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Degradation of Chondroitin Sulfate and Dermatan Sulfate with Chondroitin Lyases

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1. Introduction

Glycosaminoglycans (GAGs) are a family of complex linear polysaccharides characterized by a repeating core disaccharide structure typically comprised of an *N*-substituted hexosamine and an uronic acid residue. They can be categorized into four main structural groups: hyaluronate, chondroitin sulfate (CS)/dermatan sulfate (DS); heparan sulfate/heparin and keratan sulfate.

The biological roles of chondroitin and dermatan sulfate GAGs are poorly understood and their exact chemical structures have not been determined. Because enzymes are highly specific and act under mild conditions, enzymatic methods are often preferable over chemical methods for determining the structure of GAGs.

Enzymes that degrade GAGs have become increasingly important tools for understanding the biological roles of GAGs and the proteoglycans, including the regulation of various cellular process such as adhesion, differentiation, migration, and proliferation *(1)*. Utilizing these enzymes, design and preparation of GAG-based therapeutic agents might become possible *(2)*. Such drugs could have uses as antithrombotic agents, antiatherosclerotic agents, antiinflammatory agents, inhibitors of complement activation and regulators of cell growth, angiogenesis, and antiviral agents.

CS and DS are the most common type of GAGs in extracellular matrix proteoglycans *(1)*. CS is a hetereopolysaccharide made up largely of repeating disaccharide units, in which one sugar is *N*-acetyl-D-galactosamine and the other is D-glucuronic. These disaccharides can be sulfated at the 4- or 6-position of the *N*-acetylgalactosamine residue. The major classes are CS-A (chondroitin 4-sulfate), DS (CS-B), containing 4-sulfated *N*-acetylgalactosamine and iduronic acid, and CS-C (chondroitin 6-sulfate) (*see* **Fig. 1**).

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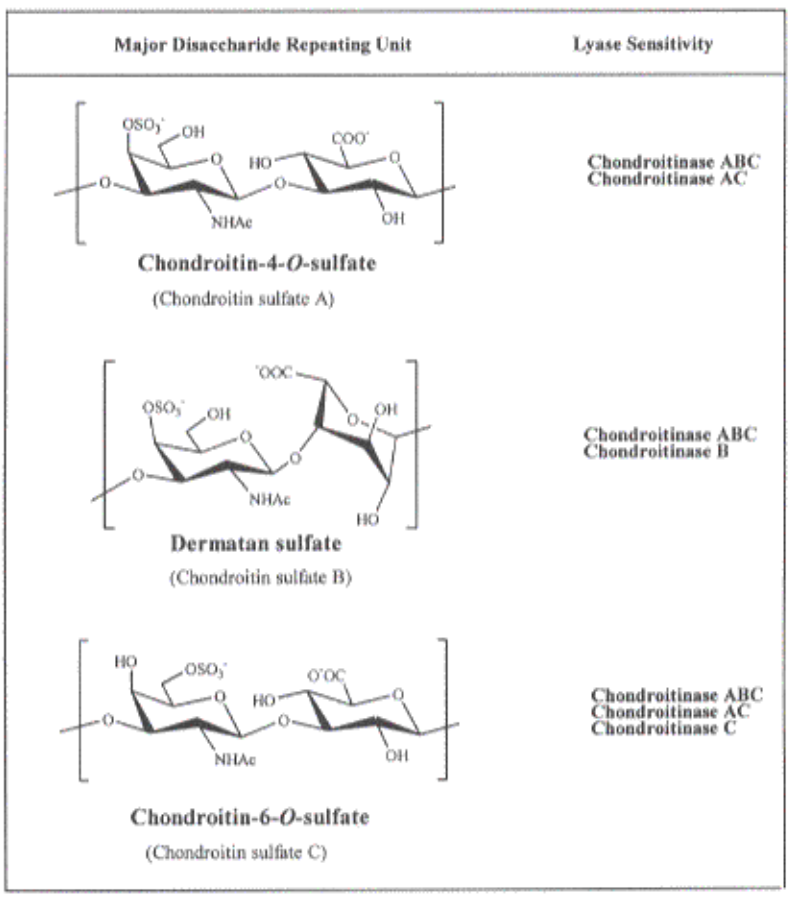

Fig. 1. Glycosidic linkages present in CS/DS and chondroitin lyases that act on these linkages.

Microorganisms are a major source of GAG-degrading enzymes *(3–5)*, particularly in the case of soil bacteria, which may depend on connective tissues in animal carcasses as a nutrient source. Based on their catalytic mechanism, GAG-degrading enzymes are divided into two distinct classes: prokaryotic enzymes, which are lyases that depolymerize GAGs by an elimination mechanism *(5)*, and eukaryotic enzymes, which act by hydrolysis *(6)* (*see* **Fig. 2**). The chondroitin lyases depolymerize the CS and DS, by an elimination mechanism, into oligosaccharides containing a $\Delta_{4,5}$-unsaturated uronic acid residue at the nonreducing end *(3–5)*. This residue exhibits an absorbance maximum at 232 nm, permitting the detection of the oligosaccharide products of the chondroitin lyases using ultraviolet (UV) spectroscopy.

Four classes of chondroitin lyases have been biochemically characterized: those that act on chondroitin, chondroitin-4-sulfate and chondroitin-6-sulfate (chondroitinase

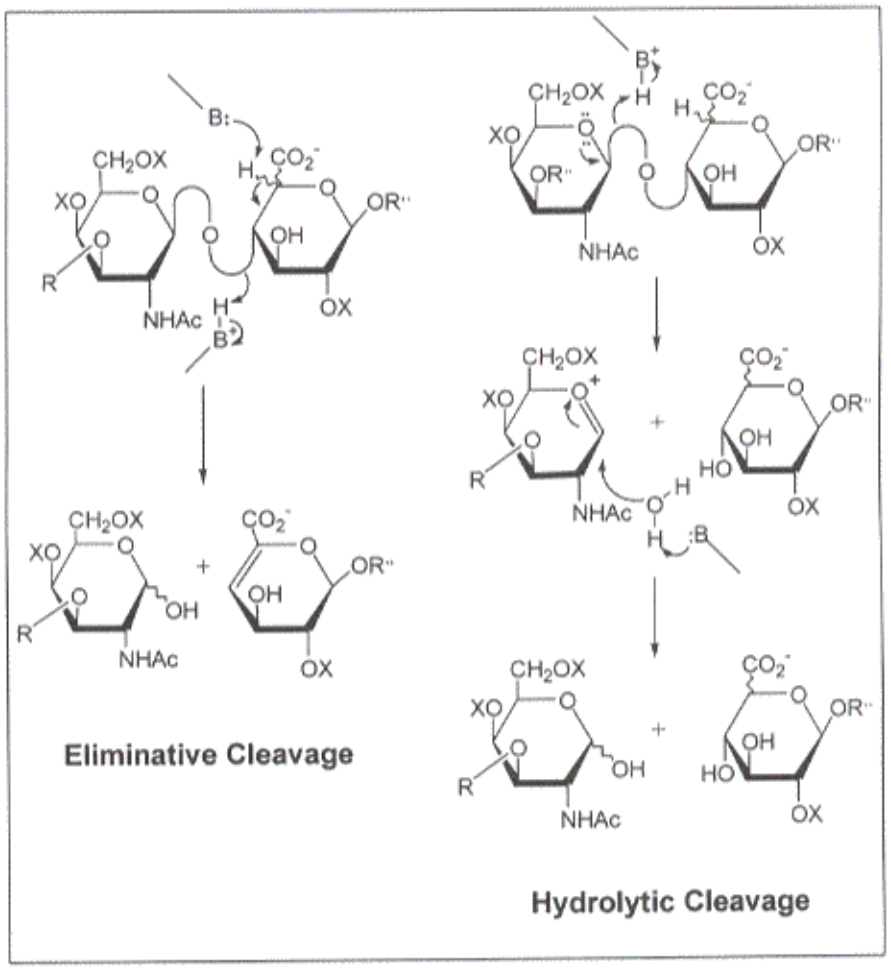

Fig. 2. Enzymatic mechanisms for chondroitin lyases (eliminative cleavage) and chondroitin hydrolases (hydrolytic cleavage), where B is a basic residue in the enzymes catalytic site, R is a monosaccharide (exolytic) or an oligosaccharide (endolytic), R' is H (exolytic) or an oligosaccharide (endolytic), and R" is an oligosaccharide or polysaccharide.

AC or chondroitin AC lyase); dermatan sulfate (chondroitinase B or chondroitin B lyase); chondroitin-6-sulfate and hyaluronate (chondroitinase C or chondroitin C lyase); and an enzyme with broad substrate specificity that acts on both chondroitin and dermatan sulfate (chondroitinase ABC or chondroitin ABC lyase) (*see* **Fig. 1**). Most commercial preparations of chondroitin ABC lyase are a mixture of two enzymes with endo (ABC endolyase) and exo (ABC exolyase) activities *(7)*. The activity of these enzymes toward small oligosaccharide substrates differs substantially. Similarly, there are two chondroitin AC lyases, AC-I (endolyase) and AC-II (exolyase) *(8–9)*.

Table 1
Sources of Commonly Used Chondroitin Lyases

Chondroitinase	Source
Chondroitinase ABC (mixture of endolyase and exolyase) from *Proteus vulgaris*	Sigma Seikagaku
Chondroitinase AC-I from *Flavobacterium heparinum*	Sigma Seikagaku
Chondroitinase AC-II from *Arthrobacter aurescens*	Sigma Seikagaku
Chondroitinase B from *Flavobacterium heparinum*	Sigma Seikagaku
Chondroitinase C from *Flavobacterium heparinum*	Sigma

Chondroitin lyases are most commonly obtained from *Proteus vulgaris*, *Arthrobacter aurescens*, *Bacteroides thetaiotaomicron*, *Bacteroides stercoris*, and *Flavobacterium heparinum*. Chondroitin lyases from *P. vulgaris*, *A. aurescens*, and *F. heparinum* have been purified to homogeneity and are commercially available (*see* **Table 1**). While little is know about the catalytic machinery of these enzymes, the recent publications of the three-dimensional structure of chondroitinase AC and B should shed light on their mechanism of action ***(10–12)***.

Determination of CS/DS oligosaccharide structure is a formidable analytical problem that has limited structure–activity relationship studies, and the development of improved methods is necessary for further progress. Current approaches involve the preparation of CS/DS oligosaccharides using chondroitin lyases followed by separation techniques including gel permeation chromatography (GPC) ***(13)***, strong anion exchange (SAX)-high-performance liquid chromatography (HPLC) ***(14)***, polyacrylamide gel electrophoresis (PAGE), ***(14)*** and capillary electrophoresis (CE) ***(13,15)***, that permit analysis of disaccharide composition. These provide important data on composition and domain structure but generally yield indirect and incomplete sequence information. Mass spectrometry (MS) has also been applied to the analysis of CS/DS oligosaccharides. Fast-atom bombardment (FAB-MS), electrospray ionization (ESI-MS), and matrix-assisted laser desorption/ionization (MALDI-MS) are capable of determining the molecular weight of oligosaccharides ***(13)***. Although nuclear magnetic resonance (NMR) spectroscopy provides for the accurate determination of the chemical fine structure of small CS/DS oligosaccharides (containing 2–14 saccharide units), it requires a large amount of material ***(13,16–18)***.

What follows in this chapter are descriptions of the materials and methods required to use chondroitin lyase enzymes in the degradation of CS/DS-containing sample and how to assay the activity of these enzymes.

2. Materials

2.1. Enzyme Preparation and Storage

1. Tris-HCl/sodium acetate buffer, 50 m*M* (*see* **Table 2** for pH).

Table 2
Properties of the Chondroitinase Lyases

Chondroitin Lyase/Organism	MW (Da)	Buffer system	Opt. pH	Opt *T* (°C)
Chondroitinase ABC from *Proteus vulgaris*	150,000	Tris-HCl/sodium acetate	8.0	37
Chondroitinase ACI from *Flavobacterium heparinum*	76,000	Tris-HCl/sodium acetate	7.5	37
Chondroitinase ACII from *Arthrobacter aurescens*	76,000	Tris-HCl/sodium acetate	6.0	37
Chondroitinase B from *Flavobacterium heparinum*	55,000	Tris-HCl/sodium acetate	7.5	25
Chondroitinase C from *Flavobacterium heparinum*	—	Tris-HCl/sodium acetate	8.0	25

2. Chondroitin lyase. The decision of which lyase to use should be based on the specificity desired (*see* **Fig. 1** and **Table 1**).
3. 500-µL polypropylene microcentrifuge tubes.

2.2. Sample Preparation and Enzymatic Digestion

1. Tris-HCl/sodium acetate buffer, 50 m*M* (for pH *see* **Table 2**).
2. Chondroitin lyase solution.
3. CS- or DS-containing sample.
4. Spectropor dialysis tubing (1000-MWCO Spectrum or Centricon (YM3, & MWCO 3000, Millipore) centrifugal filter unit.
5. 500-µL polypropylene microcentrifuge tubes.

2.3. Assay Protocol

1. Tris-HCl/sodium acetate buffer, 50 m*M* (*see* **Table 2** for pH).
2. Chondroitin lyase solution.
3. CS- or DS-containing sample.
4. 500-mL polypropylene microcentrifuge tubes.
5. UV-spectrophotometer, temperature controlled.
6. 1-mL quartz cuvet.
7. Radiolabel-containing sample.
8. Dialysis membrane (MWCO 1000) or Centricon (YM3, MWCO 3000) centrifugal filter unit.
9. 500-µL polypropylene microcentrifuge tubes.
10. Water baths at 30° and 35°C for enzyme digestion and at 100°C for inactivation of the enzyme reaction.
11. Additional reagents and equipment for product analysis, such as, SAX-HPLC, GPC, PAGE, CE, MS, and NMR.

3. Methods

3.1. Enzyme Preparation and Storage

1. Dissolve the commercial enzyme (*see* **Note 1**) by adding buffer directly to each vial to afford a 4 mU/mL final concentration (*see* **Note 2**). Cap the vials tightly and gently agitate until the solids are completely dissolved.

2. Dispense 10-µL aliquots of the enzyme solution for storage.
3. Store tubes containing enzyme at –60 to –80°C (*see* **Note 3**).

3.2. Sample Preparation and Enzymatic Digestion

3.2.1. Complete Chondroitin Lyase-Catalyzed Depolymerization of a Sample

1. Dissolve sample, containing 1 µg to 1 mg CS or DS, in 1 mL of distilled water. Exhaustively dialyze sample against distilled water using 1000-MWCO dialysis membrane. Freeze-dry the nondializable retentate. Add 50 µL of Tris-HCl/sodium acetate buffer (*see* **Note 4**).
2. Thaw and assay activity of a frozen aliquot of enzyme (*see* **Subheading 3.3.**).
3. Add 40 µL of Tris-HCl/sodium acetate buffer containing CS/DS sample to 10 µL of chondroitin lyase solution in a 500-µL polypropylene microcentrifuge tube. Add 50 µL of Tris-HCl/sodium acetate buffer to another 500-µL polypropylene microcentrifuge tube to serve as a blank control.
4. Additional enzyme (10- to 100-fold) may be required to break down small, resistant oligosaccharides ***(19,20)***.
5. Incubate 50-µL sample for 8–12 h at 37°C (*see* **Notes 5** and **6**).
6. Heat 2 min at 100°C to terminate the reaction (*see* **Note 7**). Analyze the products by a method appropriate for its purity and concentration (*see* **Subheading 1.**).

3.2.2. Complete Chondroitin Lyase-Catalyzed Depolymerization of Radiolabeled GAGs

1. Dissolve GAGs sample containing radiolabeled (^{35}S, ^{14}C, or ^{3}H) CS or DS in 1 mL of distilled water. Exhaustively dialyze sample against water using 1000-MWCO dialysis membrane. Freeze-dry nondialyzable retentate. Add 50 µL of Tris-HCl/sodium acetate buffer. Alternatively, the radiolabeled sample can be buffer exchanged using a Centricon (YM3, 3000 MWCO) centrifugal filter unit.
2. Thaw 10 µL of chondroitin lyase solution at room temperature and use immediately.
3. Add 30 µL of samples containing radiolabeled CS or DS in Tris-HCl/sodium acetate buffer to the 500-µL polypropylene microcentrifuge tube containing enzyme.
4. Add 10 µL of unlabeled CS or DS (1 mg/mL in Tris-HCl/sodium acetate buffer) or 10 mL of Tris-HCL/sodium acetate (*see* **Note 8**).
5. GPC analysis of CS/DS following complete depolymerization by the appropriate chondroitin lyase (*see* **Note 9**) affords counts in fractions corresponding to a molecular weight < 1000 daltons SAX-HPLC or PAGE can also be used with radioisotope detection.

3.3. Assay Protocol

1. Add 640 µL of Tris-HCl/sodium acetate buffer to a 1-mL quartz cuvet. Warm the cuvet to 37°C in a temperature-controlled spectrophotometer (*see* **Note 10**).
2. Thaw a 10-µL aliquot of chondroitinase lyase solution at room temperature.
3. Take the cuvet out of the spectrophotometer, remove 90 µL of warm buffer and transfer it to enzyme solution. Immediately transfer entire 100 µL (buffer plus enzyme) back to the warm cuvet.
4. Place the cuvet in the spectrophotometer and set the absorbance at 232 nm (A_{232}) to zero.
5. Remove the cuvet from spectrophotometer and add 50 µL of 20-mg/mL CS or DS solution to initiate reaction. Seal the cuvet with Parafilm and invert once or twice to mix. Remove the Parafilm and return the cuvet to the spectrophotometer. To assay for chondroitin AC lyase activity, use CS A or C as substrate. To assay for chondroitin B lyase activity, use dermatan sulfate as substrate (*see* **Tables 1** and **2**).

6. Within 30 s after adding substrate begin to measure the absorbance continuously or at 30-s intervals for 2–10 min. Plot A_{232} vs 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.
8. Enzyme activity is calculated as: Enzyme activity = $(\Delta A_{232}/\text{min})$ (700 µL)/ 3800 M^{-1}. (Calculate the number of product molecules formed per substrate molecule from the A_{232} measured at reaction completion.)

4. Notes

1. Protease contamination can also be present in the enzyme preparation. Commercial enzymes often contain bovine serum albumin (BSA) for stabilization during lyophilization, as this greatly reduces potential problems associated with proteolytic contamination.
2. Enzyme activity is defined differently by different suppliers. The definition of a milliunit used here is 1 nmol of unsaturated product formed/min (*see* **Subheading 3.3.** for assay protocol).
3. These enzymes can be stored in their lyophilized or reconstituted states at –20°C or –70°C for >1 yr. Once an enzyme is reconstituted, it should be aliquoted and frozen immediately. Single aliquots can be thawed to assay the enzyme and for use in an experiment. Chondroitinase ABC is very stable, but chondroitinase AC, B, and C are most susceptible to thermal inactivation ***(21,22)***. Lyase storage stability is enhanced by high (> 2 mg/mL) protein concentrations. This is often accomplished by addition of BSA.
4. Chondroitin lyases are compatible with a wide range of buffers, including succinate, acetate, ethylenediamine acetate, Tris-HCl, Bis-Trispropane-HCl, sodium phosphate, MOPS, TES, and HEPES ***(21)***.
5. Samples from of tissues, biological fluids, proteoglycans, and GAGs that contain microgram or greater quantities of CS/DS, which are not metabolically labeled, can be analyzed following treatment with chondroitin lyase.
6. Due to batch variations in enzymes, or the age of laboratory stocks, it is always advisable to test enzyme activities on a standard substrate before using them on valuable samples. This can easily be performed by incubating chondroitin lyase with 1.5 mg/mL of CS and monitoring the time course of the digest by the increase in absorbance at 232 nm. Once the digest appears to have ceased, a second addition of enzyme is useful to confirm that a true end point has been reached, rather than the enzyme having become prematurely inactivated. The quantity of enzyme and/or the incubation time can then be adjusted accordingly to guarantee maximal digestion of samples. The disaccharide yield using the appropriate chondroitin lyase should be > 95 %. Occasionally, CS/DS samples only partially digest, or even fail to digest at all. If the enzyme is selected correctly and is active, then the problem is in the sample quality, i.e., the presence of excess salts and/or buffer ions, certain divalent metals, denaturants, detergents, or other enzyme-inhibitory substances. Further sample clean-up is therefore necessary. Detergents should be removed by precipitation with potassium chloride or by using a detergent-removal column such as Biobeads (Bio-Rad). Urea and guanidine, salts, and metals should be removed by exhaustive dialysis using controlled-pore dialysis membrane (MWCO 1000).
7. 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, adjusted to an acidic pH. The oligosaccharide products are then 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. Also, chondroitin lyases can be immobilized using CNBr Sepharose, removed by filtration after the reaction, and reused ***(23)***.

8. When attempting to use chondroitin lyase to depolymerize radiolabeled samples that contain very small quantities of chondroitin, it is often useful to add cold substrate as a carrier to prevent sample loss.
9. Chondroitin ABC lyase can be used to completely digest a mixture of CS. 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 all GAGs ***(24,25)***. Oversulfated chondroitin sulfates including chondroitin sulfate D, E, and trisulfated chondroitin sulfate are sensitive to chondroitin ABC lyase ***(24,25)***.
10. 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.

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