin ABC lyase–chondroitin AC lyase)[a]
Hyaluronate	Hyaluronate lyase

[a]Sample is divided into two portions and each is treated with a different lyase. The amount of depolymerization (i.e., counts moving from V_o to V_t of a gel-filtration column) is determined for each portion, and the difference gives a measure of the amount of glycosaminoglycan.

Contributed by Robert J. Linhardt
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OVERVIEW OF HEPARIN LYASES

There are three well-characterized polysaccharide lyases that act endolytically on heparin and heparan sulfate (Jandik et al., 1994); these enzymes are called heparin lyases. Heparin and heparan sulfate GAGs are structurally related, linear sulfated polysaccharides. Heparin's major sequence (representing 70% to 90% of its structure) is as follows:

→4)-α-D-glucosamine-2,6-sulfate-(1→4)-α-L-iduronic acid-2-sulfate-(1→

Heparan sulfate is composed primarily of equal proportions of the following:

→4)-α-D-glucosamine-2-sulfate-(1→4)-β-D-glucuronic acid-(1→

and

→4)-α-*N*-acetyl-D-glucosamine-6-sulfate-(1→4)-β-D-glucuronic acid
(*or* α-L-iduronic acid)-(1→

These disaccharide sequences are found in differing amounts in both heparin and heparan sulfate (Desai et al., 1993a,b). Substrates for heparin lyases are illustrated in Figure 17.13B.1.

The nomenclature of the three heparin lyases is somewhat confusing. However, heparin lyase I and III have enzyme commission (EC) numbers to facilitate their identification. The decision as to which enzyme should be used for a particular application is based on both the specificity desired and the reaction conditions required. The activity of these lyases toward specific glycosidic linkages has been determined using structurally characterized oligosaccharide substrates (Desai et al., 1993a). The primary linkages cleaved by these enzymes and their relative activities toward heparin and heparin sulfate (Desai et al., 1993a, b) are presented in Figure 17.13B.1 and Table 17.13B.3. Information on the optimal conditions for the activity and stability for these enzymes is given under the description of each enzyme and summarized in Table 17.13B.2.

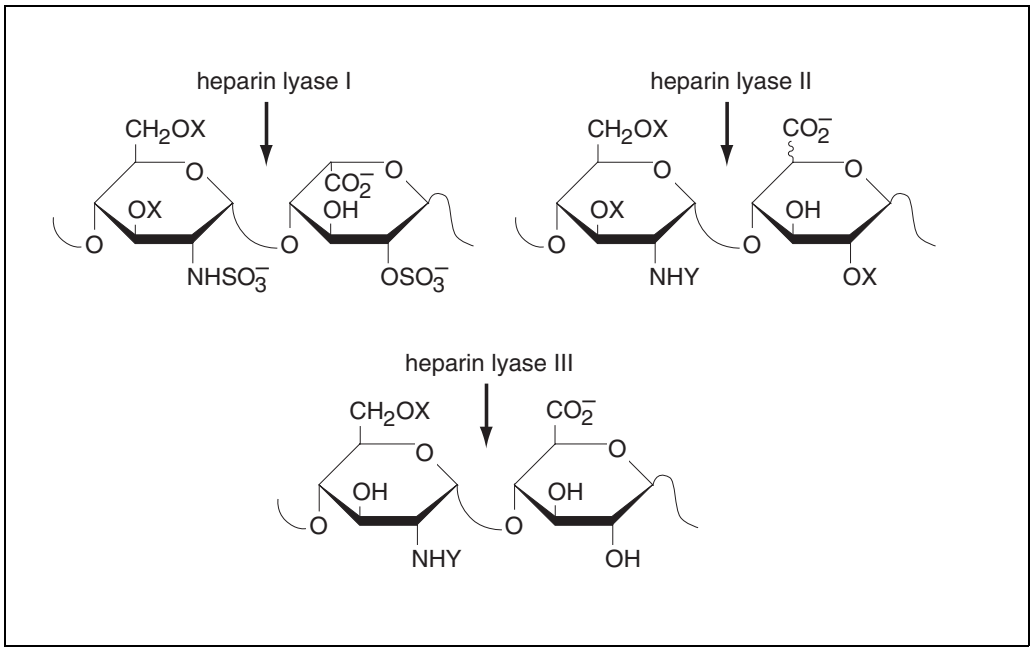

Figure 17.13B.1 Primary glycosidic linkages cleaved by heparin lyases. Abbreviations: X, H or SO_3^-; Y, CH_3CO or SO_3^-. Heparin lyase II cleaves at either glucuronic or iduronic acid residues.

Table 17.13B.2 Reaction Conditions for Polysaccharide Lyases with Optimum Buffers and Reaction Temperatures

Lyase	Buffer[a]	Optimum temperature
Heparin I	Sodium phosphate/NaCl, pH 7.1	30°C
Heparin II	Sodium phosphate, pH 7.1	35°C
Heparin III	Sodium phosphate, pH 7.6	35°C
Chondroitin ABC	Tris·Cl/sodium acetate, pH 8	37°C
Chondroitin AC	Tris·Cl/sodium acetate, pH 8	37°C
Chondroitin B	Ethylenediamine/acetic acid/NaCl, pH 8	25°C
Hyaluronate	Sodium acetate/NaCl, pH 5.2	>30°C

[a] See Reagents and Solutions for buffer recipes.

Table 17.13B.3 Activity of Heparin Lyases

Activity and substrate conversion	Heparin lyase I	Heparin lyase II	Heparin lyase III
Heparin[a]			
% Activity[b]	100	58	<1
% Conversion[c]	58 (76)[d]	85	6
Heparan sulfate[e]			
% Activity	13	100	100
% Conversion	19	39	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.

HEPARIN LYASE I (Lohse and Linhardt, 1992)

ENZYME

Heparin lyase I (EC 4.2.2.7), from *Flavobacterium heparinum* (*Cytophagia heparinia*), is commonly referred to as heparinase. The enzyme has a molecular weight of 42,800 Da and a pI of 9.1 to 9.2. Heparin lyase I has a random endolytic action pattern—i.e., it randomly acts on any site with the appropriate primary structure within the polymeric substrate (Fig. 17.13B.1; Jandik et al., 1994).

Complete Heparin Lyase–Catalyzed Depolymerization of an Unlabeled Sample

BASIC PROTOCOL

Samples consisting of tissues, biological fluids, PGs, and GAGs (*UNIT 17.2*) that contain microgram quantities of heparin and are not metabolically labeled can be analyzed using heparin lyase (see Critical Parameters for method to distinguish between heparin and heparan sulfate).

Materials

Heparin- or heparan sulfate–containing sample
Sodium phosphate/NaCl buffer (see recipe)
Heparin lyase I solution (see recipe)

Spectropor dialysis membrane, MWCO 1000 (Spectrum)
500-µl polypropylene microcentrifuge tubes
30° and 100°C water baths

Additional reagents and equipment for polysaccharide dialysis (*APPENDIX 3D*), HPLC (*UNIT 17.18*), and gel-filtration chromatography (*UNITS 10.9 & 17.17*)

1. Dissolve sample, containing 1 µg to 1 mg heparin, in 50 µl sodium phosphate/NaCl buffer. Dialyze sample against sodium phosphate/NaCl buffer using 1000 MWCO dialysis membrane.
2. Thaw and assay activity of a frozen aliquot of enzyme.
3. Thaw 10 µl heparin lyase I solution at room temperature and add 40 µl sodium phosphate/NaCl buffer to the 500-µl tube containing enzyme. Add 50 µl sodium phosphate/NaCl buffer to another 500-µl tube to serve as a blank control.

   Additional enzyme (10- to 100-fold) may be required to break down small, resistant oligosaccharides (Rice and Linhardt, 1989; Desai et al., 1993a).

   For heparin lyase II and III, the chondroitin lyases, and hyaluronate lyase, see alternative conditions listed in Table 17.13B.2 and in descriptions of individual enzymes below.

4. Add 50 µl sample to each tube and incubate 8 to 12 hr at 30°C.
5. Heat tube 2 min at 100°C to terminate the reaction. Analyze the product by a method appropriate for its purity and concentration.

   A pure sample containing >10 µg of heparin can be analyzed by measuring the difference in absorbance at 232 nm (ΔA_{232}) in 30 mM HCl between enzyme-treated sample and blank ($\varepsilon = 5500\ M^{-1}$ for oligosaccharide products in 30 mM HCl). Enzyme-treated sample is diluted with measured amounts of 30 mM HCl until the A_{232} is between 1 and 2. The blank is diluted with the same quantity of 30 mM HCl and its A_{232} is measured. The difference between the two measured A_{232} values, ΔA_{232}, is used to calculate the moles of oligosaccharide product formed. Ten moles of oligosaccharide product is obtained for each mole of heparin. If the treated sample contains substantial amounts of protein or other substances that absorb at or near 232 nm, disappearance of polysaccharide substrate can be measured using a dye-binding assay (Grant et al., 1984). Smaller quantities of samples or samples of lower purity can be analyzed by HPLC or gel-filtration chromatography using UV or conductivity detection methods.

Complete Heparin Lyase–Catalyzed Depolymerization of Very Small Amounts of Radiolabeled Glycosaminoglycans

ALTERNATE PROTOCOL

When attempting to use heparin lyase to depolymerize radiolabeled samples that contain very small quantities of heparin, it is often useful to add cold substrate as a carrier so the activity of heparin lyase can be distinguished from that of trace amounts of chondroitin lyases that are often present in heparin lyase preparations. Chondroitin lyase may pose a problem when using a heparin lyase to distinguish between heparin/heparan sulfate and chondroitin/dermatan sulfate in radiolabeled samples (see Critical Parameters and Table 17.13B.3). Therefore, it is recommended that cold carrier chondroitin/dermatan sulfate be added to block the action of minor chondroitin lyase contaminants. Alternatively, chondroitin ABC lyase from *Proteus vulgaris* (an organism free of enzymes acting on heparin and heparan sulfate) can be used to detect chondroitin sulfate chains specifically (see discussion of chondroitin lyases).

Additional Materials *(also see Basic Protocol)*

Radiolabeled heparin-containing sample (*UNIT 17.4*)
20 mg/ml chondroitin sulfate A solution (see recipe)
20 mg/ml chondroitin sulfate C solution (see recipe)
20 mg/ml dermatan sulfate solution (see recipe)

1. Dissolve GAG sample containing radiolabeled heparin in 50 µl sodium phosphate/NaCl buffer. Dialyze sample against sodium phosphate/NaCl buffer using 1000 MWCO dialysis membrane.
2. Thaw 10 µl of heparin lyase I solution at room temperature, immediately prior to use.

   For heparin lyase II and III, the chondroitin lyases, and hyaluronate lyase, see alternative conditions listed in Table 17.13B.2 and in descriptions of individual enzymes below.
3. Add 30 µl sodium phosphate/NaCl buffer to the 500-µl tube containing enzyme.
4. *Optional:* Add 1.7 µl each of 20 mg/ml chondroitin sulfate A, chondroitin sulfate C, and dermatan sulfate substrate solutions (34 µg each) to the enzyme in buffer.

   This step should be performed if there is any concern that the enzyme contains chondroitin lyase impurities.
5. Add 50 µl radiolabeled heparin solution and incubate 8 to 12 hr at 30°C.
6. Heat 2 min at 100°C to terminate the reaction.
7. Analyze depolymerized radioactive sample by HPLC or gel-filtration chromatography using radioisotope detection methods.

   In gel-filtration chromatography following treatment with heparin lyase, counts in fractions corresponding to an apparent molecular weight <1500 Da confirm the presence of heparin/heparan sulfate. Similar results are obtained with heparin lyase II and III except heparin lyase III does not act on heparin (Table 17.13B.3).

SUPPORT PROTOCOL 1

Assay of Heparin Lyase Activity

Commercial preparations of heparin lyase should be assayed before use, particularly for applications in which the level of enzyme activity is critical—e.g., when trying to distinguish between heparin and heparan sulfate or measure their relative amounts in a sample containing both.

Materials

Sodium phosphate/NaCl buffer (see recipe)
Heparin lyase I solution (see recipe)
20 mg/ml heparin solution (see recipe)

UV spectrophotometer, temperature controlled
1-ml quartz cuvette with 1-cm pathlength

Reaction conditions

A 700-µl reaction should contain:
50 mM sodium phosphate, pH 7.1
100 mM sodium chloride
10 mU heparin lyase I
1 mg heparin

Protocol

1. Add 640 µl sodium phosphate/NaCl buffer to a 1-ml cuvette. Warm the cuvette to 30°C in a temperature-controlled UV spectrophotometer.

   If a temperature-controlled spectrophotometer is not available, activity can be measured at room temperature, or sample can be incubated in a water bath and absorbance measured at fixed time points.

2. Thaw a 10-µl aliquot of heparin lyase I solution at room temperature.

3. Take cuvette out of the spectrophotometer, remove 90 µl warm buffer and transfer it to the tube containing heparin lyase I solution. Immediately transfer the entire 100 µl of buffer and enzyme back into the warm cuvette.

4. Place cuvette in the spectrophotometer and set the A_{232} to zero.

5. Remove cuvette from the spectrophotometer and add 50 µl of 20 mg/ml heparin substrate solution to initiate the reaction. Seal cuvette with Parafilm and invert once or twice to mix. Remove Parafilm and return cuvette to spectrophotometer.

6. Within 30 sec after addition of substrate, begin to measure absorbance continuously or at 30 sec intervals for 2 to 10 min. Plot A_{232} versus time.

   At room temperature (~20°C) a two-fold decrease in reaction rate is observed; this requires a 4- to 20-min assay time.

7. Calculate the enzyme activity (1 U = 1 µmol product formed/min) from the initial rate (<5% reaction completion) using $\varepsilon = 3800\ M^{-1}$ for the reaction products in sodium phosphate/NaCl buffer. Each product formed has an unsaturated uronic acid residue at its nonreducing terminus. Enzyme activity is calculated as

$$\text{Enzyme activity} = \frac{(\Delta A_{232}/\text{min})\ (700\ \mu\text{l})}{3800\ M^{-1}}$$

   The slope of the linear portion of the curve is used to calculate the initial rate of reaction.

HEPARIN LYASE II (Lohse and Linhardt, 1992)

ENZYME

Heparin lyase II (no EC number), an endolytic enzyme from *Flavobacterium heparinum*, has a molecular weight of 84,100 Da and a pI of 8.9 to 9.1. The substrate for heparin lyase II is indicated in Table 17.13B.3 and Figure 17.13B.1.

Protocols for the assay and use of heparin lyase II are identical to those for heparin lyase I, with the following modifications (see Table 17.13B.2):

1. Prepare enzyme and substrate solutions in sodium phosphate buffer, pH 7.1 (see recipes).
2. Carry out the reaction at 35°C.
3. Use 20 mg/ml heparan sulfate solution (see recipe) in Support Protocol 1 to assay for enzyme activity.

HEPARIN LYASE III (Lohse and Linhardt, 1992)

ENZYME

Heparin lyase III (EC 4.2.2.8), an endolytic enzyme from *Flavobacterium heparinum*, is commonly referred to as heparitinase and has a molecular weight of 70,800 Da and a pI of 9.9 to 10.1. Heparin lyase III can be used to confirm the presence of heparan sulfate in a sample (Fig. 17.13B.1 and Tables 17.13B.1 and 17.13B.3).

Protocols for the assay and use of heparin lyase III are identical to those of heparin lyase I, with the following modifications (see Table 17.13B.2):

1. Prepare enzyme and substrate solutions in sodium phosphate buffer, pH 7.6 (see recipes).
2. Carry out the reaction at 35°C.
3. Use 20 mg/ml heparan sulfate solution (see recipe), instead of heparin, as the substrate to assay for enzyme activity in Support Protocol 1.

OVERVIEW OF CHONDROITIN SULFATE LYASES

There are several polysaccharide lyases that act on chondroitin sulfates, dermatan sulfate, and hyaluronate. Chondroitin sulfate galactosaminoglycans are structurally related, sulfated, alternating 1→3, 1→4 linked, linear polysaccharides. The structure of the major disaccharide linkage found in each chondroitin sulfate and the enzyme that acts at each linkage are shown in Figure 17.13B.2. Hyaluronate has the same backbone structure, except that it is not sulfated and contains *N*-acetylglucosamine in place of *N*-acetylgalactosamine (Fig. 17.13B.3).

The decision of which chondroitin lyase to use should be based on both the specificity desired and the reaction conditions required (Tables 17.13B.1 & 17.13B.2).

CHONDROITIN ABC LYASE (Yamagata et al., 1968)

ENZYME

Chondroitin ABC lyase (chondroitinase ABC, EC 4.2.2.4), from *Proteus vulgaris*, has a molecular weight of 150,000 Da. This enzyme acts endolytically on chondroitin sulfates A-E (Fig. 17.13B.2; Jandik et al., 1994), slowly on hyaluronic acid, and not at all on heparin, heparan sulfate, or keratan sulfate.

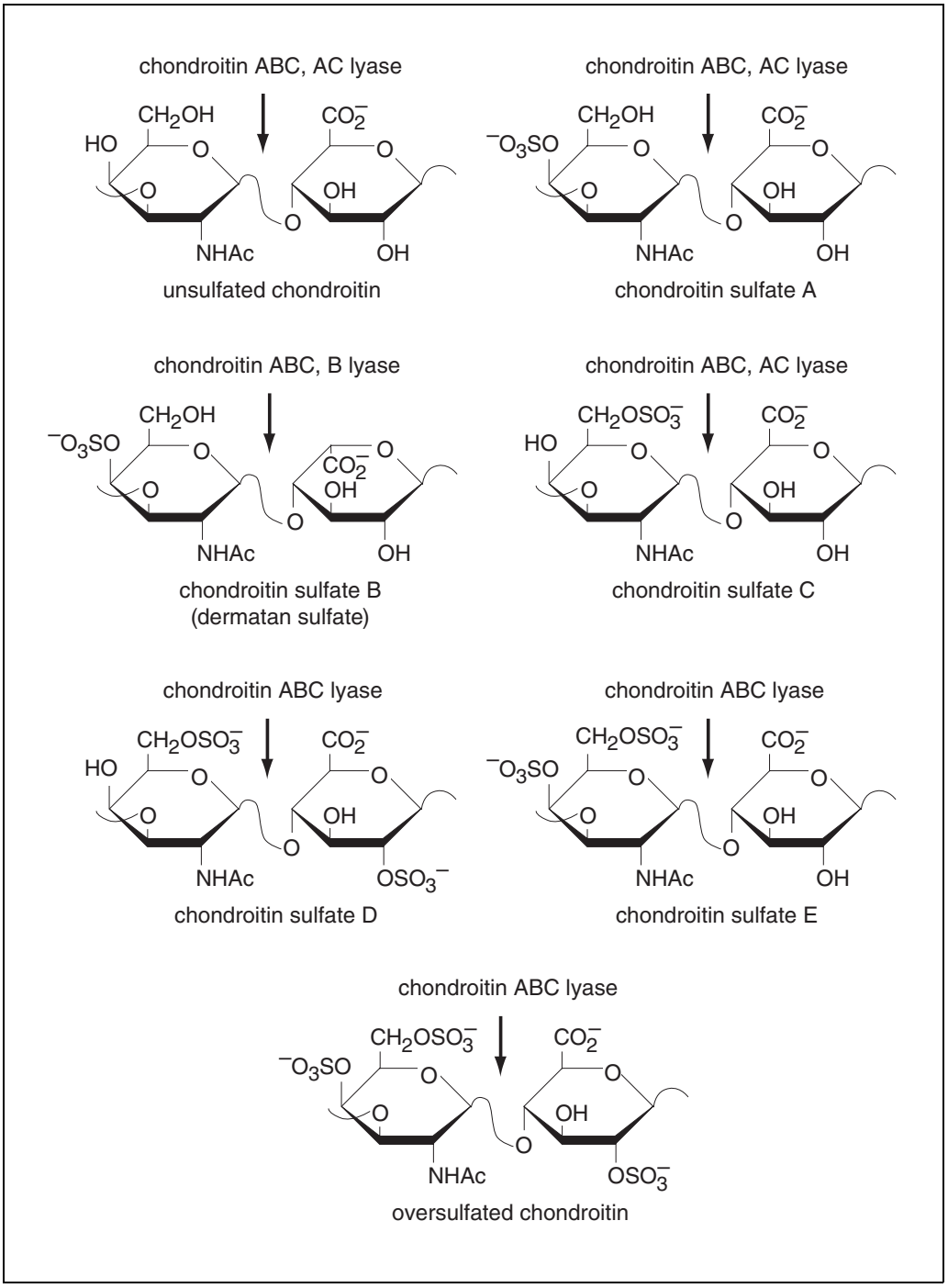

Figure 17.13B.2 Glycosidic linkages cleaved by chondroitin lyases. Abbreviation: Ac, CH_3CO.

The basic and alternate protocols for chondroitin ABC lyase are identical to those described for heparin lyase I with the following modifications:

1. Prepare enzyme and substrate solutions in Tris·Cl/sodium acetate buffer, pH 8 (see recipe).
2. Carry out the reaction at 37°C.
3. When using the alternate protocol for depolymerization of very small amounts of radiolabeled GAGs, omit step 4.

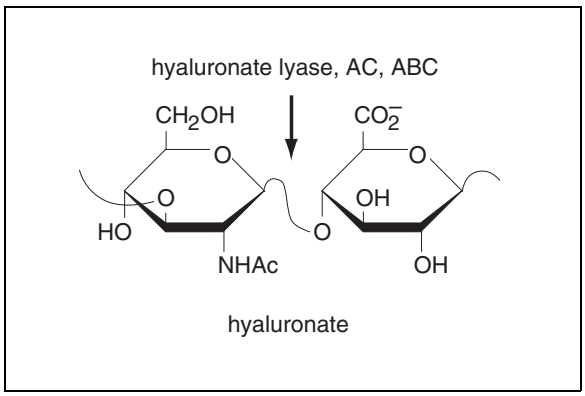

Figure 17.13B.3 Glycosidic linkage in hyaluronate cleaved by hyaluronate lyase and chondroitin AC and ABC lyases. Abbreviation: Ac, CH_3CO.

A radioactive sample containing chondroitin sulfate or dermatan sulfate can be analyzed, following chondroitin ABC lyase treatment, by HPLC (*UNIT 17.18*) or gel-filtration chromatography (*UNITS 10.9 & 17.17*) using radioisotope detection methods. In gel-filtration chromatography following treatment with chondroitin ABC lyase, counts in fractions corresponding to a molecular weight <1000 Da confirm the presence of chondroitin sulfate or dermatan sulfate.

Assay of Chondroitin ABC Lyase Activity

SUPPORT PROTOCOL 2

Commercial preparations of chondroitin lyase should be assayed before use, particularly for applications in which the level of enzyme activity is critical.

Materials

Tris·Cl/sodium acetate buffer, pH 8.0 (see recipe)
Chondroitin ABC lyase solution (see recipe)
20 mg/ml chondroitin sulfate A (see recipe), chondroitin sulfate C solution (see recipe), *or* dermatan sulfate solution (see recipe)

UV spectrophotometer, temperature controlled
1-ml quartz cuvette with 1-cm path length

Reaction conditions

A 700-µl reaction should contain:
50 mM Tris·Cl, pH 8
60 mM sodium acetate
10 mU chondroitin lyase
1 mg chondroitin sulfate A or C, or dermatan sulfate

Protocol

1. Add 640 µl Tris·Cl/sodium acetate buffer to a 1-ml quartz cuvette. Warm the cuvette to 37°C in a temperature-controlled spectrophotometer.

   If a temperature-controlled spectrophotometer is not available, activity can be measured at room temperature or the sample can be incubated in a water bath and the absorbance measured at fixed time points.

2. Thaw a 10-µl aliquot of chondroitin ABC lyase solution at room temperature.

3. Take cuvette out of the spectrophotometer, remove 90 µl warm buffer, and transfer it to enzyme solution. Immediately transfer entire 100 µl buffer plus enzyme back to the warm cuvette.

4. Place cuvette in spectrophotometer and set the absorbance at 232 nm (A_{232}) to zero.

5. Remove cuvette from spectrophotometer and add 50 µl of 20 mg/ml chondroitin A or C or dermatan sulfate solution to initiate reaction. Seal cuvette with Parafilm and invert once or twice to mix. Remove Parafilm and return cuvette to spectrophotometer.

   To assay for chondroitin AC lyase activity, use chondroitin A or C as substrate. To assay for chondroitin B lyase activity, use dermatan sulfate as substrate (see Table 17.13B.1 and description of individual enzymes below).

6. Within 30 sec after adding substrate begin to measure the absorbance continuously or at 30-sec intervals for 2 to 10 min. Plot A_{232} versus time.

7. Calculate the enzyme activity (1 U = 1 µmol product formed/min) from the initial rate (<5% reaction completion) using $\varepsilon = 3800\ M^{-1}$ for reaction products at pH 8. Enzyme activity is calculated as

$$\text{Enzyme activity} = \frac{(\Delta A_{232}/\text{min})\ (700\ \mu l)}{3800\ M^{-1}}$$

   Calculate the number of product molecules formed per substrate molecule from the A_{232} measured at reaction completion.

ENZYME

CHONDROITIN AC LYASE (Yamagata et al., 1968; Hiyama and Okada, 1975; Michelacci and Dietrich, 1975)

Two chondroitin AC lyases from *Arthrobacter aurescens* and *Flavobacterium heparinum* share the same enzyme commission number (EC 4.2.2.5). Chondroitin AC lyase from *A. aurescens* has a molecular weight of 76,000 Da (Hiyama and Okada, 1975) and a pI of 5.46. It acts exolytically on chondroitin sulfate A and C (Jandik et al., 1994) and exhibits a three-fold higher activity on hyaluronate. It can act at the glucuronic acid residues in dermatan sulfate (Fig. 17.13B.2) but does not act on heparin or heparan sulfate (Fig. 17.13B.1). Chondroitin AC lyase from *F. heparinum* acts endolytically on chondroitin sulfates A and C (Jandik et al., 1994), on hyaluronate, and at the glucuronate residues of dermatan sulfate (Fig. 17.13B.2; Gu et al., 1993). It does not act on heparin, heparan sulfate, or keratan sulfate (Yamagata et al., 1968).

The protocols for chondroitin AC lyase are identical to those described for chondroitin ABC lyase except chondroitin A or C should be used as substrate in Support Protocol 2 for assaying chondroitin lyase activity.

ENZYME

CHONDROITIN B LYASE (Michelacci and Dietrich, 1975)

Chondroitin B lyase (no EC number), from *Flavobacterium heparinum*, has a molecular weight of 55,000 Da. Chondroitin B lyase acts only on dermatan sulfate and not on the glucuronate residues of chondroitin sulfates A, C, and hyaluronate, and not on heparin or heparan sulfate (Fig. 17.13B.2).

This enzyme can be assayed as described for chondroitin ABC lyase using dermatan sulfate as substrate. The heparin lyase I protocols are used for sample analysis with this enzyme, with the following modifications (see Table 17.13B.2):

1. Prepare enzyme and substrate solutions in ethylenediamine/acetic acid/NaCl buffer, pH 8 (see recipe).
2. Carry out the reaction at 25°C.
3. Use dermatan sulfate solution (see recipe) as substrate in Support Protocol 2.

HYALURONATE LYASE (Vesterberg, 1968; Rautela and Abramson, 1973; Sasaki et al., 1982)

Hyaluronate lyases (EC 4.2.2.1 and EC 4.2.99.1) act only on hyaluronate (Hiyama and Okada, 1975) allowing hyaluronate (Fig. 17.13B.3) to be distinguished from chondroitin sulfate (Fig. 17.13B.2 and Table 17.13B.1). The enzyme from *Streptomyces hyalurolyticus* has been purified and has a pI <7 (Sasaki et al., 1982). Homogeneous *Staphylococcus aureus* hyaluronate lyase has a molecular weight of 84,000 Da (Rautela and Abramson, 1973) and a pI of 7.4 to 7.9 (Vesterberg, 1968).

Reaction conditions

A 700-µl reaction should contain:
50 mM sodium acetate, pH 5.2
125 mM sodium chloride
10 mU hyaluronate lyase
1 mg hyaluronate

The basic, alternate, and support protocols for hyaluronate lyase are identical to those described for heparin lyase with the following modifications (see Table 17.13B.2):

Protocol

1. Use sodium acetate/NaCl buffer, pH 5.2 (see recipe), to prepare enzyme (hyaluronate lyase; see recipe) and substrate solutions.
2. Perform the reaction at 30° to 60°C.

   Higher temperatures can be useful to reduce substrate viscosity—hyaluronic acid is very viscous. Higher temperatures will also increase the reaction rate, and they can be used to inhibit the activity of other lyases that may be present as contaminants of the hyaluronate lyase preparation.
3. Use hyaluronate solution (see recipe), instead of heparin solution, as substrate to assay enzyme activity (see Support Protocol 1).
4. When using the Alternate Protocol for depolymerization of very small amounts of radiolabeled GAGs, omit step 4.

Following treatment with hyaluronate lyase, a radioactive sample containing hyaluronate can be analyzed by HPLC (*UNIT 17.18*) or gel-filtration chromatography (*UNIT 17.17*) using radioisotope detection methods. In gel-filtration following treatment with hyaluronate lyase, counts in fractions corresponding to a molecular weight <500 Da confirm the presence of hyaluronate.

Testicular hyaluronidase is a hydrolase (EC 3.2.1.35) that also acts on hyaluronate (also on chondroitin sulfates A and C). Because it is not a polysaccharide lyase, testicular hyaluronidase cannot be assayed with the support protocol, but it can be used in the basic and alternate protocols with the modifications described above.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2****; for suppliers, see* ***APPENDIX 4****.*

Chondroitin lyase solutions, 1 mU/µl

Dissolve 0.1 U lyophilized enzyme in 100 µl Tris·Cl/sodium acetate buffer (see recipe) for chondroitin ABC lyase (chondroitinase ABC; Sigma or Seikagaku) and chondroitin AC lyase (chondroitinase AC from *Arthrobacter aurescens* and *Flavobacterium heparinum*; Sigma or Seikagaku) or ethylenediamine/acetic acid/NaCl buffer (see recipe) for chondroitin B lyase (chondroitinase B; Sigma or Seikagaku). Store in 10-mU aliquots in 500-µl polypropylene tubes <1 year at −70°C.

Different suppliers use different definitions for units of enzyme activity. Enzyme activity levels should be assayed and standardized, as described in the support protocols, in the laboratory before the enzymes are used in analytical studies.

Chondroitin sulfate A or C solution, 20 mg/ml

Dissolve 20 mg chondroitin sulfate A or C, sodium salt (Sigma or Seikagaku) in 1 ml Tris·Cl/sodium acetate buffer, pH 8 (see recipe) or other buffer appropriate to enzyme/substrate pair (see Table 17.13B.2). Store 1 year at <0°C.

Dermatan sulfate solution, 20 mg/ml

Dissolve 20 mg dermatan sulfate, sodium salt (Sigma or Seikagaku) in 1 ml ethylenediamine/acetic acid/NaCl buffer, pH 8 (see recipe) or other buffer appropriate to enzyme/substrate pair (see Table 17.13B.2). Store 1 year at <0°C.

Ethylenediamine/acetic acid/NaCl buffer

Dissolve 3.0 g ethylenediamine (50 mM final) and 1.7 g NaCl (30 mM final) in 900 ml H_2O. Adjust pH with glacial acetic acid to pH 8, and bring volume to 1 liter with H_2O. Store <1 month at 4°C.

Heparan sulfate solution, 20 mg/ml

Dissolve 20 mg heparan sulfate, sodium salt from bovine kidney (Seikagaku) in 1 ml sodium phosphate buffer, pH 7.6 (see recipe). Store 1 year at 0°C.

Heparin lyase solutions, 1 mU/µl

Dissolve 0.1 U lyophilized enzyme in 100 µl sodium phosphate/NaCl buffer (see recipe) for heparin lyase I or sodium phosphate buffer (see recipe) for heparin lyase II and III adjusted to the appropriate pH. Store enzyme in 10 mU aliquots in 500-µl polypropylene tubes <1 year at −70°C.

Heparin lyase I from Flavobacterium heparinum is sold as heparinase I (Sigma) and heparinase (Seikagaku). Heparin lyase II is sold as heparinase II (Sigma) and heparitinase II (Seikagaku). Heparin lyase III from Flavobacterium heparinum is sold as heparinase III (Sigma) and heparatinase or heparatinase I (Seikagaku).

Different suppliers use different definitions for units of enzyme activity. Enzyme activity levels should be assayed and standardized, as described in the support protocols, in the laboratory before the enzymes are used in analytical studies.

Heparin solution, 20 mg/ml

Dissolve 20 mg heparin, sodium salt (140 to 180 USP U/mg), from porcine intestinal mucosa or bovine lung (Sigma) in sodium phosphate/NaCl buffer, pH 7.1 (see recipe). Store 1 year at 0°C.

Different suppliers use different definitions for units of enzyme activity. Enzyme activity levels should be assayed and standardized, as described in the support protocols, in the laboratory before the enzymes are used in analytical studies.

Hyaluronate lyase solution, 1 mU/µl

Dissolve 0.1 U lyophilized enzyme (hyaluronidase from *Streptomyces hyalurolyticus*; Sigma or Seikagaku) in 100 µl sodium acetate/NaCl buffer, pH 5.2 (see recipe). Aliquot 10 mU to 500-µl polypropylene tubes and store <1 year at −70°C.

Different suppliers use different definitions for units of enzyme activity. Enzyme activity levels should be assayed and standardized, as described in the support protocols, in the laboratory before the enzymes are used in analytical studies.

Hyaluronate solution, 1.4 mg/ml

Dissolve 1.4 mg hyaluronate in 1 ml sodium acetate/NaCl buffer, pH 5.2 (see recipe). Store 1 year at <0°C.

Hyaluronate of high molecular weight is very viscous. It is best to use a hyaluronate of low to medium molecular weight to measure activity.

Sodium acetate/NaCl buffer

Dissolve 4.1 g sodium acetate (30 mM final) and 7.3 g NaCl (125 mM final) in 900 ml H_2O. Adjust pH with glacial acetic acid to pH 5.2 and bring volume to 1 liter with H_2O. Store <1 month at 4°C.

Sodium phosphate buffer

Dissolve 7.1 g dibasic sodium phosphate (50 mM final) in 900 ml H_2O. Adjust pH with concentrated phosphoric acid to pH 7.1 for heparin lyase II and pH 7.6 for heparin lyase III. Bring volume to 1 liter with H_2O. Store <1 month at 25°C.

Sodium phosphate/NaCl buffer

Dissolve 7.1 g dibasic sodium phosphate (50 mM final) and 5.8 g sodium chloride (100 mM final) in 900 ml H_2O. Adjust pH with phosphoric acid to pH 7.1 and bring volume to 1 liter with H_2O. Store <1 month at 25°C.

Tris·Cl/sodium acetate buffer

Dissolve 6.05 g Tris base (50 mM final) and 8.17 g sodium acetate (60 mM final) in 900 ml H_2O. Adjust pH to 8.0 with concentrated hydrochloric acid, and bring volume to 1 liter with H_2O. Store <1 month at 4°C.

COMMENTARY

Background Information

Proteoglycans (PGs) are primarily found in the extracellular matrix and are important in cell-cell interaction. Glycosaminoglycans (GAGs) are the dominant physical, chemical, and biological features of PGs. Lyases cleave specific glycosidic linkages at C4 of uronic acid residues present in GAGs (Figs. 17.13B.1, 17.13B.2, and 17.13B.3) through an eliminase mechanism, resulting in unsaturated oligosaccharide products that have UV absorbance spectra maxima at 232 nm (Linhardt et al., 1986). An eliminase catalyzes an elimination reaction that results in the formation of a double bond. In contrast, a hydrolase (the most common class of enzymes acting on polysaccharides) breaks down polysaccharides through the addition of water and does not result in the formation of a double bond. Lyases are useful for identifying and distinguishing the GAGs present in unlabeled or radiolabeled samples (see Table 17.13B.1).

Critical Parameters and Troubleshooting

Lyases are primarily of microbial origin and often a single organism produces multiple lyases acting on a variety of PGs and GAGs. Thus, it must be recognized that despite the high level of purity of the commercially available enzymes, they may contain small amounts of enzymatic impurities. These impurities can cause misleading results, particularly when large quantities of enzyme are used to treat very small quantities of sample—e.g., when these enzymes are used to analyze radiolabeled samples.

Protease contamination can also be present in the enzyme preparation. Commercial enzymes often contain bovine serum albumin

(BSA) for stabilization during lyophilization, and this greatly reduces potential problems associated with proteolytic contamination.

Complete degradation of a GAG. Table 17.13B.1 shows how the polysaccharide lyases can be used, alone or in combination, to determine the content of a GAG. All three heparin lyases used in combination (at equal-unit concentrations in 50 mM sodium phosphate buffer, pH 7, at 30°C) will degrade heparin or heparan sulfate to disaccharides, although a small quantity of lyase-resistant tetrasaccharide may remain. Chondroitin ABC lyase can be used to completely digest a mixture of chondroitin sulfates. If hyaluronate and chondroitin sulfate are both present, it is advisable to use an equal-unit mixture of chondroitin ABC and AC lyases. For complete degradation of GAGs, except keratan sulfate (see *UNIT 17.2*), it is advisable to treat the sample with an equal-unit mixture of heparin lyases I, II, and III and chondroitin ABC and AC lyases in sodium phosphate/NaCl buffer, pH 7, 30°C.

Heparin lyase III can be used to distinguish between samples containing heparan sulfate and those containing heparin. Although heparin lyase I (heparinase) has been used by investigators to demonstrate the presence of heparin in a sample, it also acts at some linkages present in heparan sulfate (Table 17.13B.3). Gel-filtration analysis can help distinguish between heparin and heparan sulfate because the large oligosaccharides formed from heparan sulfate elute in the void volume. In addition, oligosaccharide product compositional analysis (*UNIT 17.19*) is useful in distinguishing heparin from heparan sulfate when using heparin lyase I.

Caution is required when attempting to distinguish heparin/heparan sulfate from chondroitin/dermatan sulfate using heparin lyases. *Flavobacterium heparinum* produces both heparin and chondroitin/dermatan sulfate lyases; thus, minor contaminating activities can result in false positives. Step 4 of the Alternate Protocol for the use of heparin lyase on radiolabeled samples—incubation in the presence of nonspecific substrate—is included to eliminate false positives. The use of chondroitin ABC lyase, which contains no heparin lyase activity, is complicated by heparin's inhibition of chondroitin ABC lyase (Nakada and Wolfe, 1961). This inhibition is overcome by using excess enzyme (Linhardt et al., 1991).

Chondroitin sulfate is routinely distinguished from dermatan sulfate using chondroitin AC and ABC lyases (Saito et al., 1968). This can give slightly different results from those obtained using chondroitin AC and B lyases (Linhardt et al., 1991). Chondroitin AC lyases from *F. heparinum* and *Arthrobacter aurescens* both act at glucuronate-containing linkages in dermatan sulfates (Gu et al., 1993) and are useful for analysis of the glucuronic acid content of dermatan sulfate (Linhardt et al., 1991).

Hyaluronate content can be determined using hyaluronate lyase, which is specific for hyaluronate. Although hyaluronate lyases are inhibited by chondroitin sulfates, the addition of salt (150 mM NaCl) overcomes this inhibition (Nakada and Wolfe, 1961; Yamagata et al., 1968).

Contaminants in samples. The presence of certain metals (particularly divalent metals), proteases, polyanions, detergents (SDS and Triton X-100), and denaturants (urea and guanidine) can interfere with the activity of lyases. Before using these enzymes, 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).

Reaction conditions. Lyases are compatible with a wide range of buffers including succinate, acetate, ethylenediamine acetate, Tris·Cl, *bis*-Trispropane·HCl, sodium phosphate, MOPS, TES, and HEPES (Lohse and Linhardt, 1992). The presence of calcium may either enhance or reduce lyase activity. Its effect is probably due to changes calcium causes in the GAG substrate conformation and not through direct interaction with the enzyme. Because calcium is incompatible with certain buffers, e.g., phosphate, and can lead to variable results, its use is not recommended. The pH optima for lyases are broad—between pH 5 and 9. Hyaluronate lyase works best at pH <7, and chondroitin and heparin lyases work best at pH ≥7. Lyases can be used at temperatures between 20° and 40°C. Optimum temperatures are always a compromise between activity and stability considerations. If enzyme instability is a concern due to elevated temperature or prolonged incubation time, heparin lyase II should be used to degrade heparin or heparan sulfate, chondroitin ABC lyase should be used to degrade chondroitin sulfates and dermatan sulfates, and hyaluronate lyase should be used for hyaluronate. Optimal reaction conditions for the polysaccharide lyases are summarized in Table 17.13B.2.

Removal of lyase and lyase activity. 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 readjusted to neutral pH and analyzed (*UNITS 17.17 & 17.19*). This method can also be used to remove BSA, an excipient found in many of the commercial enzymes, from the oligosaccharide products.

Enzyme stability during storage. These enzymes can be stored in their lyophilized or reconstituted states at −20° or −70°C for ≤1 year. Once an enzyme is reconstituted, it should be aliquoted and frozen immediately. Single aliquots can be thawed to assay the enzyme or for use in an experiment. Heparin lyase II is very stable but heparin lyase III is unstable and should be used immediately after removing from frozen storage. The heparin lyases, particularly heparin lyase III, are sensitive to freeze-thawing and lyophilization. Among the chondroitin lyases, chondroitin AC lyase is most susceptible to thermal inactivation (Michelacci and Dietrich, 1975). Lyase storage stability is enhanced by high (>2 mg/ml) protein concentrations. This is often accomplished by addition of BSA.

Anticipated Results

When used correctly, an active lyase should specifically catalyze the breakdown of its GAG substrates. The types of GAGs present in a sample can be easily identified by using multiple lyases. The amount of product formed, and thus the amount of GAG in the sample, can be determined. Accuracy of analysis depends on a number of factors including the complexity of the sample and the types and concentrations of GAGs present. For a sample containing one GAG in high concentration with no contaminating proteins, salts, detergents, etc., GAG concentration can be determined to ±5%. For a complex sample containing many different GAGs at low concentrations in the presence of high levels of contaminants, it may only be possible to estimate GAG concentration to ±100%.

Time Considerations

It takes about half a day to prepare buffers and solutions and to reconstitute, aliquot, and freeze the enzyme. One aliquot of frozen enzyme can be thawed and assayed to ensure that the enzyme is active and has been stored properly. Application of the enzyme to determine the presence or type of GAG in a sample requires ~3 days for sample preparation, overnight treatment with enzyme, and analysis.

Literature Cited

Desai, U.R., Wang, H.H., and Linhardt, R.J. 1993a. Substrate specificity of the heparin lyase from *Flavobacterium heparinum Arch. Biochem. Biophys.* 306:461-468.

Desai, U.R., Wang, H.M., and Linhardt, R.J. 1993b. Specificity studies on the heparin lyases from *Flavobacterium heparinum. Biochemistry* 32:8140-8145.

Grant, A.C., Linhardt, R.J., Fitzgerald, G., Park, J.J., and Langer, R. 1984. Metachromatic activity of heparin and heparin fragments. *Anal. Biochem.* 137:25-32.

Gu, K., Liu, J., Pervin, A., and Linhardt, R.J. 1993. Comparison of the activity of two chondroitin AC lyases on dermatan sulfate. *Carbohydr. Res.* 244:369-377.

Hiyama, K. and Okada, S. 1975. Crystallization and some properties of chondroitinase from *Arthrobacter aurescens*. *J. Biol. Chem.* 250:1824-1828.

Jandik, K.A., Gu, K., and Linhardt, R.J. 1994. Action pattern of polysaccharide lyases on glycosaminoglycans. *Glycobiology* 4:000-000.

Linhardt, R.J., Cooney, C.L., and Galliher, P.M. 1986. Polysaccharide lyases. *Appl. Biochem. Biotechnol.* 12:135-177.

Linhardt, R.J., Al-Hakim, A., Liu, J., Hoppensteadt, D., Mascellani, G., Bianchini, P., and Fareed, J. 1991. Structural features of dermatan sulfates and their relationship to anticoagulant and antithrombotic activities. *Biochem. Pharmacol.* 42:1609-1619.

Lohse, D.L. and Linhardt, R.J. 1992. Purification and characterization of heparin lyases from *Flavobacterium heparinum. J. Biol. Chem.* 267:24347-24355.

Michelacci, Y.M. and Dietrich, C.P. 1975. A comparative study between a chondroitinase B and a chondroitinase AC from *Flavobacterium heparinum. Biochem. J.* 151:121-129.

Nakada, H.I. and Wolfe, J.B. 1961. Studies on the enzyme chondroitinase: Product structure and ion effects. *Arch. Biochem. Biophys.* 94:244-251.

Rautela, G.S. and Abramson, C. 1973. Crystallization and partial characterization of *Staphylococcus aureus* hyaluronate lyase. *Arch. Biochem. Biophys.* 158:687-693.

Rice, K.G. and Linhardt, R.J. 1989. Study of defined oligosaccharide substrates of heparin and heparan monosulfate lyases. *Carbohydr. Res.* 190:219-233.

Saito, H., Yamagata, T., Suzuki, S. 1968. Enzymatic methods for the determination of small quantities of isomeric chondroitin sulfates. *J. Biol. Chem.* 243:1536-1542.

Sasaki, I., Gotoh, H., Yamamoto, R., Tanaka, H., Takami, K., Yamashita, K., Yamashita, J., and Horio, T. 1982. Hydrophobic-ionic chromatography: Its application to microbial glucose oxidase, hyaluronidase, cholesterol oxidase, and cholesterol esterase. *J. Biol. Chem.* 91:1555-1561.

Vesterberg, O. 1968. Studies on extracellular proteins from *Staphylococcus aureus* III. Investigations on the heterogeneity of hyaluronate lyase using the method of isoelectric focusing. *Biochim. Biophys. Acta* 168:218-226.

Yamagata, T., Saito, H., Habuchi, O., and Suzuki, S. 1968. Purification and properties of bacterial chondroitinases and chondrosulfatases. *J. Biol. Chem.* 243:1523-1535.

Key References

Desai et al., 1993a, b. See above.

Describes the specificity of the heparin lyases in detail.

Lohse and Linhardt, 1992. See above.

Describes physical and catalytic properties of the heparin lyases.

Michelacci and Dietrich, 1975. See above.

Describes kinetic properties of chondroitin B lyase and chondroitin AC lyase.

Seikagaku Company Product Literature, 1991.

Describes general properties and assay conditions for all of the polysaccharide lyases.

Saito et al., 1968. See above.

Good example of how different chondroitin sulfates can be distinguished using chondroitin ABC lyase and chondroitin AC lyase.

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