Enzymatic and Analytical Tools for the Characterization of Chondroitin Sulfate and Dermatan Sulfate Glycosaminoglycans

by

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ABSTRACT

Glycosaminoglycans (GAGs) are complex polysaccharides that reside in the extracellular matrix and on the surfaces of all cells. The same complexity that contributes to the diversity of GAG function has also hindered their chemical characterization. Recent progress in coupling bacterial GAG-degrading enzymes with sensitive analytical techniques has led to a revolution in understanding the structure-function relationship for an important subset of GAGs, namely heparin/heparan sulfate-like glycosaminoglycans (HSGAGs). The study of chondroitin sulfate and dermatan sulfate (CS/DS), an equally important subset of GAGs, has lagged behind partially due to a lack of enzymatic and analytical tools akin to those developed for HSGAGs.

The Flavobacterial heparinas have proven indispensable in characterizing the fine structure of HSGAGs responsible for their different biological functions. As a continuation of ongoing research, a combination of chemical modification, peptide mapping, and site-directed mutagenesis was employed to explore the role of histidine in the activity of heparinase III. Of the thirteen histidines in the enzyme, His295 and His510 were found to be critical for the degradation of heparan sulfate by heparinase III.

As a first step to developing the chondroitinases as enzymatic tools for the characterization of CS/DS oligosaccharides, recombinant expression and purification schemes were developed for chondroitinase AC and B from Flactobacterium heparinum. The recombinant enzymes were characterized using biochemical techniques and kinetic parameters were determined for their respective CS/DS substrates.

By combining the modeling a tetrasaccharide substrate into the active site of chondroitinase B with site-directed mutagenesis studies, a variety of residues were identified as critical for substrate binding and catalysis. A subsequent co-crystal structure of chondroitinase B with DS-derived hexasaccharide revealed a catalytic role for a calcium ion and provided further clarity into the role of individual active site amino acids.

Additionally, using a variety of defined DS-derived oligosaccharides coupled with sensitive analytical techniques, chondroitinase B was identified as an endolytic, non-random, non-processive enzyme that preferentially cleaves longer oligosaccharides compared to shorter ones. Taken together, these studies represent a critical step in developing the chondroitinases as enzymatic tools for the characterization of CS/DS oligosaccharides in a fashion akin to the use of the heparinas to characterize HSGAGs.

Thesis Advisor: Ram Sasisekharan
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Chapter 1:

Introduction to the Glycosaminoglycan Family of Complex Polysaccharides

SUMMARY

The glycosaminoglycan family of complex polysaccharides is composed of several chemically distinct members including heparin/heparan sulfate like glycosaminoglycans (HSGAGs) and chondroitin sulfate and dermatan sulfate (CS/DS) among others. Understanding the structure-function relationships for glycosaminoglycans has traditionally been difficult due to their chemical heterogeneity and their relative scarcity from biological sources. Recent advancements in the characterization of the heparinases, enzyme that specifically degrade HSGAGs, coupled with the application of sensitive analytical techniques have led to a revolution in understanding how specific HSGAG structures mediate a variety of biological phenomena. CS/DS oligosaccharides are also emerging as important regulators biological and pathological processes. However, the biochemical characterization of the chondroitinases, bacterial enzymes that degrade CS/DS, has lagged behind the study of the heparinases. Therefore, future work in developing the chondroitinases as tools for the analysis of CS/DS oligosaccharides will help promote a better understanding of the structure-function relationships for bioactive CS/DS oligosaccharides.
1.1 General Introduction

The extracellular matrix (ECM), once thought of as an inert cellular scaffold, is emerging as an important regulator of cell function through the active modulation of cell-to-cell signaling. Some of the most important participants in this extracellular regulation are the complex polysaccharide known as glycosaminoglycans (GAGs) (Figure 1.1). Historically, these highly charged, heterogeneous polymers once known as “mucopolysaccharides” were thought of as contaminants that need to be removed as not to interfere with the important biopolymers, namely nucleic acids and proteins. The ascension of the field of glycobiology, propelled by advancements in tools and techniques for the study of GAGs, has conclusively demonstrated the biological importance complex carbohydrates in a wide variety of physiological processes and diseases.

Heparin/heparan sulfate glycosaminoglycans (HSGAGs) have led the way largely due to the fact that heparin has been administered clinically for the last half century as an anticoagulant and currently represents a billion dollar industry. Significant biochemical research has expanded the study of HSGAGs beyond its anticoagulation to demonstrate an active role for the complex carbohydrates in a diversity of processes such as cellular development, angiogenesis, and viral adhesion. Another biologically important subset of the GAG family, namely chondroitin sulfate and dermatan sulfate (CS/DS), also has been garnering recent interest due to their emerging roles in regulating anticoagulation, cartilage function, axon growth, and cell-to-cell signaling. Despite the significant advances that have been made in understanding the function of these information dense GAGs, further efforts
need to be focused on the development of better tools for the structure-function analysis of HSGAGs and CS/DS.

The research outlined in this thesis is focused on developing comparable tools for the analysis of CS/DS oligosaccharides to those that have been developed for HSGAGs. Chapter 1 provides a detailed discussion of the previous efforts in developing primarily the heparinases as tools to study the biology of HSGAGs with an accompanying discussion of the prior characterization of the chondroitinases and the emerging biology of CS/DS. The original research in Chapter 2 continues the characterization of the heparinases by exploring the role of histidine in the enzymatic activity of heparinase III. Using this study as a foundation, Chapter 3 begins the analysis of chondroitinase AC and B by developing a recombinant expression system for the enzymes, characterizing their respective substrate specificities, and assigning kinetic parameters for their catalytic activity. Chapter 4 is composed of two separate studies that detail the exhaustive characterization of the specific active site amino acids involved in the catalytic activity of chondroitinase B including the first evidence for a role of calcium in the active site of this enzyme. Chapter 5 completes the characterization of chondroitinase B by exploring the mode of action of the enzyme using sensitive analytical techniques and defined DS oligosaccharides. Taken together, the research discussed in this thesis is a significant step forward in the development and the application of the chondroitinases to the analysis of bioactive CS/DS oligosaccharides in a fashion analogous to the use of the heparinases in characterizing HSGAGs.
1.2 Glycosaminoglycan Structure

The GAG family of complex polysaccharides includes heparin, heparan sulfate, chondroitin sulfate, dermatan sulfate, keratan sulfate, and hyaluronic acid (Table 1.1) [1]. Each of these GAGs is synthesized in the Golgi as a polymer of an uronic acid linked to a hexosamine, thereby composing the common disaccharide repeat unit by which each chain is described. In addition, initiation of the synthesis each of each of these GAG chains, with the sole exception of hyaluronic acid, begins with the attachment of a linkage sequence to a core protein at a serine residue forming a proteoglycan. As synthesis of the GAG polymer proceeds, a variety of modifying enzymes refine the structure of the expanding backbone by selectively sulfating different positions of both the uronic acid and the hexosamine as well as by epimerizing the C5 position of glucuronic acid (GlcA) to iduronic acid (IdoA) [1]. The wide range of biological events regulated by GAGs is a direct consequence of the structural and chemical diversity generated during their biosynthesis.

Table 1.1: Summary of GAG structures

<table>
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<th>Glycosaminoglycan</th>
<th>Basic Disaccharide</th>
<th>Potential Modifications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chondroitin sulfate</td>
<td>-GlcA-β(1,3)-GalNac-β(1,4)-</td>
<td>4-O or 6-O sulfation</td>
</tr>
<tr>
<td>Dermatan sulfate</td>
<td>-IdoA-α(1,3)-GalNac-β(1,4)-</td>
<td>2-O, 4-O, and 6-O sulfation</td>
</tr>
<tr>
<td></td>
<td>-GlcA-β(1,3)-GalNac-β(1,4)-</td>
<td></td>
</tr>
<tr>
<td>Heparin</td>
<td>-IdoA-α(1,4)-GlcNac-α(1,4)-</td>
<td>N-deacetylation / N-sulfation, C5 epimerization,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2-O, 3-O, and 6-O sulfation</td>
</tr>
<tr>
<td>Heparan sulfate</td>
<td>-GlcA-β(1,4)-GalNac-α(1,4)-</td>
<td>N-deacetylation / N-sulfation, C5 epimerization,</td>
</tr>
<tr>
<td></td>
<td>-IdoA-α(1,4)-GlcNac-α(1,4)-</td>
<td>2-O, 3-O, and 6-O sulfation</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td>-GlcA-β(1,3)-GalNac-β(1,4)-</td>
<td>No modifications</td>
</tr>
<tr>
<td>Keratan sulfate</td>
<td>-Gal-β(1,4)-GalNac-β(1,3)-</td>
<td>6-O sulfation</td>
</tr>
</tbody>
</table>

Hyaluronic acid (HA), the simplest of all the GAGs, is composed of the basic disaccharide unit of a glucuronic acid β(1→3) linked to N-acetyl galactosamine (Table 1.1) [1]. This disaccharide unit is linked β(1→4) to form polymers of up to 25,000 disaccharides in length and does not contain any additional modifications. Keratan sulfate (KS) has the basic disaccharide repeat of galactose β(1→4) linked to N-acetyl glucosamine. These basic units are β(1→3) linked to adjacent disaccharides to form KS polymers of up to 100 disaccharides in length (Table 1.1) [1]. KS can be modified at the 6-O position of either the Gal or the GlcNac moiety. The primary structures of HSGAGs and CS/DS are discussed in detail in the following sections.
Heparin and heparan sulfate

HSGAGs are synthesized as a linear chain of 20-100 disaccharide units of N-acetylated D-glucosamine α(1->4) linked to glucuronic acid (Table 1.1) [2]. Modifications to this basic disaccharide include O-sulfation at the 6-O and 3-O positions of the glucosamine and at the 2-O position of the uronic acid. Additionally, the glucosamine can be modified by N-deacetylation followed by N-sulfation. Finally, C5 epimerization can change GlcA to IdoA (Figure 1.2) [3]. Taking into account all of the possible modifications yields 48 unique disaccharides that could be found in an HSGAG chain [4]. While all of these disaccharide combinations have not been reported in nature, this analysis still underscores the fact that HSGAGs contain more informational density than either DNA (composed of 4 bases) or proteins (composed of 20 amino acids). These modifications provide the structural heterogeneity that results in the biological diversity of HSGAG function, as well as complicates the study of the underlying structure activity relationships behind these biological functions [2].

Within a single HSGAG chain there exists regions of aggregate character that further assist in defining their biological role [3,5]. However, it is important to note that the heparin and heparan sulfate nomenclature traditionally used to describe these differentially modified regions is counterintuitive. The names would suggest that heparan sulfate is more sulfated than heparin.
when in fact the opposite is true. In a broad sense, “heparin-like” regions tend to be highly sulfated at the 2-O, 6-O, and N positions, as well as contain predominantly the IdoA epimer [1,2]. On the other hand, regions of “heparan sulfate-like” character tend to have a lower degree of 2-O and N sulfation, as well as a higher GlcA content than heparin (Figure 1.2). Therefore, a single HSGAG chain can be thought of as containing “heparin-like” and “heparan sulfate-like” regions each with different potential biological functionalities (Figure 1.2). An important additional distinction between heparin and heparan sulfate is based on their physiological location. Heparan sulfate is found on the surface of most cells and in the ECM, while heparin is synthesized solely in mast cells where it performs a distinct biological function [5]. A more complete examination of the biological roles of HSGAGs, especially heparan sulfate, is provided below.

**Chondroitin sulfate and dermatan sulfate**

Despite the chemical similarities, there are a number of differences that assist in distinguishing CS/DS from HSGAGs. Unlike HSGAGs, CS/DS oligosaccharides are synthesized as a disaccharide repeat of glucuronic acid β(1->3) linked to N-acetylated galactosamine, a defining chemical feature of CS/DS (Table 1.1) [1]. This basic repeat unit is in turn β(1->4) linked to adjacent disaccharides to form polymeric CS/DS. Similar to HSGAGs, the basic CS/DS chain is differentially modified through sulfation and epimerization (Figure 1.2). These modifications are used to divide CS/DS into 3 major subtypes [1,6]. Chondroitin 4-SO₄ is primarily sulfated at the 4-O position of the galactosamine residue and is composed of GlcA epimers at the C5 position of the uronic acid moiety. Chondroitin 6-SO₄ is sulfated at the 6-O position of the galactosamine residue and also contains primarily the GlcA epimer of the uronic acid. Dermatan sulfate, also known as chondroitin sulfate B, is defined by a 4-O sulfated galactosamine and predominantly the IdoA epimer, although GlcA is mixed in to differing degrees depending on the biological source of the DS (Figure 1.2) [1]. In addition, minor fractions of 2-O, 4-O and 4-O, 6-O disulfated disaccharides are found in DS and contribute to the biological activity of this GAG polymer [1,7,8]. Rare sulfation at the 3-O position of the uronic acid moiety has also been reported in CS/DS isolated from king crab, but has never been described for mammalian isolates [9]. While CS/DS polymers contain fewer potential structural modifications than HSGAGs, their overall diversity is nonetheless significant.
The presence of the IdoA epimer in DS is a distinguishing factor of DS from CS and actually makes DS more chemically similar to IdoA-rich regions of HSGAGs (Figure 1.2) [8]. Conformational studies of IdoA in HSGAG oligosaccharides have revealed that this epimer can exist in a variety of energetically favorable conformations while the GlcA form is relatively rigid [10,11]. Additionally, the conformation that a specific IdoA residue occupies appears to be dictated by its neighboring sulfation pattern as well as its overall location (internal vs. terminal) in the HSGAG oligosaccharide [1,10]. An initial insight into this importance was suggested by data showing that IdoA containing GAGs are more efficacious in inhibiting the proliferation of fibroblasts than GlcA containing GAGs [8,12]. A recent study relying on a combination of capillary electrophoresis (CE) and nuclear magnetic resonance (NMR) demonstrated that the flexibility of HSGAG chains at IdoA residues plays a direct role in the binding of a specific oligosaccharide sequence to basic fibroblast growth factor (FGF), a model system often used for studying GAG:protein interactions [13]. Therefore, the presence of IdoA in DS likely plays a critical role in the specific binding of proteins to sequences in the oligosaccharide [8]. This fact is underscored by the observation that HSGAGs and DS oligosaccharides are implicated in a similar set of biological phenomena as discussed below.

1.3 GAG Degrading Enzymes as Biochemical Tools

While the field of GAG biology has been expanding at an extraordinary pace, specific structure-function relationships for this important class of biopolymer has lagged behind the progress made on other important biopolymers, namely, nucleic acids and proteins. The structural complexity of GAGs and their highly charged nature (due to the presence of the carboxylate moieties and differential sulfation) has limited the extension of traditional biochemical approaches that have proven successful for DNA and proteins. An additional limitation is that, at the present time, there is no means to amplify particular GAG sequences, unlike with DNA (i.e., application of PCR) or proteins (i.e., recombinant expression). Therefore, there was an unmet biochemical need for tools tailored for the rapid and sensitive analysis of minute amounts of GAGs.

GAG-degrading enzymes, specifically those isolated from the ubiquitous soil bacterium Flavobacterium heparinum, have proven invaluable in the characterization of the structure and function of the different GAGs [1]. The most developed of the GAG-degrading enzymes are the three heparinas that specifically degrade HSGAGs. The cloning, recombinant expression and purification, and exhaustive biochemical characterization of these
enzymes has greatly increased their utility as tools for the characterization of HSGAG structure-function relationships. The chondroitinases, bacterial derived lyases that specifically degrade CS/DS, have been the focus of some initial biochemical characterization, but remain largely unexplored when compared to their heparinases counterparts. The predominant focus of this thesis work was the biochemical characterization of the chondroitinases to enable their use as tools for the analysis of CS/DS in a fashion similar to the application of the heparinases to the study of HSGAGs.

The heparinases and the chondroitinases, as well as other GAG-degrading enzymes from bacterial sources, degrade their substrates through a lytic enzymatic mechanism (Figure 1.3). The first step in this concerted β-elimination mechanism originally proposed by Gassman and Gerlt [14] is the abstraction of the C5 proton on the hexosamine moiety. This is usually performed by a basic amino acid in the enzyme and results in the formation of an enolate intermediate. The enzyme stabilizes this carbanion usually via a positively charged, hydrophilic amino acid [14,15]. The final step of reaction mechanism involves protonation of the anomic oxygen by an acidic residue with concomitant β-elimination of the uronic acid resulting in an unsaturated Δ4,5 bond [14,15]. Importantly, this double bond has UV absorbance with a $\lambda_{\text{max}}$ at 232 nm thereby providing an internal chromophore for measuring the formation of the reaction products (Figure 1.3).

The lytic mechanism outlined above is in stark contrast to the hydrolytic mechanism common to GAG-degrading enzymes isolated from mammalian sources. The majority of the mammalian GAG-degrading enzymes isolated thus far are lysosomal and are referred to as “exo-enzymes” based on their exolytic mode of action [16,17]. These enzymes are often
membrane associated and function at low pHs making their application as biochemical tools more difficult. In addition to the exo-enzymes, mammalian heparanase have also been isolated and characterized [18-21]. Heparanase is unique from the other mammalian hydrolases in that it is primarily endolytic, it is found extracellularly, and it is responsible for the degradation of heparan sulfate. Heparanase’s pattern of expression and its extracellular localization imply a role for the enzyme in the normal maintenance of tissue architecture, as well as in the progression of the cancer [18]. However, heparanase has a limited substrate specificity that has proven difficult to biochemically define thus decreasing its utility as a biochemical tool [18]. Therefore, the bacterial-derived heparinases, due to their relative amenability to biochemical characterization, have been the most useful enzymatic tools for the analysis of HSGAGs.

**Biochemical Characterization of the Heparinases**

The heparinase family of GAG-degrading lyases is composed of three members, heparinase I, II and III, each of which has a unique substrate specificity (Table 1.2) [6,22-24]. A wide-range of analytical techniques including HPLC [22], capillary electrophoresis (CE) [25,26], polyacrylamide gel electrophoresis (PAGE) [6,22], and NMR [23] have been used to examine the product profile of each of the heparinases with their respective substrates yielding a comprehensive understanding of their reaction kinetics and substrate specificities [1]. Generally speaking, heparinase I cleaves highly sulfated HSGAG sequences (traditionally characterized as “heparin”) that contain a high IdoA content [1,6]. Heparinase III is specific for regions with lower levels of sulfation that contain a higher content of GlcA and N-acetylated glucosamine (traditionally characterized as “heparan sulfate”). Heparinase II has the broadest substrate specificity of the family of enzymes capable of cleaving HSGAG oligosaccharides with varying degrees of sulfation and regardless of the presence of GlcA or IdoA [1,6] (Table 1.2).

Traditionally, the use of the heparinases as tools for the characterization of HSGAGs was impaired by the difficulty on separating the native Flavobacterial enzymes from one another [23,24]. The isolation was further complicated by the co-purification of contaminating enzymes, such as HSGAG-specific sulfatases, which could interfere with the characterization of HSGAG oligosaccharides. Therefore, an obvious need existed for the development of a purification scheme that resulted in homogeneous preparations of each of
Table 1.2: Summary of HSGAG-degrading enzyme biochemistry

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Size</th>
<th>Specificity</th>
<th>Active Site Residues</th>
<th>Mode of Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparinase I</td>
<td>41.7 kDa</td>
<td>GlcNS,6X-IdoA2S&lt;sup&gt;a&lt;/sup&gt;</td>
<td>C135, H203, K199, CB-1</td>
<td>Exolytic, Processive</td>
</tr>
<tr>
<td>Heparinase II</td>
<td>84.1 kDa</td>
<td>GlcNY,6X-UA2X&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>H238, H451, H579, C348</td>
<td>Endolytic, Non-processive</td>
</tr>
<tr>
<td>Heparinase III</td>
<td>70.8 kDa</td>
<td>GlcNY,6X–IdoA/GlcA &amp; GlcNac–IdoA/GlcA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>N.D.&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Endolytic</td>
</tr>
<tr>
<td>Heparanase</td>
<td>50.0 kDa</td>
<td>IdoA2S-GlcNS,6S-GlcA–GlcNS,3S,6S-IdoA2S-GlcNS,6S</td>
<td>N.D.&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Endolytic</td>
</tr>
</tbody>
</table>

= bond cleaved by enzyme  
<sup>a</sup> X = sulfated or unsubstituted, Y = acetylated or sulfated  
<sup>b</sup> UA = GlcA or IdoA  
<sup>c</sup> N.D. = not determined

the heparinases devoid of any contaminating enzyme activity. The cloning of the genes for each of the three heparinases from the *F. heparinum* genome enabled the recombinant expression of the enzymes in *E. coli*. Facile purification techniques relying on the presence of a N-terminal 6X His-Tag led to the production of milligram quantities of each of the recombinant heparinases. Therefore, the recombinant expression and purification of the heparinases solved the difficult problem of native isolation and yielded large amounts of pure enzyme, thus enabling the biochemical characterization of each of the three heparinases.

**Heparinase I**

The development of the recombinant expression and purification system for heparinase I facilitated the biochemical characterization of the specific residues involved in substrate binding and catalysis [27]. An exhaustive series of studies relying on specific chemical modification, peptide mapping and site-directed mutagenesis identified cysteine 135 and histidine 203 as critical for the degradation of HSGAGs by heparinase I [28-30]. In addition, heparinase I contains two positively charged clusters, named HB-1 and HB-2, respectively that were thought to bind to HSGAG substrates. Systematic site-directed mutagenesis of selected basic residues in both HB-1 and HB-2 demonstrated that the positive charge of the HB-1 region has a pronounced effect on heparinase I activity. Specifically, site-directed mutagenesis of K199A resulted in a 15-fold reduction in catalytic activity, whereas a K198A mutation produced a 2- to 3-fold reduction in heparinase I activity. Unlike with
mutations in HB-1, mutations in HB-2 had much less of an effect on heparinase I activity [31]. Therefore, Cys135 was proposed as the general base responsible for the abstraction of the C5 proton from the uronic acid moiety. His203 also appears to play a direct role in catalysis by potentially donating a proton to the leaving β-substituent. Finally, Lys199 as the third residue in the catalytic triad was proposed either to stabilize the negative charge on the uronic acid intermediate or to stabilize the unprotonated thiol group of the Cys135 (Table 1.2) [30].

Heparinase I also contains two regions that were recognized to bind calcium, a common cofactor in GAG-degrading enzymes. The first site (CB-1), which extends from Glu207 to Ala219 and is proximal to the active site, contains multiple potential chelation sites for calcium [32,33]. In addition, this site is proximate to HB-1 in heparinase I. The second site (CB-2), which is located at the C-terminus of the protein and extends from Thr373 through Arg384, also plays an important role in calcium binding and heparinase I activity [32,33]. Site-directed mutagenesis of these residues in CB-1 and CB-2 led to a decrease in calcium binding with a concomitant loss of enzyme activity [33]. CB-1 likely chelates a calcium ion that functions as a Lewis acid in stabilizing the negative charge on the carboxylate group at the C5 position of the uronic acid. This stabilization is required to reduce the pKa of the C5 proton enabling its abstraction by Cys135. The binding of calcium to CB-2 is also required for the proper catalytic function of heparinase I perhaps by assisting in the processing of polymeric HSGAG substrates (Table 1.2) [33].

To augment the identification of critical residues in heparinase I, MALDI-MS and a battery of defined HSGAG-derived oligosaccharides were used to study the mode of action of the enzyme [34,35]. The time-resolved mass identification of both the substrate and the reaction intermediates during the course of the enzymatic degradation enabled the unambiguous determination of whether heparinase I is exolytic or endolytic and whether the

![Figure 1.4: Heparinase I is an exolytic, processive enzyme. Using defined oligosaccharides, heparinase I was determined to cleave its polymeric substrate processively, starting at the non-reducing end and proceeding to the reducing end.](image-url)
enzyme processively cleaves the substrate without releasing it. Heparinase I is predominantly an exolytic, processive enzyme that cleaves HSGAG substrates from the non-reducing to reducing end (Table 1.2). Thus, in an initial step, heparinase I preferentially cleaves exolytically at the non-reducing terminal linkage of the HSGAG oligosaccharide, although it also cleaves internal linkages at a detectable rate. In a second step, heparinase I has a strong preference for cleaving the same substrate molecule processively at the next cleavable linkage toward the reducing end of the HSGAG chain (Figure 1.4) [34].

The biochemical studies outlined above have provided a more complete understanding of the catalytic function of heparinase I. A final piece to the heparinase I story would be obtaining an X-ray crystal structure of the enzyme outlining in detail the location of each of the active site residues proposed above. In fact, significant effort by several research groups has focused on obtaining such a structure without avail. Regardless, the understanding of heparinase I's biochemical activity has increased the utility of the enzyme as a tool for the analysis of bioactive HSGAG substrates.

**Heparinase II**

Similar to the exhaustive studies completed with heparinase I, the cloning and recombinant expression of heparinase II enabled biochemical studies that addressed the essential amino acid residues within the enzyme [36]. Chemical modification with DEPC, a histidine specific reagent, coupled with protease mapping studies, identified His238, His451, and His579 as solvent accessible, putative active site residues. Mutation of each of these three residues to alanine resulted in inactivation of the enzyme toward both heparin and heparan sulfate, thus identifying these residues as being critical for the proper enzymatic function of heparinase II (Table 1.2) [37].

An additional series of chemical modification experiments identified one of the three cysteines (Cys164, 189, and 348) in heparinase II as surface-accessible [38]. The cysteine, present in an ionic environment, was mapped by radiolabeling with N-[3H]ethylmaleimide and identified as Cys348. Site-directed mutagenesis of this residue to an alanine resulted in loss of activity toward the highly sulfated heparin substrate but not the under-sulfated heparan sulfate substrate, indicating that Cys348 is required for heparinase II activity toward heparin but is not essential for the breakdown of heparan sulfate (Table 1.2) [38]. This important observation implies that there is a unique set of residues within the active site of heparinase II
responsible for cleaving highly sulfated HSGAGs and a separate set for degrading heparan sulfate-like oligosaccharides.

Like heparinase I, the mode of action of heparinase II was also examined using a variety of defined HSGAG-derived oligosaccharides and MALDI-MS [39]. In contrast to heparinase I, mass spectral analysis indicates that heparinase II cleaves HSGAGs endolytically in a non-random manner. In addition, heparinase II releases the reaction intermediates, suggesting that unlike heparinase I the enzyme has a non-processive mode of action (Table 1.2). Furthermore, a comparison of the product patterns of heparinase II with the C348A mutant with both heparin and heparan sulfate as substrates confirms the observation that the enzyme has two independent sets of residues within the active site responsible for cleaving each of the respective HSGAG substrates [39].

**Heparinase III**

The heparinase III gene has been cloned from *F. heparinum* [40], but the biochemical characterization of heparinase III has lagged behind the characterization of the other two heparinases due to the lack of adequate recombinant expression system for the enzyme [36]. Therefore, biochemical studies of the enzyme have been limited to a comparison of the substrate specificity of the native and recombinant forms by directly using the crude extract from the *E. coli* [41]. While the sequence homology between the three heparinases is limited, it is important to note that heparinase III does not contain any cysteines in its primary structure [41]. This is a particularly interesting observation considering that Cys135 is required for the degradation of heparin by heparinase I and Cys348 is required for the degradation of heparin, but not heparan sulfate, by heparinase II [38]. Therefore, while cysteine is required for the degradation of heparin, this amino acid is not involved in the degradation of heparan sulfate by any of the Flavobacterial heparinases. By the same logic, the requirement of histidine for the degradation of heparan sulfate by heparinase II makes this residue a likely candidate for playing a catalytic role in heparinase III. Chapter 2 of this thesis continues the biochemical characterization of the heparinases by examining the role of histidine in the activity of heparinase III.
Biochemical Characterization of the Chondroitinas

Similar to the heparinas, significant research has focused on the isolation and characterization of native chondroitinas from a variety of different bacterial sources. As is the case with the bacterial heparinas, \textit{F. heparinum} is an important source of all four chondroitin lyases, chondroitinase AC, B, C, and ABC (Table 1.3) \footnote{The nomenclature for the chondroitinas is based on their respective substrate specificity. Therefore, chondroitinase AC is capable of cleaving chondroitin A (chondroitin 4-sulfate) and chondroitin C (chondroitin 6-sulfate). Chondroitinase C cleaves only chondroitin C, while chondroitinase B is the only known enzyme that specifically degrades dermatan sulfate (chondroitin B). Chondroitinase ABC, in an analogous fashion to heparinase II, has the broadest substrate range and can de-polymerize all of the CS/DS isomers, as well as HA. These substrates specificities were identified through biochemical studies on the native enzymes isolated directly from \textit{F. heparinum}} \footnote{6}.

**Table 1.3:** Summary of CS/DS-degrading enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Size</th>
<th>Specificity</th>
<th>Bacterial Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chondroitinase AC</td>
<td>77.7 kDa</td>
<td>GalNac,4S=GlcA &amp; GalNac,6S=GlcA</td>
<td>\textit{F. heparinum}</td>
</tr>
<tr>
<td>Chondroitinase B</td>
<td>54.0 kDa</td>
<td>GalNac,4S=IdoA2X*</td>
<td>\textit{F. heparinum}</td>
</tr>
<tr>
<td>Chondroitinase C</td>
<td>~ 70 kDa</td>
<td>GalNac,6S=GlcA</td>
<td>\textit{F. heparinum}</td>
</tr>
<tr>
<td>Chondroitinase ABC</td>
<td>&gt; 100 kDa</td>
<td>GalNac,4S=GlcA/IdoA &amp; GalNac,6S=GlcA/IdoA</td>
<td>\textit{F. heparinum, P. vulgaris}</td>
</tr>
</tbody>
</table>

= bond cleaved by enzyme
* X = sulfated or unsubstituted

While the genes for chondroitinase AC and B have been cloned, the genes for chondroitinase C and ABC still have yet to be isolated from \textit{F. heparinum} \footnote{[42,43]}. However, chondroitinase ABC from \textit{P. vulgaris} has been exhaustively characterized and represents the major commercial source for the enzyme \footnote{[44]}. Interestingly, \textit{P. vulgaris} seems to express two unique isoforms of the enzyme. The first isoform, chondroitinase ABC I (Mr = 100,000), is characterized by an endolytic mode of action while chondroitinase ABC II (Mr = 105,000) is primarily exolytic and is unable to cleave a tetrasaccharide substrate (Table 1.3) \footnote{[45,46]}. The traditional, commercial chondroitinase ABC is a mixture of the two activities while the highly purified “Protease Free” preparation contains just the exolytic chondroitinase ABC II. In
addition, one of the chondroitinase ABC enzymes has been cloned from *P. vulgaris*, although an adequate recombinant expression and purification system for chondroitinase ABC has yet to be reported [44].

The use of the chondroitinases as biochemical tools has suffered from the same initial problems as the heparinases, namely it has been difficult to isolate large quantities of pure chondroitinase without contaminating sulfatase or other chondroitinase activity [47]. The cloning of the genes for chondroitinase AC and B and the recombinant over-expression of the enzymes in *F. heparinum* represented a significant advance in the production of these enzymes [48,49]. However, the isolation of chondroitinases from Flavobacterial sources remained laborious, resulting only in approximately 300 µg of enzyme from an initial fermentation of 10 L [47]. Clearly, there was an obvious need for the development of an *E. coli*-based recombinant expression system for chondroitinase AC and B similar to those developed for the heparinases. This initiative was undertaken as part of this thesis work and is detailed in Chapter 3.

The most significant difference between the biochemical characterization of the heparinases and the characterization of the chondroitinases is the existence of published crystal structures for chondroitinase AC and B [50,51]. These structures can serve as "road maps" towards understanding the role of individual amino acids in the activity of each enzyme and obviate the need for the labor-intensive chemical modification studies such as those that were performed on the heparinases. The crystal structures of chondroitinase AC alone as well as complexed with a DS hexasaccharide, a DS tetrasaccharide, and a hyaluronic acid tetrasaccharide have led to the identification of a putative active site near the N-terminal of the protein [50]. Subsequent site-directed mutagenesis and recombinant expression in *F. heparinum* identified His225, Tyr234, and Arg288 as critical active site residues that likely play a direct role in catalysis [52]. In addition, Arg292 when mutated to alanine retained partial enzymatic activity consistent with its suspected role in substrate binding. Kinetic studies with the R292A mutant demonstrated an increase in *Km* with no change in *Vmax* when compared to the native chondroitinase AC [53]. Therefore, Arg292 while no directly involved in catalysis likely plays a role in the processing of the chondroitin sulfate substrate in chondroitinase AC.

Chondroitinase B has also been crystallized both alone and with a DS disaccharide assisting in the identification of the active site [51]. However, since the disaccharide represents a minimal reaction product of the depolymerization of DS by chondroitinase B, the authors were unable to assign the role of specific active site residues in substrate binding and catalysis. Therefore, a need existed for
either further co-crystal structures or molecular modeling to identify specific amino acids that might be involved in catalysis. Chapter 4 of this thesis describes a modeling study along with site-directed mutagenesis that was undertaken to provide clarity on the role of specific residues in the activity of chondroitinase B.

1.4 Structural Determination of HSGAGs

GAG-degrading enzymes have proven indispensable in the isolation and characterization of biologically relevant oligosaccharides. In addition, a variety of analytical techniques have been applied to the structural analysis of these isolated oligosaccharides. Compositional analysis of GAGs using HPLC or CE involves exhaustive digestion of the prepared oligosaccharide fragment using the GAG-degrading enzymes followed by detection using UV absorbance of the $\Delta^{4,5}$ ($\lambda_{232}$) bond or by fluorescent tagging and detection [54,55]. In the case of HPLC and CE, the observed retention times of disaccharides are characterized by comparison with retention times of known disaccharide standards. $^1$H NMR is one the most reliable and frequently used methods for sequencing purified GAG oligosaccharides [56]. However, NMR analysis requires large amounts of starting material (100s of micrograms to a milligram) for complete assignment of the structure. Mass spectrometry methods including electrospray ionization (ESI) [57], fast atom bombardment (FAB) [58,59] and, more recently, MALDI have been developed for fingerprinting GAG polysaccharides. The sulfate composition of the sodium salt of an oligosaccharide is obtained from its quasimolecular ion $[M - nH + (n-1) Na]^+$ that is detected, where $n$ is the number of sulfate groups. A major drawback of detecting oligosaccharides as sodium salts using ESI-MS and FAB-MS or MALDI-MS methods is the interpretation of the complex spectra of highly sulfated oligosaccharides. Further, in the case of MALDI-MS the lability of the free sulfate groups can lead to inaccuracies in the interpretation of the spectrum.

Direct Applications of the Heparinases

The biochemical characterization of the heparinases has led to the development of the degrading enzymes as tools for the biochemical characterization of HSGAGs. This section focuses on combining the heparinases with the sensitive tools of MALDI-MS and CE to develop novel technologies for the sequencing (PEN-MALDI) and characterization (SNA-MS) of biologically important HSGAGs. The information gained from the application of these two techniques has shed
light on the structure-function relationship for different HSGAG sequences in unique biological contexts.

![Diagram of disaccharide structures](image)

**Figure 1.5:** PEN scheme and mass line for HSGAG oligosaccharides. (A) Disaccharide repeat unit of HSGAGs. X indicates the site of sulfation and Y indicates site of sulfation or acetylation. (B) PEN scheme for the 32 disaccharide building blocks. (C) Mass line of all the possible di-, tetra- and hexasaccharides of HSGAGs. Y axis indicates the number of unique fragments for the corresponding mass in X axis. This demonstrates that using MALDI-MS the length and composition of an HSGAG oligosaccharide can be assigned based on its mass.

**PEN-MALDI**

The heparinases have been combined with the sensitive analytical technique of MALDI-MS and a bioinformatics framework for the identification of individual GAG disaccharides to develop the property-encoded nomenclature MALDI (PEN-MALDI) sequencing technology [60]. The methodology was developed with the express purpose of using as little sample as possible (thus requiring the sensitivity provided by MALDI-MS), while still insuring that it was practical and rapid.

The property-encoded nomenclature system identifies the differential modification in each of the 32 possible HSGAG disaccharides by assigning a binary digit to each of the 4
modifiable positions depending on its modification state (0 for no sulfate and 1 for presence of a sulfate) (Figure 1.5) [60]. The attractive feature of this coding scheme is that the HSGAG chain can be easily represented as a string of one-letter codes from the non-reducing to the reducing end (or in the opposite direction) of the polysaccharide chain in a notation that is analogous to a DNA or a protein sequence. Integration of the flexible notation scheme with the mass accuracy attainable from MALDI-MS analysis of HSGAG oligosaccharides allowed for the establishment of important mass-identity relationships, as well as a master list of all possible saccharide sequences with their theoretical molecular masses (Figure 1.5).

With this information, a unique master list is constructed for a sample based on all possible sequences that contain the disaccharide units observed in the initial compositional analysis. The masses of oligosaccharide fragments generated from enzymatic digestion are applied as experimental constraints and sequences that do not satisfy these constraints are eliminated from the initial list. In an iterative manner, moving from experimental constraints to the ever-decreasing master list of possible sequences, one can to rapidly arrive at a unique sequence solution using a minimal material. Importantly, multiple pathways, using separate experimental constraints, can be used to converge on a sequence, ensuring assignment accuracy.

This PEN-MALDI methodology was applied to the sequencing of a biologically and pharmacologically relevant HSGAG decasaccharide with known affinity for antithrombin-III (AT-III). The sequence of the decasaccharide used in this study was determined to be $\Delta U_{25}H_{NS,65}I_{25}H_{NS,65}I_{25}H_{NS,65}I_{H_{NAc,65}G_{H_{NS,35,65}} (\pm DDD4-7 \text{ in PEN}) [61]$. A surprising result was that this decasaccharide sequence did not contain an intact AT-III binding site ($I_{H_{NAc,65}G_{H_{NS,35,65}}I_{25}H_{NS,65}}$). Further studies indeed showed that this decasaccharide possessed a lower capability of binding to AT-III and, in turn, a decrease in anticoagulant activity when compared to a synthetic pentasaccharide containing an intact AT-III binding site [62].

**SNA-MS**

Previous efforts to identify the exact HSGAG sequence responsible for the binding of a specific protein, while fruitful in certain cases, often failed due to the scarcity of HSGAGs from biological sources. These techniques relied on oligosaccharides derived from heparin instead of the undersulfated heparan sulfate important for most biologically relevant
HSGAG:protein interactions. Therefore, only a select group of HSGAG sequences were actually surveyed with a specific bias to regions of high sulfation and high IdoA content. A recently developed technique referred to as surface noncovalent associated mass spectrometry (SNA-MS) has overcome these problems [63].

![Minimal HSGAG oligosaccharide binders]

**Figure 1.6:** Schematic of surface non-covalent association mass spectrometry (SNA-MS). In this scheme, avidin is adsorbed on a surface suitable for MALDI-MS analysis (gold plate or hydrophobic film). Biotinylated protein is then immobilized on the avidin. Oligosaccharides (shown as disaccharide repeat units of three for hexasaccharides) are applied to the protein spot and washed with salt to select for high affinity binders (in green). After application of (RG)_{10}R in caffeic acid matrix, the sample is directly analyzed by MALDI-MS in a one-step procedure. Oligosaccharides are detected as non-covalent complexes with (RG)_{10}R; the mass of the saccharide can be determined by subtraction of the mass of the peptide.

SNA-MS relies on the immobilization of the protein of interest directly on the hydrophobic surface of MALDI-MS plate followed by the addition of a mixture of oligosaccharides (Figure 1.6). A subsequent low salt wash is used to remove the binding of non-specific oligosaccharides. A basic peptide composed of an Arg-Gly repeat (RG_{10}R), along with the matrix, is added directly to the spot to promote stable ionization and the peptide:oligosaccharide is analyzed using MALDI-MS [63]. In addition, using enzymatic or chemical methods, the bound oligosaccharide can be depolymerized enabling its sequencing directly on the chip. Therefore, SNA-MS combines protein immobilization, oligosaccharide
selection, and sequencing into a single procedure for identifying high affinity HSGAG binding sequence (Figure 1.6). SNA-MS has been applied to the identification and sequencing of the high affinity HSGAG binders to FGF and endostatin [64].

The techniques of PEN-MALDI and SNA-MS are perfect examples of the importance of coupling GAG-degrading enzymes with analytical tools to better probe the underlying structure-function relationships of bioactive GAGs. The previous development of the recombinant expression systems for the heparinases and their subsequent biochemical characterization enabled their advanced application in PEN-MALDI and SNA-MS. Similar development of the chondroitinases will increase their utility as enzymatic tools to probe structure-function relationships for biologically relevant CS/DS oligosaccharides.

1.5 HSGAG Biology

HSGAG proteoglycans are found on the surface of nearly every cell type where they regulate a cell signaling events such as proliferation, differentiation, adhesion, migration, and programmed cell death (apoptosis). Based on this diversity of cellular functions, HSGAGs have been found to be critical regulators of biological processes ranging from embryonic development to hematostasis [4,65,66]. Their biological importance was further confirmed by direct genetic evidence indicating a direct role for HSGAGs in the modulation of cell-to-cell signaling. A series of studies in Drosophila have provided genetic evidence that the HSGAG-containing proteoglycans, dally and dally-like, are required for proper signaling of the Wnt morphogen, Wingless [67-69]. In addition, specific targeting of the murine N-deacetylase/N-sulfotransferase, a biosynthetic enzyme responsible for replacing the acetate moiety at the N- position of glucosamine with a sulfate in HSGAGs, led to a neonatal lethal phenotype, thus confirming the role of these information dense biopolymers in proper development [70,71]. The specific role of HSGAGs in a variety of biological and pathological events is discussed below.

HSGAGs in Coagulation

One of the best characterized biological functions of HSGAGs is their role in the maintaining proper blood volume and composition, known as hemostasis [4,66]. The HSGAGs found on the endothelial cells lining the lumen of the blood vasculature help regulate the activity of the various proteases involved in the coagulation cascade, such as factor Xa and factor IIa [66]. In particular, the interaction of HSGAGs with antithrombin III
(ATIII), a serine protease inhibitor, is very well characterized (Table 1.4). A defined pentasaccharide sequence \( \text{IH_{NHS,65}GH_{NS,38,65}SH_{NS,65}} \) found in HSGAGs is responsible for binding to ATIII and initiating the conformational change in the protein required for its activity [61,72]. This conformational change in ATIII provides an increase of three orders of magnitude in its binding avidity to select proteases, most notably factor Xa and IIa [4].

The interaction of the activated ATIII:HSGAG complex with both factor Xa and factor IIa occurs through two unique biochemical mechanisms that further underscore the importance of HSGAGs in the regulation of biological processes such as coagulation [4,73]. In the case of factor Xa, the conformational change in ATIII is sufficient for the formation of high affinity protein-protein complex. This complex irreversibly inhibits the protease activity of factor Xa by trapping the serpin as a reaction intermediate [74]. Conversely, the inhibition of factor IIa (thrombin) requires the binding of the protein directly to the same HSGAG chain as ATIII (Table 1.4). A HSGAG chain of at least 16 disaccharide units is required for the formation of the ATIII:IIa:HSGAG ternary complex [75]. The binding of IIa to the HSGAG polymer is thought to be toward the non-reducing end relative to the ATIII binding site and is likely mediated through non-specific ionic interactions. Therefore, in this case, the HSGAG oligosaccharide serves not only to activate ATIII, but also as a scaffold for the formation of the ATIII:IIa high affinity complex.

HSGAGs have been shown to play additional roles in coagulation such as the regulation of the release of tissue factor pathway inhibitor (TFPI), an inhibitor of additional coagulation cascade proteases [76]; as well as the modulation of the activity of heparin cofactor II (HCII) [77,78] and von Willebrand Factor (vWF) [4,79]. The multiplicity of roles of HSGAGs in coagulation has led to the effective use of these molecules as antithrombotic therapeutics. In fact, highly sulfated heparin prepared from mast cells has been used for over 50 years to treat a wide variety of thromboembolic disorders including deep vein thrombosis, arterial thromboses, and acute coronary syndromes such as myocardial infarction and unstable angina [80]. However, along with the multiple functions of HSGAGs comes a potential for unwanted side effects such as heparin-induced thrombocytopenia (HIT). Therefore, low molecular weight heparins (LMWHs) have emerged as potent anticoagulants that exhibit a reduction in deleterious side effects seen with standard heparin treatment [81].

LMWHs are defined as having a mass of less than 6 kDa and an anti-Xa:anti-IIa ratio of 1.5:1 (compared to 1:1 for traditional heparin) [82]. Given these altered chemical
parameters, LMWHs are effective for some of the clinical indications for which heparin was used such as deep vein thrombosis, but not in others, such as arterial thrombosis [4]. The understanding of structure-function relationships for LMWHs has greatly improved with the advent of the sequencing strategies for HSGAGs. The application of PEN-MALDI (as described previously) to the analysis of LMWHs led to the discovery that certain production techniques led to the cleavage of the critical intact pentasaccharide thereby decreasing the overall clinical efficacy of the preparation when compared to full length heparin [62]. This important observation prompted the development of a novel technique for producing LMWHs with tailored pharmacological and anticoagulant activities thereby increasing their overall clinical utility [83]. The LMWH story is a perfect example in how improvements in enzymatic and analytical tools has led to a dramatic increase in our understanding of the biochemical structure-function relationships that underlie a complex biological phenomena such as coagulation.

**HSGAGs in Cancer and Angiogenesis**

HSGAGs are vital components of the interface between the cell surface and the ECM and serve both static structural and dynamic signaling roles in mediating cell-to-cell communication. It is no surprise then that HSGAGs have been implicated in the onset, progression, and metastasis of tumor cells (Figure 1.7) [84,85]. Recent evidence indicates that during the process of transformation there are detectable alterations in the HSGAGs displayed on the cell surface implying that HSGAGs may be directly involved in oncogenesis [85-87]. Additionally, tailoring HSGAGs on the cell surface of tumor cells can allow for changes in how that cell responds to different cytokines and growth factors found in a

---

**Figure 1.7:** Tumor progression requires a cascade of cellular proliferation, adhesion and migration. The strategic placement of GAGs at the interface of cell and their immediate surrounding environment enables the GAGs to regulate multiple cellular functions by acting as the regulators of various GAG-binding protein molecules ranging from growth factors, adhesion molecules to coagulation factors.
growing tumor. These effects can be both stimulatory or inhibitory depending on the molecular context in which a growth factor is displayed to a specific cell [88]. Finally, removal of HSGAGs from the ECM by enzymes such as heparanase along with the degradation of matrix proteins are critical steps in metastasis, the process through which a growing tumor spreads from its original location to a secondary location in the body (Figure 1.7) [89].

A growing tumor initiates the growth of new blood vessels allowing it to connect to the body’s vasculature and receive vital nutrients, a process known as angiogenesis or neovascularization. The HSGAGs present on the surface of vascular endothelial cells help regulate the growth factor signaling responsible for the induction of angiogenesis (Figure 1.7) [90]. In fact, the direct administration of heparanase I has been shown to inhibit in vitro angiogenesis further emphasizing the role of HSGAGs in this process [91]. Fibroblast growth factor (FGF) [85,92] and vascular endothelial growth factor (VEGF) [90,93] are two of the important angiogenic players that are regulated by HSGAGs. In addition, HSGAGs are required for the activity of the potent anti-angiogenic protein, endostatin [64]. Therefore, the possibility exists that endothelial cells can alter their cell surface HSGAGs to be more or less angiogenic in response to signals from the growing tumor. Despite this biological complexity, anti-angiogenic drugs are emerging as important therapies for treating cancer.

**HSGAGs and the Regulation of FGF**

The ubiquitous distribution of HSGAGs on the cell surface and in the ECM promotes their interaction with growth factors throughout the intercellular signaling process. One of the best studied cases is the modulation of basic fibroblast growth factor (FGF-2) signaling through its specific interaction with HSGAGs (Figure 1.8 and Table 1.4) [4,94]. HSGAGs regulate the distribution and activity of FGF through a variety of mechanisms.

First, the binding of FGF to HSGAGs in the ECM appears to serve as a growth factor “sink.” This enables the facile release of FGF in response
to injury or some other disruption of the ECM thereby leading to a rapid signaling cascade without the need for novel protein synthesis (Figure 1.8) [94]. Additionally, the spatial and temporal distribution of HSGAGs in the ECM can play an important role in regulating the formation of a concentration gradient of FGF and other growth factors [65]. This differential binding of FGF in the ECM and at the cell surface creates a highly regulated diffusion of growth factor thus directly controlling the ultimate cellular response in biological processes, specifically development (Figure 1.8). Finally, HSGAGs play an active role in mediating the formation of an active signaling complex between FGF and its cell surface receptor [95]. Through the modulation of the FGF oligimerization, HSGAGs can act as molecular “rheostats” in determining the strength of the cellular response to an FGF signal [96,97]. Therefore, the sequence content and the distribution of HSGAGs can regulate FGF signaling through a variety of mechanisms (Figure 1.8).

Table 1.4: GAG binding proteins and their corresponding biological effects

<table>
<thead>
<tr>
<th>Binding Protein</th>
<th>GAGs Bound</th>
<th>Binding Sequence</th>
<th>Biology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antithrombin III (ATIII)</td>
<td>HSGAGs</td>
<td>IdoA-GlcNac6S-GlcA-GlcNS,3S,6S-IdoA2S-GlcNS,6S</td>
<td>Anticoagulation</td>
</tr>
<tr>
<td>Thrombin (IIa)</td>
<td>DS, HSGAGs</td>
<td>N.D.</td>
<td>Anticoagulation</td>
</tr>
<tr>
<td>Heparin cofactor II (HCII)</td>
<td>DS, HSGAGs</td>
<td>DS: (IdoA2S-GalNac4S)₃</td>
<td>Anticoagulation</td>
</tr>
<tr>
<td>Platelet factor 4</td>
<td>DS, HSGAGs</td>
<td>N.D.</td>
<td>Anticoagulation</td>
</tr>
<tr>
<td>Fibroblast growth factor 1 and 2 (FGF)</td>
<td>DS, HSGAGs</td>
<td>HSGAGs: highly sulfated</td>
<td>Cell proliferation &amp; tumorigenesis</td>
</tr>
<tr>
<td>Tenascin-X</td>
<td>DS, HSGAGs</td>
<td>N.D.</td>
<td>Cartilage stability</td>
</tr>
<tr>
<td>Hepatocyte growth factor/scatter factor (HGF/SF)</td>
<td>DS, HSGAGs</td>
<td>DS: (IdoA-GalNac4S)₄</td>
<td>Cellular proliferation &amp; tumorigenesis</td>
</tr>
<tr>
<td>Keratinocyte growth factor (KGF or FGF-7)</td>
<td>DS, HSGAGs</td>
<td>DS: oversulfated sequences</td>
<td>Cell proliferation in wound healing</td>
</tr>
<tr>
<td>RANTES</td>
<td>CS, DS, HSGAGs</td>
<td>N.D.</td>
<td>Inflammatory response</td>
</tr>
<tr>
<td>Midkine</td>
<td>CS, HSGAGs</td>
<td>N.D.</td>
<td>Neuronal development</td>
</tr>
</tbody>
</table>

N.D. = not determined

The FGF story is only an illustrative example of how HSGAG sequence can influence biological processes. Other growth factors and cytokines such as vascular endothelial growth factor (VEGF), tissue plasminogen activator, and interferon gamma to name a few are all regulated by diverse HSGAG sequences and though subtly different mechanisms. In addition, HSGAG sequences have also been implicated as specific targets for viral adhesion in the case
of herpes simplex virus (HSV) serotypes 1 and 2, as well as for cytomegalo virus (CMV) among others [98-100]. Clearly, specific sequences of HSGAGs regulate a wide variety of biological processes (Table 1.4). The application of enzymatic and analytical tools has and will continue to provide vital details on biologically important structure-function relationships. In turn, this information will help drive the innovative production of novel therapeutics for a variety of HSGAG-mediated pathologies.

1.6 CS/DS Biology

The chondroitinases, lyases that specifically degrade CS/DS, have been coupled, in an analogous fashion to the heparinases, with analytical tools for the characterization of biologically important CS/DS oligosaccharides. Several fingerprinting techniques have been used to determine the structure of small oligosaccharides isolated from a variety of CS/DS sources [101,102]. Notably, a recently developed technique, fluorophore-assisted carbohydrate electrophoresis (FACE), has proved useful in the compositional analysis of CS from human cartilage [103-105]. Several studies have also described specific sequences of CS/DS that modulate biologically important events, such as heparin cofactor II's regulation of anticoagulation [106] and cellular adhesion of infected red blood cells in the pathogenesis of Plasmodium falciparum [106-108]. A discussion of some of the biological roles of CS/DS oligosaccharides is provided below.

Dermatan Sulfate in Coagulation

One of the first biologically characterized roles for DS was its ability to activate heparin cofactor II (HCII), an important member of the serpin family of anticoagulant proteins (Table 1.4) [109]. A highly sulfated DS hexasaccharide composed of the IdoA2S-GalNac4S disaccharide unit has been shown to bind HCII with high affinity and produce a 50-100-fold increase in the ability of HCII to inhibit thrombin [106,110]. In a similar fashion to the HSGAG mediated activation of ATIII, a longer DS oligosaccharide containing the high affinity hexasaccharide is required for maximal activation suggesting a similar GAG template-driven ternary complex formation [111]. HCII can also be activated by a highly sulfated hexasaccharide composed of the IdoA-GalNac4S,6S disaccharide repeat, but not by a hexasaccharide composed of IdoA2S-GalNac6S (Table 1.4) [8,112]. This finding suggests that the activation of HCII is somewhat specific for a DS polymer containing both IdoA
epimer and the 4-O sulfated GalNac. In addition as its name suggests, HCII can also be activated by heparin leading to a 1000-fold increase in its antithrombin activity [109].

The non-covalent complex of DS and HCII is capable of inactivating fibrin-associated thrombin, a clinical outcome that cannot be accomplished by HSGAG oligosaccharides [113]. Therefore, significant effort has gone into developing an oversulfated DS as an anticoagulant therapy. Intimitan, a semi-synthetic, highly sulfated DS with an increased content of the IdoA-GalNac4S,6S disaccharide, has increased efficacy in inhibiting clot-bound thrombin when compared with standard heparin or DS therapy [114,115]. Additionally, DS is being pursued as a treatment for patients with heparin-induce thrombocytopenia, a disorder resulting from an IgG-mediated auto-immune response to a complex of heparin and platelet factor 4 in which heparin treatment needs to be discontinued [8,116]. Covalent complexes of HCII with heparin and DS have improved pharmacokinetics over the GAGs by themselves and may prove useful as novel anti-coagulants [117]. The regulation of HCII activity is a good example of how two different GAGs can activate a protein through a common biochemical mechanism, but produce separate clinical outcomes.

**Chondroitin Sulfate in Cartilage Function**

Based on the dry weight of the tissue, cartilage is composed of up to 90% ECM of which the most predominant GAG is CS [118]. Aggrecan, the predominant CS proteoglycan (CSPG) found in cartilage, has a molecular weight of 230 kDa and is decorated with up to 100 CS polymers [119]. The aggrecan CS chains are predominantly composed of disaccharides that are 6-O sulfated with the ratio of GalNac6S:GalNac4S increasing from infancy to adolescence [120]. The high overall charge of the carboxylate and sulfate groups present in the CS side chains of aggrecan are thought to provide the necessary electrostatic forces to promote the high water content that enables joints to withstand dynamic mechanical compression [121]. In addition, the core protein of aggrecan interacts with the collagen fibrils present in cartilage providing and additional degree of mechanical strength to the tissue [122]. Additional CS or DS containing PGs including decorin, biglycan, and perlecan are also present in cartilage and are thought to play a variety of biological roles [122].

Osteoarthritis (OA), a disease that impacts 20 million Americans annually with nearly every person over the age of 75 affected in at least one joint, is characterized by the degradation of the cartilage ECM leading to a loss of some of the mechanical properties of the
tissue. This degradation is mediated by both mechanical forces acting on the joint, as well as the enzymatic degradation of the critical cartilage components, such as aggrecan and collagen [122]. Chondrocytes, the major cell type found in cartilage, regulate the turnover and deposition of aggrecan and the other ECM molecules, and any deregulation of chondrocyte function could lead to the progression of a disease state. In addition, a decrease in the amount 6-O sulfated disaccharides with a concomitant increase in 4-O sulfated disaccharides in the CS side chains has been observed in arthritic tissue [123]. Various nutraceuticals containing chondroitin sulfate, glucosamine, and/or mannosamine have been shown to decrease the severity and progression of OA. One mechanism through which this may be occurring is the inhibition of aggrecanase, an enzyme responsible for the degradation of aggrecan [121]. Future application of the chondroitinases as enzymatic tools may help discern the exact role of CS in the pathology of OA.

Chondroitin Sulfate and Neuron Regeneration

CSPGs have also been implicated in the regulation of neuronal tracking during central nervous system (CNS) development [124]. Glial and neuronal cells, the two major cell types found in the brain and CNS, produce both unique as well as overlapping CSPGs. For example, NG2 is a large transmembrane CSPG produced only by glial cells and has been implicated in proper neuron development, as well as in the inhibition of neuron re-growth after CNS injury [125,126]. Neurocan, a secreted CSPG produced predominantly by neuronal cells, is an important regulator of growth factor signaling the ECM of the CNS [127]. In contrast, phosphocan is produced by both glial and neuronal cells and is thought to bind and regulate neuronal cell adhesion molecules [124,127].

While CSPGs are critical in the proper development of the CNS, they have also been implicated as a major player in the inhibition of the axon re-growth after an acute CNS injury [128,129]. A series of recent studies applying chondroitinase ABC directly to a variety CNS wounds have shown that removal of the CSPGs results in re-growth of the damaged axons. In a rat model of dorsal column injury, treatment with chondroitinase ABC led to the degradation of the CSPGs in the glial scar, axon regeneration, and an actual functional recovery of locomotor behavior in the animals [130]. An additional study using chondroitinase ABC treatment demonstrated that CSPGs play a critical role in proper ocular development in rats [131]. These studies and others point to the successful use of CS-
degrading enzymes as clinical tools to promote axon re-growth in response to CNS injury, a long sought after medical outcome.

**Chondroitin Sulfate & Dermatan Sulfate and Cell Signaling**

While HSGAGs have long been studied as important regulators of growth factor and cytokine signaling, CS and DS containing proteoglycans are also emerging as players in ECM-mediated cell-to-cell signaling. A variety of CSPGs have been implicated in regulating cell signaling in different biological settings. For example, CSPGs found on the cell surface of human neutrophils specifically bind to PF-4 and are responsible for cellular activation [132,133]. In addition, an artificial cell surface CSPG promoted the GAG-mediated binding of RANTES leading to the specific activation of T cells (Table 1.4) [134]. Midkine, traditionally thought of as a HSPG binding growth factor, was recently shown to interact with receptor-type tyrosine phosphatase ζ (RTPζ) and PG-M/versican, both of which are CSPGs (Table 1.4) [135]. Along with the case of HClI, this observation suggests that GAG-binding proteins likely interact with different classes of proteoglycans based on their spatial and temporal expression.

DSPGs, through direct interaction of GAG chains, have recently emerged as critical regulators of cell signaling molecules. One of the best characterized DSPGs is decorin, a small leucine rich protein secreted into the ECM of connective tissue where it is thought to regulate cell signaling along with tissue integrity [8]. Decorin has a single DS chain attached to it that has been shown to interact with tenascin-X and collagen providing a bridging action that ensures the normal tensile strength of the skin (Table 1.4) [136,137]. Decorin has also been shown to regulate fibrosis and cell proliferation through its interaction with other ECM proteins such as TGF-β, low density lipoprotein, and fibronectin, to name a few [8].

DS is also emerging as a critical regulator of extracellular growth factor signaling. Similarly to HSGAGs, DS interacts with FGF-2 and potentiates its ability to stimulate cell proliferation in vitro (Table 1.4) [138]. In addition, DS binds and activates hepatocyte growth factor/scatter factor (HGF/SF), a paracrine growth factor that signals through the transmembrane tyrosine kinase receptor (and proto-oncogene), c-met [139]. Aberrant expression of HGF/SF, which normally plays a role in cell motility, differentiation, and angiogenesis, has been linked to tumorigenesis and metastasis [140]. HGF/SF is also another
example of a protein that binds to both HSGAGs and DS, although it binds to the latter with nearly 100-fold weaker affinity (Table 1.4) [141,142]. The minimal DS binding sequence for HSGF/SF has been characterized as an undersulfated octasaccharide composed of the basic IdoA-GalNac4S disaccharide (Table 1.4) [141].

High levels of DS are found in the skin and are released in response to tissue injury, making it a likely regulator of wound healing. In fact, recent studies have implicated DS in the binding and activation of keratinocyte growth factor (KGF or FGF-7) (Table 1.4) [143]. GAG containing isolates from wound fluid that are high in DS content promoted KGF activation of a synthetic cell line expressing the FGF receptor 2 IIIb (FGFR2 IIIb), the cognate receptor for the FGF-7. In addition, DS produced a dose-dependent increase in the proliferation of normal human keratinocytes that was greater than the increase seen with the administration of heparin [143].

Given the active role of DSPGs in modulating ECM integrity and cell signaling, its not surprising that DS has also been implicated in oncogenesis and angiogenesis [144]. Treatment of melanoma or endothelial cells with chondroitinase AC and B led to a decrease in proliferation and invasion, to phenotypic hallmarks of cancer cells [145]. Additionally, chondroitinase B treatment of human dermal fibroblasts led to a reduction in their responsiveness to FGF-2, a known regulator of tumor cell proliferation [146]. Further development of the chondroitinases as enzymatic tools for the analysis of CS/DS will enable a greater understanding of the role of specific oligosaccharide sequences in normal and pathological cellular processes.
1.7 Conclusions and Thesis Goals

GAGs are complex polysaccharides that actively regulate a diversity of biological and pathological events. The chemical heterogeneity and relative biological scarcity of GAGs has hindered their characterization when compared to other biopolymers such as DNA and proteins. However, the coupled use of the heparinases as enzymatic tools with sensitive analytical tools like MALDI-MS resulted in the development of improved techniques for the characterization of the structure-function relationship for HSGAGs. This increased knowledge of HSGAG function has led to the production of improved clinical therapies for the regulation of coagulation and the treatment of cancer. CS/DS are also emerging as important regulators of a wide-range of biological events, but tools are lacking for adequately understanding structure-function relationships for this class of GAGs. The development of the chondroitinase as enzymatic tools will greatly assist in the characterization of bioactive CS/DS oligosaccharides in a fashion analogous to the use of the heparinases for studying HSGAGs. Therefore, the goals of this thesis are four-fold:

1) Continue the biochemical characterization of the heparinases by examining the role of histidine in the enzymatic activity of heparinase III;
2) Extend the previous work on the heparinases by developing a recombinant expression and purification system for the production of large amounts of pure chondroitinase AC and B;
3) Characterize the amino acids involved in substrate binding and catalysis in the active site of chondroitinase B; and
4) Explore the mode of action of chondroitinase B using defined DS-derived oligosaccharides.

Taken together the results outlined in this thesis represent a significant advancement in understanding the biochemistry of the chondroitinases. This body of work can be built upon by applying the chondroitinases along with the analytical techniques of CE and MALDI-MS discussed within to the development of a sequencing methodology for bioactive CS/DS oligosaccharides. Such advancement will likely result in a virtual explosion in the understanding of the structure-function relationships that underlie the diversity of biological and pathological events regulated by CS/DS oligosaccharides.
Chapter 2:

Histidine 295 and Histidine 510 are Crucial for the Enzymatic Degradation of Heparan Sulfate by Heparinase III

SUMMARY

The heparinases from Flavobacterium heparinum are powerful tools in understanding how heparin-like glycosaminoglycans function biologically. Heparinase III is the unique member of the heparinase family of heparin-degrading lyases that recognizes the ubiquitous cell-surface heparan sulfate proteoglycans as its primary substrate. Given that both heparinase I and II contain catalytically critical histidines, we examined the role of histidine in heparinase III. Through a series of DEPC modification experiments, it was found that surface exposed histidines are modified in a dose-dependent fashion and that this modification results in inactivation of the enzyme ($k_{\text{inact}} = 0.20 \pm 0.04 \text{ min}^{-1} \text{mM}^{-1}$). The DEPC modification was pH dependent and reversible by hydroxylamine indicating that histidines are the sole residue being modified. As previously observed for heparinase I and II, substrate protection experiments slowed the inactivation kinetics suggesting that the modified residue(s) was in or proximal to the active site of the enzyme. Proteolytic mapping experiments, taken together with site-directed mutagenesis studies, confirm the chemical modification experiments and point to two histidines, histidine 295 and histidine 510, as being essential for heparinase III enzymatic activity. This work was published in its entirety in Biochemistry and is reproduced here with the journal's consent [147].
Histidine 295 and Histidine 510 Are Crucial for the Enzymatic Degradation of Heparan Sulfate by Heparinase III

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ABSTRACT: The heparinas from Flavobacterium heparinum are powerful tools in understanding how heparin-like glycosaminoglycans function biologically. Heparinase III is the unique member of the heparinase family of heparin-degrading hydases that recognizes the ubiquitous cell-surface heparan sulfate proteoglycans as its primary substrate. Given that both heparinase I and heparinase II contain catalytically critical histidines, we examined the role of histidine in heparinase III. Through a series of diethyl pyrocarbonate modification experiments, it was found that surface-exposed histidines are modified in a concentration-dependent fashion and that this modification results in inactivation of the enzyme (k_{inact} = 0.20 ± 0.04 min^{-1} mM^{-1}). The DEPC modification was pH dependent and reversible by hydroxylamine, indicating that histidines are the sole residue being modified. As previously observed for heparinas I and II, substrate protection experiments slowed the inactivation kinetics, suggesting that the modified residue(s) was (were) in or proximal to the active site of the enzyme. Proteolytic mapping experiments, taken together with site-directed mutagenesis studies, confirm the chemical modification experiments and point to two histidines, histidine 295 and histidine 510, as being essential for heparinase III enzymatic activity.

Heparin sulfate and heparin-like glycosaminoglycans (henceforth referred to as HLGAGs),1 ubiquitous components of the extracellular matrix, are emerging as important mediators of a variety of biological processes (1, 2). These complex linear polysaccharides are composed of a disaccharide repeat unit of a hexosamine residue 1→4-linked to a uronic acid residue. These polymers of 20–100 disaccharide units can be additionally modified through N- and O-sulfation, epimerization at the C5 position of the uronic acid moiety and adding an additional microheterogeneity to these information-dense molecules (3, 4). Heparan sulfate is found as a proteoglycan on the surface of many cell types, O-linked to a variety of core proteins, and plays an active role in mediating growth factor signaling (5).

Although the structure and chemistry of HLGAGs are fairly well understood, information on how specific HLGAG sequences modulate different biological processes has proven harder to obtain. Our laboratory has recently developed a rapid sequencing methodology for polysaccharides akin to those developed previously for polypeptides and polynucleotides (6). Use of this approach to sequence HLGAGs relies on chemical and enzymatic tools to modify or degrade an unknown HLGAG polymer in a sequence-specific manner. The molecular characterization of the enzymes that act on HLGAGs is crucial for the further development of tools for this sequencing methodology. One family of enzymes that act by degrading HLGAGs in a sequence-specific manner is the heparinas.

Heparinases I, II, and III are three HLGAG-degrading enzymes produced by Flavobacterium heparinum. Each of the heparinas has its own unique HLGAG sequence at which it cleaves, making these enzymes valuable tools in obtaining sequence-specific information. Heparinase I primarily cleaves HLGAGs at the H_{N,S,X}→I_{2,5} linkage found primarily in heparin-like regions (4, 7, 8). Heparinase III cleaves at the H_{N,Y}→I and H_{N,S,X}→G1 linkages which are the major disaccharides found in heparan sulfate (4, 6, 9). Heparinase II is capable of recognizing and cleaving both sets of substrate linkages (4).

Our laboratory has also been actively investigating the enzymology of the heparinas. In the case of heparinase I, cysteine 135 and histidine 203 along with lysines 198, 199, and 132 were identified to be critical for HLGAG degradation (10–12). Also, cysteine 348 and histidines 238, 451, and 579 were determined crucial for heparinase II activity (13, 14).

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5 Abbreviations: HLGAGs, heparin/heparin-like glycosaminoglycans; DEPC, diethyl pyrocarbonate; RPHPLC, reverse-phase high-pressure liquid chromatography; IPTG, isopropyl-β-D-thiogalactopyranoside; CD, circular dichroism; ΔU, uronic acid moiety with a 4,5 double bond.

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Critical Histidines in Heparinase III

Heparinase III is unique in that it is the only member of the heparinase family that recognizes and preferentially cleaves heparan sulfate. Heparinase III is also unique among its heparin-degrading family members in that it contains no cysteines in its amino acid sequence (15, 16). Heparinase III, however, does contain 13 histidines of which 1 or several might be involved in the activity of the enzyme. Therefore, through a combination of chemical modification, peptide mapping, and site-directed mutagenesis studies, we sought to examine the potential role of histidine in the catalytic activity of heparinase III.

MATERIALS AND METHODS

Chemicals and Materials. Hydroxylamine hydrochloride and urea were from EM Science (Gibbstown, NJ). The chemical modification reagent diethyl pyrocarbonate (DEPC) was purchased from Sigma and used as received (Milwaukee, WI). Heparan sulfate was purchased from Celsus Laboratories (Cincinnati, OH). Lys-C from Lysobacter enzymogenes (EC 3.4.21.50) was from Roche Molecular Biochemicals (Indianapolis, IN). Heparinase III from Flavobacterium heparinum (EC 4.2.2.8) was purified as described previously (11, 17) and was a gift from IBEX Technologies (Montreal, Canada).

Heparinase III Activity Assay. The activity of heparinase III was measured using a UV 232 nm assay similar to those reported for hepariiniase I and heparinase II (11, 14, 17). Briefly, the increase in absorbance at 232 nm as a function of time was monitored under saturating substrate conditions. All assays were performed with heparan sulfate at a concentration of 2 mg/mL in 50 mM sodium phosphate, pH 7.6. The temperature for enzymatic activity measurements was kept constant at 35 °C.

Chemical Modification of Heparinase III with DEPC. (A) DEPC Inactivation of Heparinase III. At pH values ranging from 6.0 to 8.0, heparinase III (50 μg/mL) was incubated with DEPC in 50 mM sodium phosphate buffer at 25 °C. The DEPC stock solution (6.9 M) was diluted with ethanol. Control reactions contained an equivalent amount of ethanol without DEPC, which did not affect enzymatic activity over the experimental time range. At each pH, three reactions were run using different concentrations of DEPC, ranging from 50 μM to 2.5 mM. At fixed time intervals, 25 μL aliquots were withdrawn from the reaction mixtures for the UV 232 nm activity assay.

The kinetics of DEPC inactivation of heparinase III were determined by plotting the natural log of percent activity versus an adjusted time term (to account for the decomposition of DEPC). Briefly, this adjusted time term (t') was calculated according to the equation:

\[ t' = \frac{1 - e^{kt}}{k} \]

In this equation, k is the first-order rate constant for DEPC hydrolysis and t is the measured time after addition of DEPC to the heparinase III solution. At each pH, the order of the reaction in DEPC was determined by plotting the log of the observed rate constants of inactivation at each pH vs log [DEPC]. The slope of this line is n, the order of the reaction with respect to DEPC (18).

(B) Reactivation of DEPC-Modified Enzyme with Hydroxylamine. Similar to what was completed with heparinases I and II (11, 14), heparinase III (50 μg/mL) was incubated with 0.97 mM DEPC, pH 6.5, until its enzymatic activity was reduced to 50% of its initial value. Hydroxylamine was then immediately added to the reaction mixture to a final concentration of 0.5 M. The reaction was incubated at room temperature for 6 h. Aliquots were withdrawn every hour for the activity assay. The control mixture contained no DEPC but the same concentration of hydroxylamine to account for the nonspecific loss of activity. The ratio of the activity of the reaction mixture over the activity of the control was calculated to determine recovery of enzymatic activity.

(C) Substrate Protection of Heparinase III against DEPC Inactivation. Heparinase III (50 μg/mL) was preincubated with 2 mg/mL heparan sulfate in 50 mM sodium phosphate, pH 7.6, for 30 min at room temperature prior to the addition of 1.5 mM DEPC. A control reaction with no prior incubation of substrate was also completed. The time course of inactivation for both was determined using the heparinase III activity assay.

(D) Quantification of the Number of Histidines Modified by DEPC. The extent of modification of an enzyme by DEPC can be determined by monitoring the formation of the N-carbethoxyhistidyl adduct at 240 nm. At time zero, DEPC was added to a final concentration of 1.5 mM to the cuvette containing heparinase III in sodium phosphate buffer, pH 7.0. The change in absorbance at 240 nm was monitored every minute for 10 min. The number of modified residues was determined using a molar extinction coefficient of 3200 M⁻¹ cm⁻¹ (18).

Peptide Mapping Studies. To determine which histidine residues were modified by DEPC, mapping studies using the protease Lys-C were completed. Heparinase III (1 nmol) was incubated with 4 mM DEPC for 15 min, denatured with 6.5 M urea at 55 °C, and diluted with water. The enzyme was digested with 1.6 μg of Lys-C at 37 °C for 12 h.

Peptides derived from heparinase III digestion were separated by RP-HPLC and monitored at 210, 277, and 240 nm. Peptide peaks not present in the control digest were collected and sequenced using an Applied Biosystems Sequencer model 477 with an on-line model 120 PTH amino acid analyzer (Biopolymers Laboratory, MIT).

Site-Directed Mutagenesis. Each of the 13 histidine residues of heparinase III was mutated to alanine using overlap extension PCR for 15 cycles (19). The PCR reactions were separated on a low-melt agarose gel, and the band corresponding to the proper length was excised. The DNA was extracted from the gel using a Gel Purification Kit (Qiagen, Valencia, CA), the insert was subcloned into pCR 2.1 (Invitrogen, Carlsbad, CA), and the plasmid was prepared using a Miniprep kit (Qiagen, Valencia, CA). The validity of all the point mutations and the integrity of the rest of the gene were verified by sequencing (data not shown). The 13 mutant heparinase III clones, along with recombinant heparinase III, were excised from pCR2.1 using NdeI/BamHI (New England Biolabs, Beverly, MA) and subcloned into a pET-15b expression vector (Novagen, Madison, WI) that had been previously digested with these same enzymes. The pET-15b plasmid contains an NH₂ -terminal His-Tag for Ni²⁺ -column purification. Recombinant heparinase III was also
expressed and compared to the native heparinase III isolated directly from Flavobacterium heparinum.

Expression and Purification of r-Heparinase III and Mutants in E. coli. Overnight cultures of Luria–Bertani (LB) broth (5 mL) containing 0.02 mg/mL ampicillin (amp) were used to inoculate 500 mL LB/amp cultures at an initial OD$_{600}$ of 0.1. The cultures were induced with 1 mM isopropyl-$\beta$-thiogalactopyranoside (IPTG) in mid-log phase (OD$_{600}$ 0.7–0.9) and incubated for another hour at 37 °C. To harvest the cells, the cultures were spun at 5000 rpm, and the supernatant was discarded.

The cell pellet was resuspended in 20 mM Tris, 500 mM NaCl, 5 mM imidazole hydrochloride, pH 7.9 (1/50 of the initial culture volume). The resuspended cells were placed on ice and sonicated as described previously (20). The soluble protein of the cell lysate was isolated by centrifugation at 12,000 rpm for 20 min at 4 °C. The supernatant was filtered through a 0.45 μm filter and loaded onto a nickel POROS column using a Biocad Perfusion Chromatography system (PerSeptive Biosystems, Framingham, MA). The column was washed, and the protein was subsequently eluted in 20 mM Tris, 500 mM NaCl, 500 mM imidazole hydrochloride, pH 7.9. SDS–polyacrylamide gel electrophoresis analysis using precast 12% gels, the Mini-Protein II apparatus, and the Silver Stain Plus kit (Bio-Rad, Hercules, CA) was performed to determine the concentration, by using known amounts of heparinase III as standards, and the purity of the individual proteins. The concentrations of the individual proteins were confirmed using the Micro BCA Assay (Bio-Rad, Hercules, CA).

HPLC Analysis of Saccharide Products of Heparinase III Activity. Exhaustive digests of 3 mg/mL heparan sulfate in 50 mM sodium phosphate buffer, pH 7.6, were performed overnight at 37 °C for each of the mutants (20 μg of protein). The reactions were loaded onto a Spherisorb S5 SAX column (Waters) and eluted using a linear gradient of 0.2–1.0 M NaCl, pH 3.5. The products were monitored at 232 nm, and each of the major peaks was collected. To identify the products, the collected fractions were analyzed by capillary electrophoresis and identified by comigration with known standards.

Circular Dichroism (CD). Recombinantly expressed heparinase III and the heparinase III mutants, H295A and H510A, were concentrated and buffer-exchanged into 50 mM sodium phosphate, pH 7.0, using a Centricon 30 Filter (Millipore, Watertown, MA). CD spectra were collected on an Aviv 62DS spectropolarimeter equipped with a thermostatic static temperature controller and interfaced to an IBM microcomputer. Measurements were performed in a quartz cell with a 1 mm path length. Spectra were recorded at 25 °C, in an average of 10 scans between 205 and 260 nm, with a 1.0 nm bandwidth and a scan rate of 3 nm/min. CD band intensities are expressed as molar ellipticities, $\theta_{222}$, in deg cm$^2$ dmol$^{-1}$.

RESULTS

DEPC Inactivates Heparinase III. As a first step toward identifying histidines that are critical for the enzymatic activity of heparinase III, we determined the effect of the modification reagent DEPC on the enzymatic activity of heparinase III. DEPC is a common reagent used for the modification of solvent-accessible histidines in enzymes. As stated in early publications (11, 14), DEPC is useful for the determination of catalytically critical histidines that are solvent-accessible; however, care needs to be taken to ensure that other nucleophilic residues, namely, tyrosines, lysines, and cysteines, are not modified.

For heparinase III, we find that, similar to heparinases I and II, DEPC inhibits in a concentration-dependent fashion. A measured second-order rate constant of 0.20 ± 0.04 min$^{-1}$ mM$^{-1}$ (Figure 1) is obtained by varying the concentration of the inhibitor. Consistent with this reaction being first order in both heparinase III and DEPC, a plot of log $k_{max}$ vs log [DEPC] yielded a line with a slope of 1 (Figure 2).

To ensure that the interaction of DEPC with heparinase III is through histidine modification, we investigated whether other nucleophilic amino acids of heparinase III interact with DEPC. First, unlike with heparinase I or II, there is no possibility for cysteine modification since heparinase III contains no cysteines in its primary amino acid sequence. Furthermore, there was no loss of absorbance at 278 nm upon incubation of DEPC with heparinase III as would be expected if tyrosines were modified. Finally, addition of hydroxylamine to DEPC-modified heparinase III reversed most of the inactivation, indicating that strongly nucleophilic residues, such as lysine, were not modified by DEPC (Table 1).

In an attempt to further define the interaction of DEPC with the histidines of heparinase III, we examined the effect of the pH on the inactivation kinetics. Examination of the

![Figure 1: DEPC inactivation of heparinase III. (Inset) Heparinase III was incubated with 0.31 (○), 0.54 (●), 0.97 (▲), 1.5 (●), and 1.9 (△) mM DEPC at pH 6.5 and at 25 °C. The natural log of the percent activity remaining was plotted versus an adjusted time term (t) to account for the decomposition of DEPC. The slope of each of the lines at the various DEPC concentrations represents the pseudo-first-order rate constants of inactivation. Plotting these pseudo-first-order rate constants versus the respective DEPC concentrations yields a second-order rate constant of inactivation of 0.20 ± 0.04 mM$^{-1}$ min$^{-1}$.](image)
Critical Histidines in Heparinase III

**Figure 2:** pH dependence of the second-order rate constant of inactivation. Heparinase III was incubated with 50 µM to 2.5 mM DEPC at pH's 6.0–8.0 at 25 °C. The second-order rate constant of inactivation was calculated for each pH.

The rate of inactivation as a function of pH has been used to derive a pKa for a modified residue, since, in the case of histidine, the unprotonated form is much more readily modified than is the protonated form. With heparinase III, increasing the pH of the reaction from 6 to 7.5 results in an increase in the inactivation kinetics without changing the order of the reaction (Figure 2). However, at pH 8.0 and higher, the reaction is no longer first order in DEPC, indicating other residues (possibly lysines) are interacting with DEPC at this pH. Consistent with this interpretation, hydroxylamine is no longer able to reverse inactivation at pH 8.0. Therefore, the mapping studies and substrate protection experiments discussed below were conducted at pH 7.0 which maximized the reactivity while ensuring that only histidines were the target of DEPC modification.

Consistent with the idea that DEPC is interacting with a histidine residue in heparinase III, there is an increase in absorbance at 240 nm as a function of time, resulting from N-carbethoxyhistidylation derivatives. The number of modified histidines was quantified (Figure 3). Over the course of 10 min, 1.8 histidines are modified per enzyme molecule, resulting in a loss of greater than 90% activity. Thus, it appears that one or possibly two histidines, modified by DEPC, result in loss of enzymatic activity for heparinase III.

Preincubation of heparinase III with heparan sulfate substrate before addition of DEPC resulted in lower inactivation kinetics (Figure 4), suggesting that the histidine(s) modified by DEPC is (are) proximate to the substrate binding and/or active site of heparinase III, similar to what was observed for heparinases I and II (11, 14).

**Peptide Mapping of the Histidine Modified by DEPC.** To identify the histidine(s) modified by DEPC that resulted in the loss of enzymatic activity, DEPC-modified heparinase III was digested with Lys-C. Peptides that had altered retention times and an increased in absorbance at 240 nm as compared to a control digest were collected and sequenced (Figure 5). Three peptides that had altered retention times and increased absorbance at 240 nm were isolated and sequenced. Two of the peptides contained histidine 295, and one contained no modified histidine residues (see Discussion).

**Figure 3:** Quantification of DEPC-modified histidine residues in heparinase III. At time zero, 1.5 mM DEPC was added to a cuvette containing heparinase III (540 µg/mL) in sodium phosphate buffer, pH 7.0. The change in absorbance at 240 nm was monitored at time intervals for 10 min. The number of modified histidines were calculated using ε = 3200 M⁻¹ cm⁻¹. At the beginning and end of the experiment, aliquots of heparinase III were withdrawn and tested for activity. Less than 5% of the initial activity remained after 10 min incubation with DEPC.

**Figure 4:** Substrate protection of heparinase III inactivation by DEPC. Heparinase III (50 µg/mL) was incubated with 2 mg/mL heparan sulfate for 30 min. 1.5 mM DEPC was added to the reaction, and the time course of inactivation was completed using the heparinase III activity assay (O). A control reaction without preincubation with heparan sulfate was also done (■).

**Site-Directed Mutagenesis of Heparinase III.** In parallel to the mapping studies and to confirm the results of the chemical modification experiments, each of the 13 histidine residues present in heparinase III was mutated to alanine. The recombinant heparinase III mutant proteins were expressed, purified, and assessed for enzymatic activity toward heparan sulfate (Table 2). As a control, the r-heparinase III construct without its putative signal sequence was expressed. The concentration and purity of all recombinant enzyme preparations were determined using SDS–PAGE and a Micro BCA assay (data not shown). All of the enzyme preparations were of sufficient purity for the subsequent experiments. The recombinant heparinase III was also compared to the heparinase III isolated from *F. heparinum* to ensure that they were the same molecular weight. Both enzymes displayed similar kinetic activity toward heparan
sulfate and yielded the same degradation profiles as determined by SAX-HPLC (Figure 6). The products of the exhaustive digests were then analyzed using capillary electrophoresis. The first major peak (5 min) observed in the SAX-HPLC chromatograms has a migration time that is identical to $\Delta U$-H$_{\text{Nas}}$. The second peak (7.5 min) has a migration time that is identical to $\Delta U$-H$_{\text{Nas}}$ (data not shown). Thus, the heparin sulfate degradation by recombinant heparinase III produces an identical product profile to that of wild-type heparinase III, indicating that, at least functionally, these enzymes are the same.

The replacement of histidine 295 and histidine 510 with alanine residues completely eliminated the activity of heparinase III toward heparan sulfate (Table 2). Kinetic data for the wild-type heparinase III are included to demonstrate its similarity to the recombinant heparinase III. The H295A and H510A mutant enzymes showed no differences in terms of expression level or molecular weight. However, both the kinetic data and the exhaustive digest profile for H295A and H510A suggest that the enzymes are completely inactive (Figure 6). Nineteen of the histidine mutants (H36A, H152A, H225A, H234A, H241A, H469A, H424A, H510A, and H539A) showed no significant changes in recombinant protein yield, enzyme activity, or kinetic parameters when compared with r-heparinase III. Interestingly enough, 3 (H105A, H110A, and H195A) of the 13 histidine mutants yielded much less recombinant protein than either recombinant heparinase III or the other mutants. Despite lower protein yields, the H105A and H195A mutant proteins were amenable to kinetic analysis whereas the H105A mutant protein was not. However, SAX-HPLC analysis of overnight heparan sulfate digests confirmed that despite lower levels of recombinant expression, all three of these underexpressed enzymes retain their catalytic activity. The results for H105A are shown in Figure 6.

The wild-type heparinase III, the recombinantly expressed heparinase III, the H295A mutant, and the H510A mutant were compared using circular dichroism (CD). The possibility remained that the histidine 295 and/or histidine 510 were somehow responsible for the folding or the tertiary structure of the enzyme and not directly involved in catalysis. However, the CD spectra for H295A and H510A were nearly identical to those of wild-type and recombinant heparinase III (Figure 7). While the near-identity of the CD profiles does not preclude the possibility that there are perturbations in the local environment surrounding histidine 295 and histidine 510 that are not represented in the CD profile, it does suggest there are no gross conformational changes induced by mutating histidine 295 and histidine 510 to alanine.

**DISCUSSION**

We have shown through a combination of chemical modification, peptide mapping, and site-directed mutagenesis experiments that two histidines, histidine 295 and histidine 510, are critical for the enzymatic degradation of heparan sulfate by heparinase III. To the best of our knowledge, this represents the first biochemical characterization of an enzyme that exclusively degrades heparan sulfate.

**Chemical Modification.** We find that DEPC inactivates heparinase III in a pseudo-first-order, concentration-dependent manner (Figure 1). This suggests that DEPC is directly modifying a residue involved in the catalytic degradation of heparan sulfate. The second-order rate constant of inactiva-
Figure 6: SAX analysis of exhaustive heparinase III digests of heparan sulfate. Heparinase III (20 μg/mL) was incubated with 4 mg/mL heparan sulfate overnight at 37 °C. The reaction was loaded onto a SAX column, and the saccharide products were eluted using a gradient of 0.2–1.0 M NaCl, pH 3.5, over 30 min and monitored at 232 nm. (A) Heparan sulfate digested with heparinase III from F. heparinum. (B) Heparan sulfate digested with recombinant heparinase III. (C) Heparan sulfate digested with the H295A mutant enzyme. (D) Heparan sulfate digested with the H510A mutant enzyme. (E) Heparan sulfate digested with the H105A mutant enzyme.

Consistent with the observation that DEPC is modifying a histidine residue, there is an increase in the absorbance at 240 nm as a function of time. This is indicative of formation of an N-carbethoxyhistidyl derivative, the product of a reaction between DEPC and a histidine residue. Over the course of 10 min, 1.8 histidine residues are modified, and the enzymatic activity is decreased by 90% (Figure 3). Also, preincubation with heparan sulfate resulted in lower inactivation kinetics of heparinase III by DEPC (Figure 4). These data suggest that DEPC specifically modifies a critical histidine residue proximate to the substrate binding/active site of heparinase III, inactivating the enzyme.

An apparent discrepancy arises from these results in that the reaction of DEPC with heparinase III follows pseudo-first-order kinetics, yet two histidines appear to be independently modified. It is possible that two surface-accessible histidines react with DEPC at identical rates. It could be the case that either one or both of the modified residues is responsible for inactivating the enzyme. The limits of these chemical modification experiments prevent us from distinguishing between these two scenarios. However, the site-directed mutagenesis experiments discussed below point to
II, several histidines, specifically histidines 238, 451, and 579, were shown to be catalytically active in the depolymerization of heparan sulfate (14). It is also worth noting that when the catalytically critical cysteine of heparinase II, cysteine 348, is mutated to alanine, the enzyme loses the ability to depolymerize heparin but not heparan sulfate (13). This observation, along with the fact that there are no cysteines in the primary sequence of heparinase III, suggests that cysteine is not important in the degradation of heparan sulfate.

Although the role of histidine has not been clearly elucidated for heparinase II, a model exists for the role of histidine in heparinase I (12). It has been proposed that histidine 203 forms part of a catalytic triad also consisting of lysine 199 and cysteine 135 in heparinase I. In this model, histidine acts to stabilize the unprotonated form of the cysteine residue, allowing the thiolate anion to abstract the C5 hydrogen of the uronic acid moiety in the heparin chain. Lysine 199, along with calcium, is thought to stabilize the developing negative charge on the carboxylate of the uronic acid. Histidine may also directly protonate the anomic oxygen in the leaving group. Histidine 295 or histidine 510 may be playing a similar acid-base catalysis role in the active site of heparinase III with another residue acting as the base directly involved in the abstraction of the C5 hydrogen. Future studies will hopefully identify other residues critical for heparinase III activity. This paper represents the first step in an effort to fully understand the mechanism by which heparinase III depolymerizes heparan sulfate.

ACKNOWLEDGMENT

We acknowledge Ulf Ekernas for his help with the recombinant expression of heparinase III, IBEX Technologies for its gift of native heparinase III, and Professor Peter Kim for use of his CD equipment.

REFERENCES

Critical Histidines in Heparinase III

CONCLUSIONS AND SIGNIFICANCE

This chapter is focused on developing an understanding of the role of specific histidine residues in the degradation of heparan sulfate by heparinase III. Employing a series of chemical modification and peptide mapping studies along with site-directed mutagenesis, we have shown that histidine 295 and histidine 510 are critical for the catalytic activity of heparinase III. Histidine has also been implicated in the activity of both heparinase I and II [29,37]. In heparinase I, His203 has been proposed to be a member of a catalytic triad of residues (along with Cys135 and Lys199) that act together to carry out the eliminative cleavage of the heparin substrate [30]. Specifically, His203 is thought to either stabilize the unprotonated form of the cysteine residue allowing for the abstraction of the C5 proton or to protonate the anomic oxygen in the leaving group (see Chapter 1 for an in depth discussion). While three unique histidines have been implicated in the activity heparinase II, the exact role of each residue is not known at this time [37]. The results presented in this chapter have extended our biochemical understating of the heparinases by demonstrating that histidine is also required for the activity of heparinase III.

The results of this study are significant for several reasons. To begin with, these results represent the first biochemical characterization of an enzyme that degrades heparan sulfate, the most biologically relevant HSGAG. Subsequently, heparinase III was used as a critical enzymatic constraint in the development of the PEN-MALDI sequencing strategy [60]. In addition, both heparinase III, directly and heparinase III-generated HSGAG fragments were shown to have anti-cancer effects in a melanoma mouse model [88]. Finally, heparinase III has recently been used in the enzymatic synthesis of novel low molecular weight heparins that have tailored anticoagulation profiles [62,83]. Therefore, this initial study increased the utility of heparinase III as both a potential clinical therapeutic, as well as an enzymatic tool for the generation and characterization of biologically important HSGAGs.

This initial study of heparinase III also helped formed the foundation for the subsequent work presented in this thesis. Our laboratory's exhaustive biochemical characterization of the heparinases and their subsequent application to the study of HSGAGs helped elucidate the structure-function relationships for a variety of biologically important HSGAG sequences [61,63]. Around the same time, CS/DS oligosaccharides began emerging as important regulators of a diverse array of biological processes. Therefore, we sought to extend the paradigm of enzymatic tool development driving the characterization of
biologically relevant polysaccharides by developing the chondroitinases as tools for the characterization of biologically important CS/DS oligosaccharides. The subsequent chapters of this thesis focus on the biochemical characterization of the chondroitinases to enhance their utility as tools for understanding structure-function relationships for CS/DS oligosaccharides in a fashion akin to our previous efforts with the heparinases.
Chapter 3:

Recombinant Expression, Purification, and Kinetic Characterization of Chondroitinase AC and Chondroitinase B from Flavobacterium heparinum

SUMMARY

Two of the important lyases that degrade chondroitin sulfate and dermatan sulfate, chondroitinase AC (E.C. 4.2.2.5) and chondroitinase B (no E.C. #), have been isolated and cloned from Flavobacterium heparinum. The focus of this chapter is the development of an improved methodology for the recombinant expression and purification of these chondroitinases, thus enabling the functional characterization of the recombinant form of the enzymes for the first time. Utilizing an N-terminal 6x histidine tag, the recombinant chondroitinases were produced by two unique expression systems, each of which can be purified to homogeneity in a single chromatographic step. The products of exhaustive digestion of chondroitin-4SO₄ and chondroitin-6SO₄ with chondroitinase AC, and dermatan sulfate with chondroitinase B were analyzed by strong-anion exchange chromatography and a novel reverse polarity capillary electrophoretic technique. In addition, the Michaelis-Menten parameters were determined for these enzymes. With chondroitin-4SO₄ as the substrate, the recombinantly expressed chondroitinase AC has a $K_m$ of 0.8 $\mu$M and a $k_{cat}$ of 234 s⁻¹. With chondroitin-6SO₄ as the substrate, the enzyme has a $K_m$ of 0.6 $\mu$M and a $k_{cat}$ of 480 s⁻¹. Recombinantly expressed chondroitinase B has a $K_m$ of 4.6 $\mu$M and a $k_{cat}$ of 190 s⁻¹ for dermatan sulfate as its substrate. Efficient recombinant expression of the chondroitinases facilitated the structure-function characterization of these enzymes and will help promote the development of the chondroitinases as enzymatic tools for the fine characterization and sequencing of CS/DS. This work has been published in the Biochemical and Biophysical Research Communications and is reproduced here with the editor's consent [148].
Recombinant Expression, Purification, and Kinetic Characterization of Chondroitinase AC and Chondroitinase B from Flavobacterium heparinum

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Glycosaminoglycans (GAGs) are a family of complex polysaccharides involved in a diversity of biological processes, ranging from cell signaling to blood coagulation. Chondroitin sulfate (CS) and dermatan sulfate (DS) comprise a biologically important subset of GAGs. Two of the important lyases that degrade CS/DS, chondroitinase AC (EC 4.2.2.5) and chondroitinase B (no EC number), have been isolated and cloned from Flavobacterium heparinum. In this study, we outline an improved methodology for the recombinant expression and purification of these chondroitinases, thus enabling the functional characterization of the recombinant form of the enzymes for the first time. Utilizing an N-terminal 6× histidine tag, the recombinant chondroitinases were produced by two unique expression systems, each of which can be purified to homogeneity in a single chromatographic step. The products of exhaustive digestion of chondroitin-4SO4, and chondroitin-6SO4, with chondroitinase AC and dermatan sulfate with chondroitinase B were analyzed by strong anion exchange chromatography and a novel reverse-polarity capillary electrophoretic technique. In addition, the Michaelis–Menten parameters were determined for these enzymes. With chondroitin 4SO4, as the substrate, the recombinantly expressed chondroitinase AC has a $K_m$ of 0.8 μM and a $k_{cat}$ of 234 s$^{-1}$. This is the first report of kinetic parameters for chondroitinase AC with this substrate. With chondroitin-6SO4, as the substrate, the enzyme has a $K_m$ of 0.6 μM and a $k_{cat}$ of 480 s$^{-1}$. Recombinant expressed chondroitinase B has a $K_m$ of 4.6 μM and a $k_{cat}$ of 190 s$^{-1}$ for dermatan sulfate as its substrate. Efficient recombinant expression of the chondroitinases will facilitate the structure–function characterization of these enzymes and allow for the development of the chondroitinases as enzymatic tools for the fine characterization and sequencing of CS/DS.

Key Words: chondroitinase AC; chondroitinase B; chondroitin sulfate; dermatan sulfate; recombinant protein expression.

Chondroitin sulfate (CS) and dermatan sulfate (DS) are generally composed of a disaccharide repeat of uronic acid (1→3)-linked to N-acetylated galactosamine. This repeat unit is (1→4)-linked to adjacent disaccharides to form the CS/DS polymers found in nature (1). Similar to heparin-like GAGs, modifications to the backbone sequence of CS/DS provide its fine structure. On the basis of differential modifications of the backbone structure, CS/DS can be divided into 3 major subtypes. Chondroitin-4SO4 is primarily sulfated at the 4-O position of the N-acetylgalactosamine residue and contains all glucuronic epimers at the C5 position of the uronic acid moiety. Dermatan sulfate is primarily composed of 4-O-sulfated galactosamine and a mixture of the iduronic and glucuronic epimers. Chondroitin-6SO4 is sulfated at the 6-O position of the N-acetylgalactosamine residue and contains primarily the glucuronic epimer of the uronic acid moiety (2, 3). Rare sulfation at the 2-O and the 3-O position of uronic acid moieties in CS/DS has also been reported (4–6).

Chondroitin and dermatan sulfate, found predominantly as proteoglycans, have been implicated in a variety of biological processes. For example, a specific heparinase found in dermatan sulfate composed of a disaccharide repeat of 2-O-sulfated iduronic acid and
4-O-sulfated N-acetylgalactosamine modulates the antithrombin activity of the serpin, heparin cofactor II (7–9). Unlike the interaction with heparin cofactor II, the exact role of specific sequences of CS and DS in other biological processes, including various pathologies, remains elusive. The CS-containing proteoglycans, NG2 and neurocan, produced by astrocytes in response to nerve damage, have been shown to have an inhibitory effect on axon regeneration (10–12). In this case, understanding the exact polysaccharide sequences involved in this inhibition could facilitate efforts towards successful nerve regeneration after CNS injury. The degradation of CS/DS proteoglycans has also been implicated in the mechanical decay of cartilage, making the polymers a potential therapeutic treatment for osteoarthritis (13–15).

Enzymes that selectively degrade polysaccharides have proved useful in the isolation and sequencing of these polymers (16, 17). For example, the cloning, recombinant expression, and characterization of the heparinases greatly increased the utility of these enzymes as tools for the study heparin/heparan-like GAGs (18–21). Chondroitinase AC, which selectively degrades chondroitin-6SO₄ and chondroitin-4SO₄, and chondroitinase B, which is the only known enzyme that specifically degrades DS, could be used in a similar fashion to the heparinases to characterize CS/DS (1, 2). While both of these enzymes have been directly isolated and cloned from F. heparginum, obtaining a high yield of either enzyme after expression and purification has remained elusive (3, 22).

In this study, we report an efficient system for the bacterial expression and purification of both chondroitinase AC and B. The kinetic properties of the recombinant enzymes were analyzed along with the major products of the respective degradation reactions. The efficient production of chondroitinase AC and B reported here, along with the published crystal structures of both enzymes (23, 24), will facilitate future structure-function characterization of the enzymes, as well as provide contaminant-free enzymatic tools for analysis of the fine structure of CS/DS proteoglycans and their specific roles in biology.

MATERIALS AND METHODS

Materials. Chondroitin A, chondroitin-6SO₄, chondroitin C, dermatan sulfate, and the disaccharide standards were from Sigma (St. Louis, MO). Chondroitin-4SO₄ was from Seikagaku America (Falmouth, MA). Sinapinic acid, ampicillin and isopropyl-β-D-thiogalactopyranoside (IPTG) were also from Sigma. The sources of all other reagents used are noted in the experimental section.

Cloning of chondroitinase AC and B from F. heparginum. The native forms of chondroitinase AC and B are expressed with a N-terminal signal peptide that is proteolytically cleaved to produce the active form of each enzyme. To produce active recombinant enzymes, the genes for chondroitinase AC (cAC) and chondroitinase B (cB) were cloned without their respective N-terminal signal sequences. The published sequences of each gene were used to design PCR primers that truncated the first 66 bases of cAC and the first 75 bases of cB (22). To facilitate cloning into pET15b (Novagen, Madison, WI), a Ndel restriction site was engineered at the 5' end and a BamHI site at the 3' end of each gene. The N-terminal and C-terminal primers for cAC have the sequences 5'-CATATGGAGCAAGCTCAGTCA-3' and 5'-GGATC-
CCTATTTACTCAACCCTG-3', respectively. The N-terminal and C-terminal primers for cB have the sequences 5'-CATATG-CAGTTGTGCTGTCAAT-3' and 5'-GGATCCGATGTCCTTT-AATTC-3', respectively.

The PCR products were ligated into the TOPO/TA vector and transformed into TOP10 E. coli cells (Invitrogen, Carlsbad, CA). Plasmid DNA was isolated from positive colonies using a DNA mini-prep kit (Qiagen, Valencia, CA). The genes were excised from the TOPO/TA vector using the engineered Ndel and BamHI restriction sites and subcloned into a pET15b vector that had been digested with the same restriction enzymes. The ligation reactions were transformed into DH5α E. coli cells and screened for positive colonies. The plasmid DNA was isolated and transformed into BL21 (DE3) E. coli for expression.

In an attempt to circumvent the subcloning of the chondroitinase AC and B genes through an intermediate vector, the PCR products were cloned directly into the pCR7/NT expression vector and transformed into TOP10F' cells (Invitrogen). Positive colonies containing the inserted gene in the proper orientation were identified using PCR. Plasmid DNA was isolated from positive colonies and was used to directly transform BL21 (DE3) E. coli. By eliminating the intermediate cloning step, this pCR7/NT expression system will facilitate the rapid cloning of specific mutant chondroitinases in future structure-function studies.

Protein expression and purification. For the facile expression of cAC and cB, both pET15b and pCR7/NT expression systems contain an inducible T7 promoter, as well as a N-terminal 6× His tag to facilitate purification. Overnight cultures of Luria-Bertani (LB) broth (5 ml) containing 0.02 mg/ml ampicillin (amp) were used to inoculate 500 ml LB/amp cultures at an initial OD₆₀₀ of 0.05. The cultures were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) in mid-log phase (OD₆₀₀ 0.6–0.8) and incubated at room temperature (22°C) for 18–20 h. The cells were harvested by centrifugation and the supernatant was discarded.

The cell pellet was resuspended in 20 mM Tris, 500 mM NaCl, 5 mM imidazole, pH 7.9 (1/20 of the initial culture volume). The resuspended cells were placed on ice and lysed by sonication. The soluble protein of the cell lysate was isolated by centrifugation at 13,000 rpm for 20 min at 4°C. The supernatant was filtered through a 0.45-μm filter and loaded onto a 1-ml or 5-ml Hi-Trap Metal Chelate column (Pharmacia-Biotech, Piscataway, NJ) that was charged with 200 mM nickel sulfate. The column was washed and the protein was subsequently eluted in 20 mM Tris, 500 mM NaCl, 250 mM imidazole, pH 7.9. SDS-polyacrylamide gel electrophoresis analysis using precast 12% gels, the Mini-Protean II apparatus, and the Silver Stain Plus kit (Bic-Rad, Hercules, CA) was used to assess the purity of the individual proteins. The Bradford assay (Bio-Rad, Hercules, CA) was used to calculate protein concentration with bovine serum albumin (BSA) as a standard.

The 6× His tag from chondroitinase AC and B that were recombinantly expressed in pCR7/NT were removed using an enterokinase kit from Novagen. Briefly, the recombinant enzyme (500 μg) was incubated with biotinylated enterokinase (5 U) at 25°C for 6 h. Streptavidin beads were used to selectively bind the biotinylated enterokinase by following the manufacturer’s protocol. The beads along with the bound enterokinase were removed by centrifugation. The 6× His tag from the chondroitinases recombinantly expressed in pET15b were removed using a thrombin kit from Novagen (Madison, WI). In a similar fashion to the chondroitinases from pCR7/NT, chondroitinase AC and B (500 μg) were individually incubated with biotinylated thrombin (5 U) for 8 h at 25°C. Streptavidin beads were used to remove the biotinylated thrombin by following the manufac-
turer’s protocol. The beads along with the bound biotinylated thrombin were removed by centrifugation.

MALDI-MS of chondroitinases. The molecular weight of chondroitin AC and chondroitin B were confirmed by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) using a Voyager Elite time-of-flight instrument (PerSeptive Biosystems, Framingham, MA). Prior to mass analysis, the N-terminal histidine tag of the recombinant proteins was removed using a thrombin capture kit as described above. The enzymes were then buffer exchanged into 50 mM Tris–HCl, pH 8.0 and concentrated to 0.3 mg/ml using a YM-10 Centricron column (Millipore, Bedford, MA).

For MALDI-MS analysis, 0.5 μl of an aqueous protein solution was added to the target followed by the addition of 1 μl of a saturated sinapinic acid matrix solution in 50% acetonitrile/water. The sample was allowed to air dry and then placed into the instrument. Mass spectra were collected in the linear mode using delayed extraction. Instrument parameters were adjusted to maximize signal. Mass spectra were calibrated externally using a mixture of ovalbumin and bovine serum albumin.

SAX-HPLC analysis. To identify the major products of chondroitin AC digestion, exhaustive digests of 2 mg/ml chondroitin A and C in 50 mM Tris–HCl, pH 8.0, were performed overnight at 37°C (25 μg protein). The reactions were loaded onto a SphereClone 5μ SAX column (Phenomenex) and eluted using a linear gradient of 0.2–2.0 M NaCl, pH 3.5. Similar experiments were performed with chondroitin B (30 μg) using 2 mg/ml derrman sulfate in 50 mM Tris–HCl, pH 8.0, as a substrate. The major peaks were identified by comparison with AUA-GaINAC6s and AUA-GaINAC4s disaccharide standards.

Capillary electrophoresis. The exhaustive overnight digests of the CS/DS subunits by recombinant chondroitinase AC and B were also analyzed using capillary electrophoresis. Capillary electrophoretic analysis of the chondroitinase digestions was completed using a Hewlett Packard 3D CE instrument with an extended pathlength cell. A voltage of 30 kV was applied using reverse polarity. Saccharides were injected into the capillary using hydrodynamic pressure and were detected using an ultraviolet detector set at 232 nm. The running buffer consisted of 50 mM Tris, 10 μM dextran sulfate that had been brought to a pH of 2.5 using phosphoric acid. The identity of each peak was determined by comparison with known disaccharide standards.

Kinetic analysis. The activity of chondroitinase AC was determined by adding 10–50 μl of the sample to a 1 ml cuvette containing 1 mg/ml of chondroitin-6SO4 in 50 mM Tris–HCl, pH 8.0, at 35°C. Similar assays for the heparinases, product formation was monitored as an increase in absorbance at 232 nm as a function of time (19, 21). The observed activity was converted to units (μmol of product formed/min) using the molar absorption coefficient for the C4–C5 double bond formed in the reaction (3800 cm−1 M−1) (19). The activity of chondroitinase B was determined by adding 10–50 μl of sample to a 1 ml cuvette containing 1 mg/ml derrman sulfate in 50 mM Tris–HCl, pH 8.0, at 30°C. Activity units were calculated in the same fashion as for chondroitinase AC.

The kinetic parameters, K<sub>m</sub> and k<sub>cat</sub>, were calculated for both chondroitinase AC and chondroitinase B by obtaining the initial reaction rate (v<sub>i</sub>) as a function of substrate concentration. For chondroitin AC, approximately 1 μg (13 pmol) of enzyme was added to a 1 ml of chondroitin-6SO4 and chondroitin-4SO4 at concentrations ranging from 0.010 μg/ml to 1 mg/ml. The initial rate was measured for 4–10 s at 35°C in the same buffer used for the activity assay. The slope of the resulting line was plotted versus the substrate concentration in SigmaPlot (SPSS, Inc., Chicago, IL). The K<sub>m</sub> (μM) and V<sub>max</sub> (μM/s) were calculated using the Michaelis–Menten equation: v<sub>i</sub> = (V<sub>max</sub> × [S])/(K<sub>m</sub> + [S]). The k<sub>cat</sub> (s<sup>−1</sup>) was calculated by dividing the V<sub>max</sub> by the concentration of enzyme in the reaction. The K<sub>m</sub> and V<sub>max</sub> values for both chondroitin-6SO4 and -4SO4, calculated using a double-reciprocal plot of 1/[substrate] vs 1/V<sub>i</sub>, were virtually identical to the values obtained with the Michaelis–Menten equation.

RESULTS

Expression and Purification

The genes for cAC and cB were cloned from the F. heparinum genome as the mature form of the enzymes without their putative leader sequences. NdeI and BamHI sites were engineered 5′ and 3′, respectively, to facilitate subcloning of both genes into the multicloning site of pET15b. A PCR product of approximately 2 kb was produced for cAC and a product of 1.5 kb was produced for cB (Fig. 1A). These products were directly cloned into the pCRT7/NT expression vector and subsequently subcloned into pET15b. The genes were sequenced to insure that no mutations had been introduced during cloning. Both the pCRT7/NT and the pET15b containing cAC and cB were transformed into the BL21 (DE3) E. coli for expression.

Cultures were grown to an OD<sub>600</sub> of 0.6–0.8 and induced with 1 mM IPTG. After induction, the cultures were initially grown at 37°C for 2 h. Under these conditions, no soluble enzyme was produced from any of the constructs (data not shown). However, an overnight induction at 22°C yielded soluble active enzyme from all of the constructs (Fig. 1B). The final OD<sub>600</sub> of 4.5 was reached for the cultures that were grown overnight at 22°C.

After the cells were lysed, the recombinant enzymes with the N-terminal 6× His tag were bound to a charged Ni<sup>2+</sup> column and eluted in a sharp peak with 250 mM imidazole. The 6× His tag enables purification of both chondroitinase AC and chondroitinase B to homogeneity in a single chromatographic step (Fig. 1B). After purification, the 6× His tag was proteolytically removed from the pCRT7/NT expressed proteins using enterokinase and from the pET15b expressed proteins using thrombin. Removal of the 6× His tag did not influence the activity of either of the enzymes (data not shown).

Chondroitinase AC produced from pET15b gave the highest yield with 24.4 mg of protein and a total activity of 8820 U (a Unit is defined as the amount of enzyme required to degrade 1 μmol of substrate per min) from 1 L of culture (Table 1). The chondroitinase AC produced from pCRT7/NT had a lower yield of 7.0 mg of protein and a total activity of 3790 U. Chondroitin-
nase B produced with pET15b yielded 5.2 mg of protein and a total activity of 420 U from 1 L of culture. Unlike the case with the chondroitinase AC clones, chondroitinase B in pCRT7/NT produced more protein than the pET15b clone, yielding 10.4 mg of enzyme and a total activity of 880 U (Table 1).

In addition to the gel analysis, to insure that the proper recombinant proteins were being expressed and purified, the 6× His tags were proteolytically removed from the recombinantly expressed chondroitinas and their masses were examined using MALDI-MS. The observed mass of chondroitinase AC was 78,033 Da, in close agreement with the calculated expected mass of 77,669 Da (Fig. 2A). The observed mass of chondroitinase B was 54,067 Da, also in close agreement with the calculated mass of 54,037 Da (Fig. 2B). The lack of contaminating peaks in the mass spectra indicates that the recombinant proteins samples are pure with no residual thrombin or enterokinase present.

**SAX and CE Analysis of Degradation Products**

Recombinant chondroitinase AC (25 μg) was incubated with 2 mg/ml chondroitin-4SO₄, chondroitin-6SO₄, or "chondroitin A" (Sigma), a mixture of the two components, overnight at 37°C. The products of the reactions were separated on a SAX column using a linear gradient of NaCl from 0.2 to 2.0 M. The products

<table>
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<td>cB/T7</td>
<td>6.3</td>
<td>84.6</td>
<td>13</td>
<td>10.4</td>
<td>880</td>
</tr>
<tr>
<td>cB/15b</td>
<td>1.2</td>
<td>80.8</td>
<td>69</td>
<td>5.2</td>
<td>420</td>
</tr>
</tbody>
</table>

* Specific activity is 1 μmol of product formed/min · mg of protein.
* Total enzyme is reported as mg/1 L of culture.
* Total activity is reported as Units (1 μmol of product formed/min) for 1 L of culture.

**FIG. 2.** MALDI MS analysis of recombinantly expressed chondroitinas. After cleavage of the N-terminal 6× His tag, approximately 2–3 pmol of either chondroitinase AC (A) or chondroitinase B (B) in a sinapinic acid matrix (30% acetonitrile) was subjected to MALDI analysis using a time-of-flight instrument in the positive mode. For each, two ions; the singly charged ([M]⁺) and the doubly charged ([M]²⁺) species, are observed. No other signals are observed, further indicating the purity of the samples used in this analysis. In each case the observed value for the [M]²⁺ ion is in close agreement with the expected value. For chondroitinase AC, the measured value is 78,032.8 (expected 77,669). For chondroitinase B, the measured value is 54,067.1 (calculated 54,036).
products of this reaction were also analyzed using SAX chromatography. The digest of dermatan sulfate also yielded one major peak (Fig. 3D). Dermatan sulfate was also exhaustively digested with recombinant chondroitinase AC and analyzed by SAX-HPLC. A small peak representing the 4-sulfated disaccharide was observed (data not shown). Since dermatan sulfate is composed of a small percentage of 4-sulfated, iduronic acid-containing disaccharides, this observation is consistent with the known substrate specificity of chondroitinase AC.

The exhaustive overnight digests were also analyzed using capillary electrophoresis and the major peaks identified by comigration with known standards. The main peak in the chondroitin-6SO₄ digest and the first major peak in the “chondroitin A” digest had migration times of 15.41 and 15.39 min, respectively. These values are close to the migration time for the ΔUA-GalNAc6S disaccharide of 15.32 min (Figs. 4A and 4C) and well within the 4% error margin calculated for CE (25). The major peak in the chondroitin-4SO₄ digest and the second peak in the “chondroitin A” digest had migration times of 15.60 and 15.95 min, respectively, comparable to the 15.68 min migration time of the ΔUA-GalNAc4S disaccharide (Figs. 4B and 4C). Based on the comigration results, the major peak in the chondroitin-6SO₄ digest and the first peak in the “chondroitin A” digest are likely ΔUA-GalNAc6S. The major peak in the chondroitin 4SO₄ digest and the second peak in the “chondroitin A” digest are likely ΔUA-GalNAc4S.

The major peak in the chondroitinase B digest of dermatan sulfate had a migration time of 15.78 min, once again, similar to that of the ΔUA-GalNAc4S disaccharide, indicating that the peak is most likely this disaccharide (Fig. 4D). Several peaks eluted later in the SAX and earlier in the CE analysis and most likely represent disulfated disaccharides and tetrasaccharides.

**Kinetic Analysis**

The specific activity of each of the enzyme fractions was calculated by dividing the total activity of the fraction by the amount of enzyme present. The specific activity of chondroitinase AC produced by pCRT7/NT and pET15b are 541 and 362 U/mg, respectively (Table 1). The specific activity of the purified chondroitinase B from pCRT7/NT and pET15b were 84.6 and 80.8 U/mg, respectively (Table 1).

To obtain kinetic parameters for the recombinantly expressed enzymes, the initial reaction rates for their various substrates were plotted using the Michaelis–Menten equation (Fig. 5). Chondroitinase AC was analyzed kinetically using both chondroitin-4SO₄ and chondroitin-6SO₄ as substrates in 50 mM Tris, pH 8.0 at 35°C. The kinetic properties of chondroitinase AC towards chondroitin-6SO₄ from both the pCRT7/NT...
and the pET15b are comparable (Table 2). The $K_{\text{cat}}$'s for chondroitinase AC from pCRT7/NT and pET15b with chondroitin-6SO$_4$ as the substrate are 800 and 600 nM, respectively, and the $k_{\text{cat}}$'s are 300 and 480 s$^{-1}$, respectively (Table 2). The kinetic properties of chondroitinase AC toward chondroitin-4SO$_4$ are also comparable between the two expression systems. The $K_{\text{cat}}$'s for chondroitinase AC from pCRT7/NT and pET15b are 600 and 800 nM, respectively, and the $k_{\text{cat}}$'s are 233 and 234 s$^{-1}$, respectively. No products were observed over the time course of the reaction when recombinant chondroitinase AC was incubated with dermatan sulfate. However, as indicated above, exhaustive digestion of dermatan sulfate with chondroitinase AC produced a small amount of product as seen by SAX-HPLC indicating that dermatan sulfate is a poor substrate for this enzyme.

The kinetic parameters for both the pCRT7/NT and pET15b clone of chondroitinase B are also comparable (Table 2). Chondroitinase B expressed from the pCRT7/NT construct had a $K_{\text{m}}$ of 2.8 μM and a $k_{\text{cat}}$ of 87 s$^{-1}$. Chondroitinase B from the pET15b clone had a $K_{\text{m}}$ of 4.6 μM and a $k_{\text{cat}}$ of 190 s$^{-1}$. Over the time course of the reaction, no products were observed when recombinant chondroitinase B was incubated with either chondroitin-5SO$_4$ or chondroitin-6SO$_4$. Exhaustive digests of these two substrates also did not yield any products when analyzed by SAX-HPLC (data not shown).

**DISCUSSION**

The genes for chondroitinase AC and chondroitinase B have been cloned from *F. heparinum* without their
TABLE 2

Kinetic Analysis of Chondroitinase AC and B

<table>
<thead>
<tr>
<th>Clone</th>
<th>Substrate</th>
<th>$K_a$ (μM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cAC/T7</td>
<td>Chondroitin 6-SO$_4^-$</td>
<td>0.8 ± 0.15</td>
<td>423 ± 64</td>
</tr>
<tr>
<td>cAC/15b</td>
<td>Chondroitin 6-SO$_4^-$</td>
<td>0.6 ± 0.05</td>
<td>480 ± 50</td>
</tr>
<tr>
<td>cAC/T7</td>
<td>Chondroitin 4-SO$_4^-$</td>
<td>0.6 ± 0.03</td>
<td>233 ± 23</td>
</tr>
<tr>
<td>cAC/15b</td>
<td>Chondroitin 4-SO$_4^-$</td>
<td>0.8 ± 0.10</td>
<td>234 ± 5</td>
</tr>
<tr>
<td>cB/T7</td>
<td>Dermatan sulfate</td>
<td>2.8 ± 0.20</td>
<td>87 ± 10</td>
</tr>
<tr>
<td>cB/15b</td>
<td>Dermatan sulfate</td>
<td>4.6 ± 0.31</td>
<td>190 ± 80</td>
</tr>
</tbody>
</table>

*Kinetic parameters are reported as the mean of 3 experiments ± SE.

FIG. 5. Kinetic analysis of chondroitinase AC and chondroitinase B. The initial reaction rates were plotted against substrate concentration for chondroitinase AC and chondroitinase B with their respective substrates using the two parameter, nonlinear Michaelis–Menten equation. (A) Chondroitinase AC degradation of chondroitin-6SO$_4^-$ (Inset) A double reciprocal plot of 1/[chondroitin-6SO$_4^-$] vs 1/$v_0$ for chondroitinase AC. (B) Chondroitinase AC degradation of chondroitin-4SO$_4^-$ (Inset) A double reciprocal plot of 1/[chondroitin-4SO$_4^-$] vs 1/$v_0$ for chondroitinase AC. (C) Chondroitinase B degradation of dermatan sulfate. (Inset) A double reciprocal plot of 1/[dermatan sulfate] vs 1/$v_0$ for chondroitinase B.

The methods outlined in this study for the recombinant production of chondroitinase AC and B are significantly more effective than isolating the enzymes directly from F. heparinum. Gu et al. were able to isolate 300 μg of chondroitinase AC and 340 μg of chondroitinase B from a 10 L culture (3). Starting with 20-fold less initial culture volume, we were able to express and purify three orders of magnitude more of both chondroitinase AC (24.4 mg from the pET15b clone) and chondroitinase B (10.4 mg from the pCRT7/NT clone) than was purified through direct isolation (Table 1). Also, the specific activities for the recombinant chondroitinase AC (362 and 573 U/mg) are three to fivefold higher than the specific activity reported from the direct isolation of native chondroitinase AC from F. heparinum (121 U/mg) (Table 1) (3). Most importantly, as can be seen with SDS–polyacrylamide gel analysis, the recombinant chondroitinase AC and B from both expression vectors were purified to homogeneity in a respective leader sequences into two expression systems, pCRT7/NT and pET15b. The enzymes are expressed with a N-terminal 6× His tag at high levels in BL21 (DE3) E. coli. The 6× His tag binds selectively to a Ni$^{2+}$ affinity column and allows for the purification of the enzymes from the soluble crude extract in one chromatographic step. After purification, the 6× His tag can be removed by proteolysis, leaving behind the mature forms of chondroitinase AC and chondroitinase B. While these enzymes have been isolated directly from F. heparinum, and their genes have been previously cloned and recombinantly expressed, the results of this study dramatically improve on previous yield and activity (3, 22). This study also clearly delineates for the first time the kinetic properties and substrate specificities of the recombinantly expressed chondroitinase AC and chondroitinase B.
single chromatographic step, whereas five or six steps were required for the isolation of pure enzymes directly from \textit{F. heparinum} (Fig. 1B) (3). In the previous effort to recombinantly express these enzymes, specific activities and yields were not reported by the authors (22).

To ensure that the proper proteins were being expressed, the 6× His tags were proteolytically removed from chondroitinase AC and chondroitinase B, and the proteins were analyzed using MALDI-MS. The observed mass for chondroitinase AC was 78,033 kDa, a difference of +364 Da from the expected molecular weight of 77,669 kDa. The observed mass for chondroitinase B was 54,067 kDa, a difference of +30 Da from the expected mass of 54,036 kDa (Fig. 2). These expected masses for the recombinantly expressed enzymes include the four amino acids (GSHM) that remain on the N-terminal of the proteins after proteolytic cleavage of the 6× His tag in the pET15b expression system. The lack of contaminating peaks in the MALDI-MS analysis confirms the purity of the recombinant enzymes (Fig. 2).

The products of exhaustive digestion of the chondroitin-6SO₄ with chondroitinase AC and dermatan sulfate with chondroitinase B were analyzed using SAX-HPLC and CE (Figs. 3 and 4). In both cases, the major peaks were identified by comigration with defined disaccharide standards. As expected, the chondroitin-6SO₄ exhaustively digested with chondroitinase AC yielded primarily ΔUA-GalNAc6S and the exhaustive digest of dermatan sulfate yielded primarily ΔUA-GalNAc4S (Figs. 3 and 4). The peaks eluting later in the SAX-HPLC chromatogram and earlier in the CE electropherogram are most likely disulfated disaccharides and/or tetrasaccharides. The novel, reverse polarity capillary electrophoretic technique we employ here provides an increased level of detection and resolution of chondroitin and dermatan disaccharides than previously described techniques.

Interestingly, most of the commercial preparations of chondroitin-4SO₄ contain a large amount of contaminating chondroitin-6SO₄ (unpublished observation, K. Pojascek and R. Sasishekar). One of these preparations, “chondroitin A” from Sigma, was exhaustively digested with recombinant chondroitinase AC and the products were separated using SAX-HPLC and CE. Two nearly equal peaks representing ΔUA-GalNAc6S and ΔUA-GalNAc4S were observed in the SAX chromatogram (Fig. 3). The CE electropherogram also contains two major peaks, although the ΔUA-GalNAc4S peak is noticeably larger than the ΔUA-GalNAc6S peak (36% of the sample). However, we found that the super special grade of chondroitin-4SO₄ (Seikagaku of America), when exhaustively digested with chondroitinase AC and analyzed by SAX-HPLC and CE (Figs. 3 and 4), is primarily made up of ΔUA-GalNAc4S (95% pure). Therefore, we used the pure chondroitin-4SO₄ for the accurate chondroitinase AC kinetic measurements.

The kinetic parameters obtained for the recombinantly expressed chondroitinases are similar to those of the native chondroitinases (Fig. 5). Gu \textit{et al.} report a $K_m$ of 9.3 μM and a $k_{cat}$ of 149 s⁻¹ for the native chondroitinase AC with chondroitin-6SO₄ in phosphate buffer, whereas we observe a $K_m$ of 0.6 μM and a $k_{cat}$ of 480 s⁻¹ for the recombinant enzyme (expressed in pET15b) in the Tris buffer (3). The lower $K_m$ and the slightly higher $V_{max}$ observed for the recombinant enzyme are most likely due to these differing buffering conditions. For the native chondroitinase B, Gu \textit{et al.} report a $K_m$ of 7.4 μM and a $k_{cat}$ of 192 s⁻¹ for the degradation of dermatan sulfate (3). The $K_m$ and $V_{max}$ for the recombinant chondroitinase B (expressed in pET15b) are virtually identical at 4.9 μM and 190 s⁻¹, respectively (Table 2). In this case, the reaction conditions for the kinetic analysis of the recombinant enzyme are nearly identical to those used in the characterization of the native enzyme. The authors of the previous recombinant expression study for chondroitinase AC and B state that the kinetics of the recombinant enzymes are comparable to the native enzymes, without presenting $K_m$ and $k_{cat}$ values. Therefore, we can only compare our kinetic values to those published for the native enzymes.

In addition to analyzing the kinetics of recombinant chondroitinase AC, we are the first to report kinetics for chondroitinase AC with pure chondroitin-4SO₄ (Fig. 5). With the chondroitin-4SO₄ as a substrate, the recombinant chondroitinase AC (expressed in pET15b) has a $K_m$ of 0.8 μM and a $k_{cat}$ of 234 s⁻¹. Despite having the same $K_m$ for chondroitin-4SO₄, the lower $k_{cat}$ suggests that chondroitin-6SO₄ is a better substrate for chondroitinase AC, thus providing insight into the substrate specificity of the enzyme.

The results of this study provide an easy means for producing large amounts of pure recombinant chondroitinase AC and chondroitinase B from two unique expression systems. The pET15b constructs yields the most enzyme and could be a promising source for the commercial production of chondroitinase AC and chondroitinase B, while the pCRT7/NT constructs will be useful for the rapid screening of molecular engineered mutants of the enzymes. Our novel reverse polarity capillary electrophoresis technique along with traditional SAX-HPLC allow for reproducible analysis of the products of chondroitin and dermatan sulfate degradation. We are the first to report the kinetics of chondroitinase AC with chondroitin-4SO₄ as its sole substrate. We also enumerate, for the first time the kinetics of recombinant chondroitinase AC and B with their respective substrates, chondroitin-6SO₄ and dermatan sulfate. As a whole, this study represents a seminal effort in future structure–function studies of chondroitinase AC and chondroitinase B.
ing the function of these enzymes will allow for the
development of enzymatic tools to better probe the
intricacies of chondroitin sulfate and dermatan sulfate
biology.

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Conclusion and Significance

The research discussed in this chapter focused on the development of a recombinant expression and purification system for chondroitinase AC and B from *F. heparinum*. The genes for each of the chondroitinases were subcloned into *E. coli*-based expression plasmids and recombinantly expressed as N-terminal 6x-His tag fusion proteins, thus enabling the one step purification of the enzymes using a Ni\(^{2+}\) affinity column. The identity and purity of each of the recombinant chondroitinases were confirmed using SDS-PAGE and MALDI-MS. In addition, we calculated the kinetic parameters (K\(_m\) and k\(_{cat}\)) for each enzyme with its respective substrates. Finally, the product profile generated by the exhaustive degradation of the CS/DS substrates by chondroitinase AC and B was analyzed using SAX-HPLC and CE. Taken together, these results form the biochemical foundation for the further characterization of chondroitinase AC and B.

An important step in developing the heparinases as tools for the analysis of HSGAGs was the development of recombinant expression systems for the production of milligram amounts of pure enzyme devoid of contaminating sulfatase activity [47,149,150]. Therefore, as a first step in developing the chondroitinases as tools for the analysis of CS/DS oligosaccharides we began by developing a similar *E. coli*-based expression system. The existing recombinant expression system relied on using *F. heparinum*, which did not address the potential for contaminating sulfatases and a required laborious purification system [47]. While improvements on this Flavobacterial system have recently been published, the yield and the purity described herein remain significantly better [48]. As whole, the results presented in this chapter represent the foundation for the future work on the biochemical characterization of chondroitinase B presented in the subsequent chapters. The characterization of chondroitinase AC has been pursued by another research group [52,53] and is summarized in Chapter 1.
Chapter 4:

**Part I: Biochemical Characterization of the Chondroitinase B Active Site**

**Summary**

A recent co-crystal structure of chondroitinase B with a disaccharide product of DS depolymerization provided some insight into the location of the active site and suggested potential roles of some active site residues in substrate binding and catalysis. However, this co-crystal structure may not be representative of the actual enzyme-substrate complex since the disaccharide product did not have either the right length or chemical structure of the minimal substrate (tetrasaccharide) that can be cleaved. Therefore, only a limited picture of the functional role of active site residues in DS depolymerization was possible in previous structural studies. This chapter is focused on identifying novel roles of specific active site amino acids in the catalytic function of chondroitinase B by docking a DS tetrasaccharide into the proposed active site of the enzyme. The catalytic residues, Lys250, Arg271, His272, and Glu333 along with the substrate binding residues, Arg363 and Arg364, were mutated using site-directed mutagenesis, and the kinetics and product profile of each mutant were compared to recombinant chondroitinase B. Mutating Lys250 to alanine resulted in inactivation of the enzyme, potentially attributable to the residue’s role in stabilizing the carbanion intermediate formed during enzymatic catalysis. The His272 and Glu333 mutants showed diminished enzymatic activity that could be indicative of a possible role for one or both residues in the abstraction of the C5 proton from the galactosamine. In addition, the Arg364 mutant had an altered product profile after exhaustive digestion of DS suggesting a role for this residue in defining the substrate specificity of chondroitinase B. These results were published in the Journal of Biological Chemistry [151].
Chondroitinase B from Flavobacterium heparinum is the only known lyase that cleaves the glycosaminoglycan, dermatan sulfate (DS), as its sole substrate. A recent co-crystal structure of chondroitinase B with a disaccharide product of DS depolymerization has provided some insight into the location of the active site and suggested potential roles of some active site residues in substrate binding and catalysis. However, this co-crystal structure was not representative of the actual enzyme-substrate complex, because the disaccharide product did not have the right length or the chemical structure of the minimal substrate (tetrasaccharide) involved in catalysis. Therefore, only a limited picture of the functional role of active site residues in DS depolymerization was presented in previous structural studies. In this study, by docking a DS tetrasaccharide into the proposed active site of the enzyme, we have identified novel roles of specific active site amino acids in the catalytic function of chondroitinase B. Our conformational analysis also revealed a unique, symmetrical arrangement of active site amino acids that may impinge on the catalytic mechanism of action of chondroitinase B. The catalytic residues Lys-250, Arg-271, His-273, and Glu-333 along with the substrate binding residues Arg-363 and Arg-364 were mutated using site-directed mutagenesis, and the kinetics and product profile of each mutant were compared with recombinant chondroitinase B. Mutating Lys-250 to alanine resulted in inactivation of the enzyme, potentially attributable to the role of the residue in stabilizing the carbanion intermediate formed during enzymatic catalysis. The His-272 and Glu-333 mutants showed diminished enzymatic activity that could be indicative of a possible role for one or both residues in the abstraction of the C-5 proton from the galactosamine. In addition, the Arg-364 mutant had an altered product profile after exhaustive digestion of DS, suggesting a role for this residue in defining the substrate specificity of chondroitinase B.

Dermatan sulfate (DS)† and chondroitin sulfate (CS) are related glycosaminoglycans that are composed of a disaccharide repeat unit of uronic acid α(1→3) linked to N-acetyl-d-galactosamine (GalNAc) These disaccharide repeats are β(1→4) linked to each other to form polysaccharides of CS or DS. Epimerization at the C-5 position of the uronic acid moiety during the biosynthesis of DS leads to a mixture of l-iduronic and d-glucuronic acid epimers (1). In addition to C-5 epimerization, C-4 sulfation of GalNAc is another hallmark modification of the DS backbone. Rare sulfation at the 2-O and 3-O positions of the uronic acid moiety has also been reported (2, 3).

CS/DS polysaccharides have been implicated in a variety of biological phenomena ranging from anticoagulation to osteoarthritis (4–6). In fact, specific sequences of highly sulfated DS from a variety of invertebrate and mammalian sources are being pursued as pharmacologically viable treatments for specific blood coagulation disorders (7–9). Changes in the DS side chain of the small proteoglycan, decorin, have been observed in human colon cancer (10), and modification of existing glycosaminoglycan sequences by chondroitinase B and chondroitinase AC may inhibit angiogenesis and tumor metastasis (11). Overall, the role of glycosaminoglycans as specific mediators of tumorigenesis and other biological events is an emerging field that offers great potential for the development of novel therapeutics (12, 13).

Flavobacterium heparinum is a common source for glycosaminoglycan-degrading lyases, producing both the extensively characterized heparin-degrading heparinasenses (14–16) and the DS/CS-degrading chondroitinasenses (17). Chondroitinase B is the only member of the chondroitinase family that degrades DS as its sole substrate (18, 19). We have recently developed a large scale recombinant expression and purification scheme for chondroitinase AC and B as a first step toward using these enzymes as tools for the characterization of CS/DS oligosaccharides (19). Extensive biochemical characterization of the catalytic mechanism and substrate specificities of the heparinasenses enabled their application as tools to sequence biologically important heparin oligosaccharides (13, 20).

Chondroitinase B, like the other glycosaminoglycan-degrading lyases from F. heparinum, is thought to cleave its DS substrate through a concerted β-elimination mechanism originally proposed by Gerlt and Gassman (21). The first step in the proposed reaction is the abstraction of the C-5 proton on the uronic acid moiety by a basic amino acid forming an enolate intermediate. The enzyme stabilizes this carbanion intermediate usually via a positively charged, hydrophilic amino acid (21, 22). The final step of the reaction mechanism involves protonation of the anomeric oxygen by an acidic residue with con-
Characterization of Chondroitinase B

comitant β-elimination of the uronic acid, resulting in an unsaturated Δ^4,5 bond (21, 22).

A recently solved co-crystall structure of chondroitinase B with a disaccharide product of DS degradation, ΔUA-GalNAc-4S (23), provided the location of the active site and suggested residues that are potentially involved in substrate binding and catalysis based on their interactions with the disaccharide product. Although this structure is a good starting point to understand the location and topology of the active site, the functional role of the specific active site residues could not be directly ascertained. To begin with, the co-crystal structure represents an enzyme-substrate complex, not an enzyme-substrate complex. In fact, the minimum substrate length required for catalysis is a tetrasaccharide, as opposed to the disaccharide observed in the co-crystal structure. In addition, the ΔUA-containing disaccharide in the co-crystal structure has a unique planar carboxyl group not present in a viable substrate, thereby altering the interactions of active site residues with this disaccharide.

Therefore, we sought to address these issues through conformational studies with an actual substrate. A DS tetrasaccharide structure obtained from the co-crystal structure with chondroitinase B (24) was docked into the active site of chondroitinase B (24). This conformational analysis study uncovered several significant differences in the identification of specific roles for certain amino acids and identified a symmetrical distribution of active site residues that may impinge on the mechanism of action of chondroitinase B. Based on this analysis, we chose a subset of active site residues and by selectively mutating these amino acids to alanine using site-directed mutagenesis we provided evidence for the proposed roles of the catalytic and substrate binding residues. Our study provides the first molecular basis for understanding how chondroitinase B depolymerizes DS, a critical requirement for the future use of this enzyme in the sequencing and characterization of bioactive DS oligosaccharides.

MATERIALS AND METHODS

Materials—Porcine intestinal mucosa dermatan sulfate, chondroitin 4-sulfate, and chondroitin 6-sulfate were purchased from Sigma. The disaccharide standards were from Seikagaku/Associates of Cape Cod (Talmouth, MA). Oligonucleotide primers for PCR mutagenesis were from Invitrogen. All other reagents used are from common sources or are as noted under "Materials and Methods."

Docking of Dermatan Sulfate Tetrasaccharide into Chondroitinase B Active Site—The structure of the DS tetrasaccharide was obtained from a recently solved co-crystall structure of a chondroitinase AC mutant enzyme with a DS hexasaccharide (PDB, 1HM2). Only four of the sugar units in this hexasaccharide were defined in the co-crystal structure (24). Therefore, we used the defined tetrasaccharide region, ΔUAβ(1→4)GalNAc4Sβ(1→4)IdoUAβ(1→3)GalNAc4S, in our docking study. The initial orientation of this DS structure relative to chondroitinase B was obtained by superimposing the non-reducing end of the tetrasaccharide onto the disaccharide in the co-crystal structure. This preliminary orientation was modified by manually manipulating the tetrasaccharide structure to optimize favorable contacts between the active site amino acids and the tetrasaccharide. All the manipulations of the structures and docking were done using the viewer and docking modules of INSIGHT II.

The manually modified docked tetrasaccharide was subjected to an energy minimization process in which the potentials of the enzyme and the oligosaccharide were set using the AMBER force field modified to include carbohydrates (25) with sulfate and sulfamate groups (26). The enzyme-substrate complex was subjected to 300 steps of steepest gradient minimization without including charges, keeping most of the enzyme fixed and allowing only the regions close to the substrate to move. A force constant of 5,000 kcal was applied to each of the ring torsion angles, ensuring that the ring geometries of the sugar units in the tetrasaccharide were not significantly distorted.

Each of the subsequent orientations of the tetrasaccharide substrate was evaluated for steric contacts and non-bonded interactions with the active site of the enzyme. The optimal orientation with reasonably low steric hindrance was selected for further energy minimization. The refined structure was further subjected to 300 steps of conjugate gradient minimization including charges. A distance-dependent dielectric with a scaling factor of 4.0 and a 1.4 non-bonding scaling factor of 0.5 was set while using the AMBER force field as recommended by the software manual.

PCR Site-directed Mutagenesis of Chondroitinase B—Lys-280, Arg-271, His-272, Gly-533, Arg-565, and Arg-364 were mutated to alanine using overlap extension PCR with 15 cycles (16). The primer sequences for each of the mutants are listed below. The R727A mutant primers have the sequences KαACCTGTCGCTGGGACTGATC-3′ and 5′-ATGACCCCGCCGACCAAGTGT-3′. The E339A mutant primers have the sequences 5′-ATGGCTGGCGCGGATCCGGCC-3′ and 5′-AGAGCTGTCGCGGCGAAGCCAT-3′. The R250A mutant primers have the sequences 5′-ATCCACACACCCCGG-3′ and 5′-TTTCTGCGGACGACCAAGTCT-3′. The R271A mutant primers have the sequences 5′-ATGAACCCTTTCGGTCAAGT-3′ and 5′-ATACCCGTGACGAAATTGCT-3′. The R363A mutant primers have the sequences 5′-TTGGATAGTGGCGACGAAAGAA-3′ and 5′-TCTTCTTTTCTGCGCTCATA-3′. The R2564A mutant primers have the sequences 5′-GATGGCGGCGGACTGATC-3′ and 5′-ATATCTTCTTTTGCCTGTCATC-3′. The N- and C-terminal primer sequences are as previously described (19).

The PCR reaction products were separated on an agarose gel, and the band corresponding to the proper length was excised. DNA was extracted from the gel using a gel purification kit (Qiagen, Valencia, CA). The insert was subcloned into pCR7/NT (Invitrogen) and the plasmid was prepared using a maxi preparation kit (Qiagen). Each of the clones was sequenced to verify the presence of the individual alanine point mutations. Each chondroitinase B mutant was excised from pCR7/NT using an NdeI and BamHI (New England Biolabs, Beverly, MA) enzyme mixture and subcloned into a pET15b expression vector (Novagen, Madison, WI), which had been digested previously with the same enzymes. Recombinant chondroitinase B that had been cloned in a similar fashion was also expressed and compared with each of the alanine mutants.

Protein Expression and Purification—Recombinant chondroitinase B and the site-directed mutants were expressed and purified as previously described (19). The purity of the recombinant chondroitinase B and site-directed mutants was assessed by SDS-polyacrylamide gel electrophoresis analysis using precast 12% gels, the Mini-Protein II apparatus, and the silver stain-plus kit (Bio-Rad). A relative protein concentration was calculated using the Bradford assay (Bio-Rad) with bovine serum albumin as a standard.

Kinetic Analysis—The activity of chondroitinase B and various site-directed mutants was determined by adding 10–50 μl of the sample to a 1-ml cuvette containing 1 mg/ml DS in 50 mM Tris-HCl, pH 8.0, at 30°C. Product formation was monitored as an increase in absorbance at 322 nm as a function of time (19).

The kinetic parameters, K_m and V_max, were calculated for chondroitinase B and the site-directed mutants by obtaining the initial reaction rate (v), as a function of DS concentration. 1 μg (15 pmol) of enzyme was added to 1 ml of DS at concentrations ranging from 0.100 μg/ml to 2 mg/ml. The initial rate was measured for 4–10 s at 30°C in the same Tris-HCl buffer used for the activity assay. The slope of the resulting line, assuming zero order kinetics, was plotted versus the substrate concentration using SigmaPlot (SPSS, Inc., Chicago, IL). The K_m (μM) and V_max (pmol/min) were calculated using the Michaelis-Menten equation:

\[ v = \frac{V_{max} \times [S]}{K_m + [S]} \]

The k_{cat} (s^{-1}) was calculated by dividing the V_max by the concentration of enzyme in the reaction.

Dermatan Sulfate Digestion and Capillary Electrophoresis—To examine changes in the product profile of each site-directed mutant compared with recombinant chondroitinase B (50 μg), digests of 1 mg/ml DS 50 mM Tris-HCl, pH 8.0, were performed for 12–14 h at 30°C. The digests were analyzed using capillary electrophoresis as previously described (19). Briefly, the chondroitinase B and site-directed mutant digests were diluted 2-fold and analyzed with an extended path length cell and a voltage of 30 kV applied using reverse polarity. The running buffer consisted of 50 mM Tris, 10 mM NaOH, and the buffer was brought to a pH of 2.5 using phosphoric acid, and the saccharide products were detected by monitoring at 232 nm.

The total peak area for the recombinant chondroitinase B and mutant digest profiles was calculated by adding the areas of the ΔUA-GalNAc-4S, ΔUA-GalNAc-4S-6S, and ΔUA-GalNAc-4S peaks. The total peak area for the R364A mutant also included the sum of the area of the three additional oligosaccharide peaks. The ratio of the ΔUA-GalNAc-4S peak area to the total peak area was then calculated for the
Characterization of Chondroitinase B

Fig. 1. Docking of the dermatan sulfate substrate in the active site of chondroitinase B. A, stereoview of a Connolly surface rendering of the active site of chondroitinase B with the docked dermatan sulfate tetrasaccharide (green) and disaccharide product (orange) with orientation replicated from the co-crystal structure (22). Although the direction of both tetrasaccharide and disaccharide is the same from non-reducing end (close to the C terminus above the active site) to reducing end (close to the N terminus below the active site), the tetrasaccharide is positioned to completely occupy the active site. B, stick representation of the dermatan sulfate tetrasaccharide in the active site of chondroitinase B, colored according to the atoms (green, C; blue, N; red, O; and yellow, S) (left) and the two-dimensional schematic distribution of the active site residues (right) in the linear mode with delayed extraction and similar instrument parameters to those described previously (27).

Results and Discussion

Interactions between Chondroitinase B and Dermatan Sulfate Substrate—The structure of a previously crystallized DS tetrasaccharide was docked into the chondroitinase B active site. The direction of the tetrasaccharide relative to the enzyme was the same as the ΔUA-GalNAC-4S disaccharide product in the crystal structure, with the non-reducing end of the tetrasaccharide toward the C terminus and the reducing end toward the N terminus of the enzyme. However, the orientation of the tetrasaccharide relative to the parallel β-helical axis of the enzyme was different from that of the disaccharide (Fig. 1A). When the non-reducing end of the tetrasaccharide was superimposed with the disaccharide product from the co-crystal structure, the orientation of the tetrasaccharide was such that its reducing end collided with a wall of the active site cleft (Fig. 1A). Also, in this orientation the reducing end was too far
from the basic cluster of residues His-116, Arg-184, and Arg-218, previously implicated to provide a binding site for an additional 4-O-sulfate group located at the reducing end of GalNAc (24). Our docking and energy minimization resulted in repositioning of the tetrasaccharide substrate to achieve maximum contact with the active site cleft of the enzyme (Fig. 1A). In the final orientation, the tetrasaccharide completely occupied the -2, -1, +1, and +2 subsites of the active site of chondroitinase B.

Active Site Residues—Because the docked tetrasaccharide occupied all of the chondroitinase subsites, our theoretical enzyme-substrate complex provided a better picture of the interaction between the DS substrate and the active site residues compared with what was observed in the co-crystal structure (23). Glu-333, Lys-250, Arg-271, and His-272 were identified as key residues involved in catalysis based on proximity to the -1 and +1 subsites containing cleavable -GalNAc4Sβ(1-4)IdoUA linkage (Fig. 1B). This cluster of charged residues in the catalytic site suggests that there may be more than the prototypical triad of residues that are involved in the proton abstraction and donation mechanism resulting in the β-elimination cleavage. Glu-333 is positioned proximal to the O-1 of GalNAc-4S in such a way that it could potentially mediate proton abstraction via a water molecule. This interaction is consistent with the earlier observation from the co-crystal structure that implicated Glu-333 as a general base for proton abstraction based on the distance from its OE1 to the reducing end O-1 (4.4 Å) of the ΔUA-GalNAc-4S (24). The proximity of His-272 and Lys-250 to the C-5 proton (Fig. 1B) indicates that these residues are also positioned to act as a general base for proton abstraction. However, Lys-250 is the only residue in proximity to the carboxylate moiety of the IdoUA monosaccharide. This strongly supports its involvement in neutralizing the charge of the carboxylate group, which is a key step required for β-elimination (21). Arg-271 is proximal to both the ring oxygen and O-1 of the GalNAc residue and thus is positioned to protonate the leaving O-1 atom of the GalNAc after cleavage.

Because the co-crystal structure did not contain any monosaccharide units in the +1 and +2 subsites, the authors could only speculate on the roles of most of the above residues from the co-crystal structure (23). For instance, Lys-250 was suggested as a likely candidate for charge neutralization. However, its role was not definitive from the co-crystal structure because its only interaction was with the reducing end O-1 of the disaccharide via a water molecule. In addition, His-272 was described as an unlikely candidate in proton abstraction because it was not close enough to the reducing end of the disaccharide to act as a general base, although our analysis indicates that this may not be the case.

Substrate Binding Residues—Several residues involved in substrate binding were identified from our theoretical chondroitinase B-tetrasaccharide complex. These include basic residues Arg-318, -363, and -364 and pyrroline ring stacking aromatic residues Phe-296 and Trp-298. Phe-296 provides a parallel stacking interaction with the IdoUA in the -2 subsite, and Trp-298 stacks perpendicularly with the IdoUA and GalNAc in subsites -2 and -1, respectively (Fig. 1B). Arg-364 is positioned to interact with both the 4-O-sulfate of the GalNAc-4S and the carboxyl group of the non-reducing end IdoUA (Fig. 1B), consistent with what was observed in the co-crystal structure (23). Because the 4-O-sulfate group of GalNAc-4S and IdoUA represents hallmark modifications of DS, the Arg-364 residue is most likely to be involved in substrate specificity of the enzyme. Arg-318 interacts with the IdoUA in the -2 site, and Arg-363 is positioned to interact with an additional GalNAc-4S moiety on the non-reducing end in what would potentially be subsite -3. Finally, Asn-213 interacts with the N-acetyl group of GalNAc in the -1 subsite (Fig. 1B).

In the product release site (subsites +1 and +2), the side chains of Arg-184 and His-116 are oriented to provide favorable ionic interactions with the GalNAc-4S residue at the reducing end of the DS tetrasaccharide (Fig. 1B). These interactions provide a more definitive meaning to the speculated role of these two basic residues in binding to the 4-O-sulfate group at the reducing end of the DS substrate. Taken together, our enzyme-substrate complex, when compared with the earlier co-crystal structure, provides a clearer framework of the various residues involved in substrate binding and product release.

Active Site Symmetry—In addition to providing further insight into the exact role of each residue in the chondroitinase B active site, our conformational study has also uncovered a chemical symmetry of amino acid side chains in this region. In fact, there appears to be an internal 2-fold symmetry of the positively charged, negatively charged, and hydrophobic residues in the active site about an axis passing through the cleavage site (-1 and +1) and perpendicular to the axis of the β-helix (Fig. 2). Specifically, the proposed residues that are involved in the substrate binding site (2-1 and -1), including Phe-296, Arg-318, and Arg-364, resemble a corresponding residues in the product release site (+1 and +2), including Tyr-222, Arg-184, and Arg-219 that are related by this symmetry. In addition, Glu-245 is in proximity to the catalytic site and

![Fig. 2. Apparent internal symmetry in the active site of chondroitinase B. The grasp-rendered view of the active site is shown on the left with the basic residues (H, K, R) in blue, acidic residues (D, E) in red, and bulky hydrophobic residue (F, Y, W) in pink. The right panel is a two-dimensional schematic of the residues with their sequence numbers encircled using the same color coding scheme as on the left. Also shown on the right is an gray arrow indicating the assumed direction of the dextran sulfate (point of arrow indicates the reducing end). There is an approximate 2-fold symmetry in the distribution of the acidic, basic, and hydrophobic residues about an axis perpendicular to the helix of the dextran sulfate oligosaccharide.](image-url)
Fig. 3. Capillary electrophoretic analysis of the dermatan sulfate reaction products for the catalytic mutations. A, recombinant chondroitinase B (20 μg), B, H272A, C, E333A, and D, K250A were incubated with 1 mg/ml dermatan sulfate for 12 h at 30 °C. Capillary electrophoretic analysis was performed using an extended path length cell and a voltage of 30 kV applied using reverse polarity. Saccharides were injected into the capillary using hydrodynamic pressure and were detected using an ultraviolet detector set at 232 nm. The running buffer consisted of 50 mM Tris, 10 μM dextran sulfate that had been brought to a pH of 2.5 using phosphoric acid. The disulfated disaccharides, ΔUA2S-GalNAC-4S and ΔUA-GalNAC-4S,6S, are indicated by one asterisk and two asterisks, respectively. Inset, electropherogram of the ΔUA-GalNAC-4S (ΔDi4S) disaccharide standard.

It appears to be related to the Glu-333 residue by the same 2-fold symmetry (Fig. 2).

Understanding the significance of the active site symmetry will provide valuable insights into the mechanism by which chondroitinase B depolymerizes its DS substrate. Based on our current observations, we can offer several plausible explanations regarding the importance of this active site symmetry. To begin with, the distance between the carbonyl oxygens of both Glu-245 and Glu-333 is about 9.5 Å, a distance comparable with the diameter of the structure of the DS substrate projected along the helical axis. Thus, if both of these negatively charged glutamic acids are involved in catalysis, their symmetrical arrangement would facilitate the translation of the substrate through the active site cleft without the need for its rotation, leading to more efficient DS depolymerization. In addition, this active site symmetry may be involved in accommodating the
perturbations in the DS chain caused by the conformational flexibility of iduronic acid, a common component of DS (28).

The symmetry of the active site may also be involved in defining the direction in which the substrate is processed through the active site. Interestingly, the DS-derived disaccharide in the co-crystal structure that is an actual product of chondroitinase B action is in the substrate binding site, not the product release site. This observation, coupled with the active site symmetry, raises the issue that the directionality of the active site might be more complex than originally thought. In fact, the reducing end of a genuine substrate may be potentially oriented toward the C-terminal end of an enzyme (a pattern of binding common among other polysaccharide lyases, Refs. 29 and 30) and not toward the N-terminal end as seen in the co-crystal structure (23). The directionality of substrate binding within the active site of polysaccharide lyases is usually unambiguously defined by a structural feature similar to the presence of a Ca++ ion at one end of the cleft, as is the case with pectate lyase C from Erwinia chrysanthemi (30). This underscores the uniqueness of the chondroitinase B active site symmetry and the need for further characterization.

**Mutagenesis and Active Site Characterization**—Having identified the key substrate binding and catalytic residues using our theoretical enzyme-substrate complex, we sought to establish their functional roles using site-directed mutagenesis. The basic residues, Lys-250, Arg-271, and His-272, were chosen based on their location in the active site of chondroitinase B. In addition, the acidic residue Glu-333 was chosen because of its possible role in proton abstraction. We also mutated two of the residues implicated in substrate binding and specificity, namely Arg-363 and Arg-364, to alanine. These site-directed mutants were cloned into pET15b and expressed alongside the recombinant chondroitinase B.

Both H272A and E333A showed altered kinetics when compared with the recombinant chondroitinase B (Table I). For instance, the $K_m$ and $k_{cat}$ for the H272A chondroitinase B mutant are 2.7 μM and 29 s⁻¹, respectively, compared with a $K_m$ of 4.6 μM and a $k_{cat}$ of 190 s⁻¹ for the recombinant enzyme (19). The E333A mutant had similar alterations in $K_m$ and $k_{cat}$ (Table I). Both of these mutations lead to a slight reduction in $K_m$ while drastically reducing $k_{cat}$. In fact, when compared with the recombinant chondroitinase B, the H272A and the E333A mutants have a 4- and 26-fold decrease in $k_{cat}/K_m$, respectively (Table I).

In addition to kinetic analysis, each of the mutant enzymes and the recombinant chondroitinase B was allowed to exhaustively digest DS to determine changes in product profile that may belie alterations in substrate specificity. These digests were diluted and analyzed using capillary electrophoresis. Complete digestion of the dermalan substrate was seen with the chondroitinase B reaction, as indicated by a major disaccharide peak (Fig. 3). This prominent disaccharide peak in all of the electrophoregrams was identified as ΔUA-GalNAc-4S (referred to as ΔDi4S) through co-migration of the known DS disaccharide standards. The two minor peaks that elute around

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**TABLE II**

Ratio of ΔDi4S area to total peak area for chondroitinase B and mutants

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>ΔDi4S/Total peak area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chondroitinase B</td>
<td>0.93</td>
</tr>
<tr>
<td>R250A</td>
<td>n.d.*</td>
</tr>
<tr>
<td>H272A</td>
<td>0.94</td>
</tr>
<tr>
<td>E333A</td>
<td>0.93</td>
</tr>
<tr>
<td>R363A</td>
<td>0.93</td>
</tr>
<tr>
<td>R364A</td>
<td>0.39</td>
</tr>
</tbody>
</table>

* No peaks were observed for the K250A digest.

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**FIG. 4.** Capillary electrophoretic analysis of the reaction products for the substrate binding mutations. A. R363A and B. R364A were incubated with 1 mg/ml dermatan sulfate for 12 h at 30 °C and analyzed by capillary electrophoresis. The length and sulfate composition of the additional peaks in the R364A digest (B) were determined using MALDI-MS. Peak 1 is an octasaccharide (1922.4 Da) with five sulfates. Peak 2 is a hexasaccharide (1539.7 Da) with five sulfates. Peak 3 is a tetrasaccharide (999.2 Da) with three sulfates. The disulfated disaccharides, ΔUA2S-GalNAc-4S and ΔUA-GalNAc-4S,6S, are indicated by one asterisk and two asterisks, respectively.

10 min were identified as ΔUA2S-GalNAc-4S (*) and ΔUA-GalNAc-4S,6S (**), respectively (Fig. 3). A comparison between the ratio of the ΔUA-GalNAc-4S peak to the total peak area of the mutant digests and the recombinant enzyme showed that H272A and E333A demonstrate full enzymatic activity over the 12-h time course of the reaction (Table II). This suggests that although His-272 and Glu-333 are important in the active site chemistry, chondroitinase B can still
**Characterization of Chondroitinase B**

Fig. 5. CD spectra of chondroitinase B and the K250A mutant. The recombinant chondroitinase B and the K250A mutant were concentrated and buffer-exchanged into 50 mM sodium phosphate buffer, pH 7.0. Proteins were analyzed in a quartz cell with a 1-mm path length at 25 °C. CD spectra were recorded between 205 and 270 nm with an average of five scans. The bandwidth was set at 1.0 nm, and the scan rate was 3 nm/min. The CD band intensities are expressed as molar ellipticities, $\theta_m$, in deg cm$^2$ dmol$^{-1}$.

function without one of them, albeit at a much slower catalytic rate.

In contrast, changing Lys-250 to alanine completely ablated the activity of chondroitinase B (Table I and Fig. 3). To ensure that the mutating Lys-250 did not influence the overall stability of the protein, the CD spectrum of K250A was compared with the spectrum of recombinant chondroitinase B. Although the virtual identity of the CD profiles does not preclude the possibility that there are perturbations in the local environment surrounding Lys-250 that are not represented in the CD profile, it does suggest there are no gross conformational changes induced in chondroitinase B by mutating Lys-250 to alanine (Fig. 5). Therefore, Lys-250 is essential for the catalytic activity of chondroitinase B.

Along with the active site residues discussed above, we mutated Arg-271 to alanine. Interestingly, the R271A mutant was expressed at comparable levels to the recombinant chondroitinase B but was completely insoluble. Several attempts to denature and refold the mutant using different methods including a strong chaotropic agent (4 M guanidinium HCl) proved unsuccessful (data not shown). The insolubility of the R271A mutant could implicate this residue in the active site chemistry of chondroitinase B. Another possibility is that removing the side chain of Arg-271 somehow interferes with the hydrophobic stacking interactions of Phe-296 and Trp-298, leading to a dramatic decrease in the stability of chondroitinase B (Fig. 1B).

Further mutagenesis studies in which this amino acid is altered to residues other than alanine will be necessary to help elucidate the exact role of Arg-271 in the active site of chondroitinase B. In addition to the catalytic residues discussed above, two basic residues proximal to subsites -2 and -1, Arg-368 and Arg-364, were selected for mutagenesis based on their potential role in substrate binding. The R363A mutant had a $k_{cat}$ of 404 s$^{-1}$, leading to a slight increase in $k_{cat}/K_m$ compared with the recombinant chondroitinase B (Table I). This 2-fold increase in $k_{cat}/K_m$ suggests that removal of Arg-363 allows for a slight increase in catalytic efficiency in chondroitinase B. The R363A mutant produced a similar profile to chondroitinase B after exhaustive digestion of DS (Fig. 4).

In contrast to the R363A results, mutating Arg-364 to alanine led to a complete loss of activity in the real time kinetic assay and an altered product profile after exhaustive digestion of DS (Table I and Fig 4). In fact, the ratio of the AUA-GalNAc-4S peak area to the total peak area was only 0.39, significantly lower than the ratio for the recombinant chondroitinase B (Table II). In addition, the AUA-GalNAc-4S peak was not the only prominent peak in the electropherogram (Fig. 4).

To further characterize the novel peaks seen in the R364A digest of DS, the sample was analyzed using MALDI-MS. Peak 3 had a mass of 999.2 Da, which identifies it as a tetrasaccharide containing three sulfates. Peak 2 had a mass of 1539.7 Da, which identifies it as hexasaccharide containing five sulfates. Finally, peak 1 had a mass of 1922.4 Da, which classifies it as an octasaccharide, also containing five sulfates. Adding more of the R364A mutant enzyme to the sample did not result in a significant decrease of these higher order peaks, suggesting that these oligosaccharides are the end products of the reaction. As suggested by our structural analysis, Arg-364 is critical for proper substrate binding and digestion of DS by chondroitinase B.

Compositional analysis of the DS starting material revealed that the AUA2S-GalNAc-4S and AUA-GalNAc-4S,6S disaccharides are 2.3 and 4.6% of the total disaccharide content (data not shown). Interestingly, there is a shift in the percentages to 5.5 and 2.3% for the AUA2S-GalNAc-4S and AUA-GalNAc-4S,6S disaccharides, respectively, when DS is digested by the R364A mutant, suggesting that the oversulfation of the higher order oligosaccharides is at the 6-O position (data not shown). Therefore, it appears that Arg-364 is involved in the ability of chondroitinase B to recognize and cleave regions containing AUA-GalNAc-4S,6S in DS. This interesting insight into the specificity of chondroitinase B is currently being pursued and will prove useful in the generation of biologically important DS oligosaccharides.

Taken together, these results for the first time directly implicate Lys-250, His-272, Glu-333, and possibly Arg-271 in the catalytic degradation of DS by chondroitinase B. Because the H272A mutation shows a 6.5-fold decrease in $k_{cat}$, this residue can potentially be involved in proton abstraction (Table I). Histidine has been implicated in the enzymatic degradation of other glycosaminoglycan-degrading enzymes, including Group B streptococcal hyaluronidase and heparinases I, II, and III (16, 31, 32). However, because the enzyme activity is not completely ablated, another residue may also be involved in abstraction of the C-5 proton. Glu-333, another candidate for C-5 proton abstraction, showed a nearly 40-fold decrease in $k_{cat}/K_m$ when mutated to alanine (Table I). Nevertheless, because the enzyme still retains close to full activity over a 12-h period (Fig. 3), Glu-333 may not be the sole residue involved in the C-5 proton abstraction. One possibility is that Glu-333 and His-272 work in concert to lower the $pK_a$ of the C-5 proton and to abstract it. A mutant chondroitinase B in which both residues are mutated will help further elucidate the roles of these residues. Another possibility is that Glu-245, the symmetrical active site residue to Glu-333, may also play a part in proton abstraction (Fig. 1B).

Mutating Lys-250 to alanine led to a complete loss of enzymatic activity of chondroitinase B toward the DS substrate. Because the e-NH$_2$ of the lysine ($pK_a$ of 10.5) is mostly protonated in the reaction buffer (pH 8.0), it seems unlikely that this residue would be involved in proton abstraction. Also, our conformational study points to the involvement of Lys-250 in stabilizing the charge of the carboxylate moiety. This charge stabilization is required in the proposed $\beta$-elimination mechanism to lower the $pK_a$ of the C-5 proton for base abstraction (21). Therefore, the complete loss of enzymatic activity in the K250A
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CONCLUSIONS

Biochemical characterization of polysaccharide lyases is a challenging task because of the complex steps involved in their catalytic process. In addition, the wide range of pH optima for many of these enzymes complicates the determination of the precise role of active site residues. Several x-ray co-crystal structures of polysaccharide lyases with their respective substrates or products have been solved (23, 24, 30). These structures provide static descriptions of inert enzyme-substrate complexes that are potentially valuable for identifying active site residues. However, because the co-crystallized substrate is not a native substrate for the enzyme because it would be degraded during the crystallization, these crystal structures do not provide sufficient information for definitively establishing the role of these residues in activity. For example, even after obtaining several crystal structures of active site chondroitinase AC mutants with different substrates, three different scenarios were proposed for the specific role of active site residues in catalysis (24). In the case of the chondroitinase B, there is even less information on the functional roles of the active site residues, because it was co-crystallized with a disaccharide product that is chemically different from the DS substrate and does not have the minimum substrate length required for catalysis.

Our study provides a first step toward defining the substrate binding and catalytic functions of the active site residues in chondroitinase B. Based on the interactions with the DS tetrasaccharide and the kinetics of the alanine mutants, we have provided substantial evidence on the involvement of Lys-250, His-272, and Glu-333 in catalysis. Lys-250 is a critical residue most likely involved in stabilizing the carboxylate moiety allowing for proton abstraction. In contrast to the previous suggestion of the involvement of a single Glu-333 residue in proton abstraction, our results demonstrate that both His-272 and Glu-333 could potentially be involved in the proton abstraction process. In addition to defining the roles of the catalytic residues, we have also used a battery of biochemical studies to define the role of Arg-364 in conferring substrate specificity. Mutating Arg-364 to alanine produced an altered product profile after exhaustive digestion of DS.

Unlike the typical situation for lyases, there appears to be more than a triad of residues involved in the degradation of DS by chondroitinase B. In addition, we have observed a 2-fold symmetry in the distribution of the active site residues with similar chemical properties. This symmetry has not been observed in other polysaccharide lyases, and we are currently investigating the significance of the symmetry in the recognition and mechanistic processing of substrate.

Acknowledgment—We thank Zachary Shriver for helpful comments and critical reading of the manuscript.

REFERENCES

Chapter 4:

Part II: The Role of Calcium in the Activity of Chondroitinase B

SUMMARY

As a follow up to our initial studies of the active site of chondroitinase B, we collaborated with Dr. Mirek Cygler's Group in studying the role of calcium in the activity of chondroitinase B. Two new co-crystal structures of chondroitinase B with a chondroitin 4-O sulfate tetrasaccharide and a dermanan sulfate derived hexasaccharide, respectively, have shed further light on the biochemistry of the active site. Of primary importance was the discovery of the presence of a calcium ion that was chelated by three amino acids in chondroitinase B as well as by the C5 carboxylate moiety of an iduronic acid. Increasing the calcium concentration produced an increase in the activity chondroitinase B with a maximal activity reached at 5 mM calcium. Kinetic analysis revealed that the presence of increasing calcium resulted in a decrease in $K_m$ and an increase in $k_{cat}$. In addition, mutating Glu243 and Glu245 to alanine confirmed the importance of these residues in the activity of chondroitinase B likely through their role in chelating calcium. These novel co-crystal structures along with the new biochemical and site-directed mutagenesis data provide further insight into the role of specific active site residues in the activity of chondroitinase B. The data presented here is a portion of two manuscripts that are being prepared for submission to the Journal of Molecular Biology (2003).
4.2.1 Introduction and Motivation

The first portion of this chapter outlined our important first step in identifying the critical amino acids involved in substrate binding and catalysis in the active site of chondroitinase B [151]. The initial co-crystal structure was of chondroitinase B complexed with a disaccharide reaction product [51]. While this structure helped identify the topological location of the active site within the protein, it provided little insight into the role of individual residues in catalysis. Building on this initial co-crystal structure of the enzyme, we were able to identify, mutate and characterize a set of residues that we believed were involved in the activity of chondroitinase B based on modeling a substrate into the active site cleft.

While our previous study did address some of the issues surrounding the role of residues in the active site, there remained a number of unresolved questions. For example, we observed a two-fold symmetry of residues with similar chemical properties throughout the active site cleft, but were unable to determine if this had any functional importance. Additionally, our initial attempts to mutate Arg271 to alanine resulted in unstable mutants that could not be purified from the crude lysate of *E. coli* [151]. Therefore, we were unable to ascertain if this residue played a significant role in catalysis as our modeling indicated. Additional co-crystal structures with longer DS derived oligosaccharide substrates would greatly assist in further defining the role of individual residues in the activity of chondroitinase B.

4.2.2 Summary of Co-crystal Structures

Two novel co-crystal structures of chondroitinase B with a 4-O sulfated chondroitin tetrasaccharide (CS$^{tetra}$) and with a DS-derived hexasaccharide (DS$^{hca}$) were recently solved by our collaborators in Mirek Cygler’s laboratory (Biotechnology Research Institute, Montreal, Quebec, Canada). The results discussed herein are designed to provide an overview of the important aspects of the co-crystal structures as they pertain to the ongoing biochemical characterization of the chondroitinase B active site and therefore are not comprehensive in their scope (Figure 4.2.1).

The CS$^{tetra}$ co-crystal revealed the 4-O sulfated chondroitin tetrasaccharide occupying subsites –1 and –2 of the chondroitinase B active site (The location of the subsites in the chondroitinase B active site depicted in Figure 4.2.2). The GalNac4S at the reducing end of this disaccharide unit is superimposable with the GalNac4S from the DS-derived disaccharide
Figure 4.2.1: Stick and ribbon rendition of the chondroitinase active site showing the location of the calcium ion. Atoms in the substrate are color-coded (C: green, N: blue, O: red and S: yellow). For the enzyme, basic residues (Lys, Arg, Asn, His) are colored blue and acidic residues (Glu) are colored red. The calcium ion is purple and its interaction with the C5 carboxyl group of IdoA is in red.

observed in the original co-crystal structure [51]. There is a slight shift in the GlcA in the CS$_{letra}$ structure compared to the ΔUA in the original co-crystal structure likely resulting from both the presence of the unsaturated double bond, as well as the β-conformation of the internal bond of the disaccharide. The disaccharide on the non-reducing of the tetrasaccharide extends toward the solvent perpendicular to the β-helix backbone of chondroitinase B and helps define subsite −3 and −4 which are likely involved in binding longer oligosaccharides.

In the case of the DS$_{hexa}$ co-crystal structure, two disaccharide reaction products occupy subsites −2 and −1 and subsites +2 and +1, respectively suggesting that chondroitinase B retained activity in its crystalline form (Figure 4.2.1 and 4.2.2). Similar to the CS$_{letra}$ and the previous co-crystal structure, a GalNac4S-ΔUA disaccharide occupies subsites −1 and −2 (Figure 4.2.1 and 4.2.2). The ΔUA of the disaccharide in subsite +1 is a distance of 2.96 Å from the C4 position of the GalNac4S in subsite −1, a distance comparable to the β(1,4) linkage found in an intact DS substrate (Figure 4.2.1). Both disaccharides are oriented with their reducing ends pointed toward the N-terminus of the protein further suggesting that they represent the cleavage products of an intact oligosaccharide. Another disaccharide unit is
bound in the opposite orientation of the other disaccharides at a location removed from the active site and will not be considered further.

An additional spherical electron density representing the presence of a calcium ion was noted in the DS$_{\text{hexa}}$ co-crystal that had not been seen in any of the previous structures (Figure 4.2.1). This ion establishes contacts with the carboxyl oxygen atoms of Glu243 and Glu245, the carbonyl oxygen of Asn213, and the one of the oxygen atoms of the C5 carboxyl group of the ΔUA. The coordination is completed by two water molecules and has an octahedral geometry. The presence of the calcium ion in the active site was quite surprising due to the apparent lack of a requirement for the divalent in chondroitinase B activity [149,150]. The role of calcium in the activity of chondroitinase B was explored though a variety of biochemical experiments and is discussed below.

In addition to discovery of the calcium ion, the DS$_{\text{hexa}}$ structure reveals a variety of important contacts between the enzyme and the bound disaccharides. The guanidinium group on the side chain of Arg271 is hydrogen bonded to the anomeric hydroxyl group of the GalNac moiety in subsite –1 (Figure 4.2.1 and 4.2.2). The C3 hydroxyl group of the DUA in
subsite +1 is hydrogen bonded with both His272 and Glu333. Additional contacts between the disaccharides and the enzyme are present in the co-crystal structure, but are not relevant to the biochemical studies outlined below and therefore are not discussed.

4.2.3 The Role of Arg271 in Chondroitinase B Activity

In our initial study of the biochemical role of active site amino acids in chondroitinase B, we mutated Arg271 to alanine and attempted to recombinantly express the mutant enzyme. However, this mutant was very unstable and we were unable to purify the recombinant enzyme from the soluble, crude *E. coli* lysate. We speculated that removing this arginine resulted in a decrease in the stability of hydrophobic stacking interactions within the active site. Therefore, a more conservative site-directed mutagenesis strategy might produce a more soluble enzyme and help elucidate the role of this residue in degradation of DS by chondroitinase B.

In the current study, we used PCR site-directed mutagenesis to create R271E and R271K. The conversion of the arginine to a glutamic acid (R271E) replaces the positively charged guanidino group with a carboxylate group, thereby significantly altering the biochemistry of this residue. The mutation of Arg271 to lysine (R271K) replaces the guanidino group with a side chain of comparable length that contains a terminal amine. Therefore, while the R271K mutant is a more conservative mutation than the R271E mutant, the combination of the two should assist in assigning the role of this residue in catalysis.

The R271E and R271K mutants were created using PCR site-directed mutagenesis and were recombinantly expressed in *E. coli*. Similar to the R271A mutant, the soluble expression level of each of these mutants was relatively low with most of the protein forming insoluble inclusion bodies. However, we were able to purify enough of each of the mutants to test their activity by monitoring the increase in absorbance at 232 nm, the $\lambda_{\text{max}}$ of the $\Delta^{4,5}$ bond formed in the product, using the real time kinetic assay. It is important to note that in light of the role of calcium in the activity of chondroitinase B, the activity assays were performed in the presence of 5 mM calcium (see below). Neither R271K nor R271E demonstrated any
detectable change in absorbance at 232 nm over the time course of the reaction (Table 4.2.1). However, when R271K was incubated with 1 mg/ml DS for 16 hr and analyzed by CE, small product peaks were detected implying that the enzyme retains residual activity (Figure 4.2.3). The exhaustive digestion of DS by R271E did not produce any discernable peaks in the capillary electropherogram (Figure 4.2.3). No significant differences were observed in the CD spectrum of the mutants when compared to the recombinant chondroitinase B implying that the mutant enzymes are properly folded. Therefore, Arg271 is required for the proper catalytic activity of chondroitinase B.

The anomic oxygen of the GalNAc4S moiety in subsite −1 is hydrogen bonded to the side chain of Arg271 in the co-crystal structure of chondroitinase B with the DS derived hexasaccharide (Figure 4.2.1 and 4.2.4). This side chain is also stacked against Trp298 and forms a weak hydrogen bond with Glu333, which when mutated to alanine demonstrates a 41-fold decrease in $k_{cat}$ [151]. This suggests that if the guanidium side chain of Arg271 is protonated (pKa of 12.5) at the beginning of the reaction, the stability of this proton is not reinforced by the microenvironment of the amino acid. Therefore, given its location and its biochemical environment, Arg271 is a likely candidate for the donation of a proton to leaving group at the anomic oxygen completing the β-elimination cleavage of a DS substrate.

Figure 4.2.3: CE analysis of the dermatan sulfate digested by the R271K and R271E mutants. (A) R271K retains partial activity after a 16 hr. digest at 30°C. (B) However, the R271E mutant is completely inactive.
Figure 4.2.4: Schematic representation of a dermatan sulfate tetrasaccharide substrate in the active site of chondroitinase B. The critical active site amino acids (blue: basic, red: acidic, purple: uncharged) are depicted in their relative orientation to the DS substrate. Glu243 and Glu245, along with Asn213 chelate a calcium ion in the proper orientation to interact with the C5 carboxylate of IdoA. This lowers the pKa of the C5 proton that is likely abstracted by Lys250. Arg271 is in position to donate a proton to the glycosidic oxygen in the leaving group, thus completing the lytic mechanism. Glu333 and His272 (not shown for simplicity) may be involved in stabilizing the other catalytic residues. And Arg364 is likely involved in binding and orienting the DS substrate for proper catalysis.

4.2.4 Calcium and Chondroitinase B Activity

The surprising discovery of the calcium ion in the active site of the DS\textsuperscript{hexa} co-crystal led us to examine the effect of calcium on the activity of chondroitinase B. The enzyme was incubated with 1 mg/ml DS at 30°C and the appearance of product was monitored as an increase in absorbance at 232 nm in the real time activity assay. Increasing the calcium concentration led to a notable increase in the activity of the enzyme with a maximal activity at approximately 5 mM calcium (Figure 4.2.5). In addition, in the presence of 5 mM EDTA, a chelator of calcium and other divalent cations, the enzyme’s activity was ablated. The addition of increasing amount of other divalents ions such as magnesium, manganese and zinc had no significant effect on the activity of enzyme. Therefore, chondroitinase B demonstrates an absolute and specific requirement for calcium for its maximal activity.

<table>
<thead>
<tr>
<th>[Ca\textsuperscript{2+}] (mM)</th>
<th>K\textsubscript{m} (µM)</th>
<th>kcat (s\textsuperscript{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>4.3</td>
<td>60</td>
</tr>
<tr>
<td>0.1</td>
<td>2.0</td>
<td>210</td>
</tr>
<tr>
<td>5.0</td>
<td>0.3</td>
<td>270</td>
</tr>
</tbody>
</table>
The kinetic parameters $K_m$ and $k_{cat}$ were determined for chondroitinase B at three different calcium concentrations (Table 4.2.2). Increasing the calcium concentration led to a 14-fold decrease in $K_m$ and a 4.5-fold increase in $k_{cat}$, thereby dramatically increasing the catalytic efficiency ($k_{cat}/K_m$) of chondroitinase B. These results are consistent with the observation that both the substrate and the enzyme chelate the Ca$^{2+}$ ion within the active site in the co-crystal structure (Figure 4.2.1 and 4.2.4). Therefore, calcium appears to play a significant role both in substrate binding, as well as a direct role in catalysis likely through the stabilization of the negative charge on the C5 carboxylate moiety.

![Absorbance vs Calcium Concentration](image.png)

**Figure 4.2.5:** Effect of calcium on the activity of chondroitinase B. The activity of chondroitinase B toward dermatan sulfate (1 mg/ml) was measured in the presence of increasing calcium concentrations. Chondroitinase B demonstrated maximal activity in the presence of ~5 mM [Ca$^{2+}$].

To further examine the role of calcium in the active site of chondroitinase B, the two major residues responsible for chelating the divalent cation, Glu243 and Glu245, were mutated to alanine. Both E243A and E245A demonstrated no detectable change in the absorbance at 232 nm as a function of time in the presence of 5 mM calcium (Table 4.2.1). However, when each of the mutants was incubated with DS for 16 hr and analyzed by CE, small yet detectable peaks were observed suggesting that each mutant retained partial enzymatic activity (Figure 4.2.6). This detectable activity coupled with the fact that the CD spectra for E243A and E245A are identical to that of recombinant chondroitinase B suggests that the two mutants were properly folded. Therefore, given the location of these glutamates
in the active site, the observed decrease in the activity of E243A and E245A is likely due to a decrease in the ability of the chondroitinase B mutants to chelate calcium. However, the residual activity of each of the mutants suggests that the Ca$^{2+}$ ion can still be partially chelated by chondroitinase B and the DS substrate in the absence of one of these glutamates.

Early studies of the activity of the native chondroitinase B revealed no effect of calcium on the activity of the enzyme [149,150]. This observation remained unchallenged through the recent biochemical characterization of the recombinant enzyme produced both in *F. heparinum* and in *E. coli* [148]. However, the discovery of the calcium ion in the co-crystal structure of chondroitinase B with the DS derived hexasaccharide is not entirely surprising given the requirement of the divalent cation in other polysaccharide degrading lyases.

As discussed in Chapter 1, calcium is required for the activity of heparinase I with a maximal activity observed at 10 mM calcium [32]. The calcium coordination motifs in heparinase I are loosely described by the EF hand motif, whereas the calcium coordination in chondroitinase B is more similar to that seen in the pectate lyase family [152]. In fact, polysaccharide degrading enzymes from both the PL1 and PL10 families of polysaccharide degrading enzymes have calcium ions coordinated by a substrate carboxylate group and an invariant, acidic amino acid at the −1 and +1 subsites [153]. Interestingly, despite the similar requirement of calcium for chondroitinase B (PL6 family member) and the pectate lyases from the PL1 and PL10 families, the active sites of the different enzymes are not superimposable implying that the enzyme structure is influenced more by the structure of the respective substrates than by the overall protein fold. Regardless, the requirement of calcium for enzymatic activity is emerging as a common motif for the different members of the
polysaccharide degrading families of enzymes.

4.2.5 Overview of Chondroitinase B Active Site

The location of the Ca$^{2+}$ ion in the active site of the DS$_{\text{hexa}}$ co-crystal structure provided significant insight into the role of specific residues in the active site of chondroitinase B. Glu243 and Glu245 clearly are important for chelating the Ca$^{2+}$ ion as indicated by both the co-crystal structures and the site-directed mutagenesis data (Figure 4.2.1 and Table 4.2.1). These residues position the Ca$^{2+}$ ion for a specific interaction with the C5 carboxyl group on the iduronic acid in subsite +1. Therefore, the Ca$^{2+}$ ion, not Lys250 as previously proposed, serves to neutralize the negative charge on the carboxylate group effectively lowering the pKa of the C5 proton (Figure 4.2.4). This is the requisite first step in the β-elimination mechanism by which chondroitinase B degrades DS. Interestingly, calcium bound within the active site of heparinase I (CB1) was proposed to play a similar role in the degradation of its heparin substrate [33].

The ε-NH$_3$ moiety of Lys250 is the only functional group that is proximal to the C5 proton of the iduronic acid in subsite +1 (Figure 4.2.4). Therefore, given the complete lack of activity of the K250A mutant, this lysine likely acts as a base in the abstraction of the C5 proton from the iduronate. The theoretical pKa of the terminal amine of the lysine side chain is 10.5, relatively high for the residue to be acting as a general base. However, an arginine residue (theoretical pKa of 12.5) has been shown to act as a base in the β-elimination of pectin substrates by members of the PL1 and PL10 pectate lyase families [153]. Therefore, it is not unlikely that the local microenvironment stabilizes the deprotonated form of Lys250 allowing it to abstract the C5 proton from the iduronic acid.

Finally, the location of Arg271 in the co-crystal structure suggests that it may contribute a proton to the anomeric oxygen of the GalNac4S leaving group in subsite −1 (Figure 4.2.4). This function for Arg271 was also predicted from our previous modeling study, but remained unconfirmed due to the lack of solubility of the recombinant R271A mutant. The lack of activity of K271E and the diminished activity of R271K in the current study are consistent with this arginine acting as a general acid in the β-elimination mechanism.
The current study also shed some additional light onto some of the findings of our earlier modeling study. To begin with, the active site symmetry reported in our previous study, while structurally interesting, does not appear to have any functional consequence in the processing of DS by chondroitinase B [151]. In addition, it appears unlikely that Glu333 and His272 act as general bases in the abstraction of the C5 proton. In the current structure, both residues are a significant distance from the C5 carbon, but they are hydrogen bonded to the hydroxyl group at C3 of the iduronic acid in subsite +1 (Figure 4.2.5). Therefore, the decrease in activity seen when these residues were mutated to alanine suggests they contribute to the positioning of the substrate in active site, but not to the abstraction of the C5 proton. In addition, Glu333 may play a role in stabilizing Arg271 for the transient protonation of the anomeric carbon in the GalNac4S leaving group. Taken together, the studies outlined in the two parts of this Chapter provide a comprehensive understanding for the individual contribution of specific amino acids in the catalytic activity of chondroitinase B. This information, while interesting from an enzymology stand point, will also help increase the practical utility of chondroitinase B and the site-directed mutants in the characterization of bioactive DS oligosaccharides.
Chapter 5:

Direct Examination of the Action Pattern of Chondroitinase B Using Defined Dermatan Sulfate Oligosaccharides

SUMMARY

This chapter explores the mode of action of chondroitinase B on defined dermatan sulfate derived oligosaccharides using capillary electrophoresis (CE) and matrix assisted laser desorption ionization mass spectrometry (MALDI-MS). The defined DS oligosaccharide, ranging from tetra- to dodecasaccharides, were produced first through partial enzymatic digestion and isolated through a series of chromatography steps. CE and MALDI-MS were used in tandem to confirm the identity and purity of the each oligosaccharide. Digesting a decasaccharide with chondroitinase B and monitoring the products by CE revealed a non-processive, endolytic mode of action for the enzyme. Further analysis using a semi-carbazide labeled hexasaccharide and decasaccharide confirmed these initial results and demonstrated that chondroitinase B is also non-random; preferentially cleaving endolytic bonds proximal to the reducing end at a higher rate than other potential sites. Additionally, chondroitinase B preferentially cleaves longer oligosaccharides at a higher rate than shorter oligosaccharides. The Arg364Ala mutant of chondroitinase B, previously shown to alter the enzyme's kinetic activity likely through changes in substrate binding, was shown to have an altered mode of action pattern further confirming this residue's role in substrate processing. These results have been submitted to Biochemistry (2003) for publication.
5.1 Introduction and Motivation

A previous study identified the mode of action of chondroitinase B as random, endolytic using by examining changes in the viscosity of the reaction and by analyzing the reaction products using gel electrophoresis [6]. However, these mechanism studies were completed using a heterogeneous polymeric DS and did not allow for the time-resolved determination of the relative amount of individual defined products during the course of the enzymatic reaction. With the recent production of defined oligosaccharides derived from DS by partial enzymatic digestion, it is now possible to directly explore the mechanism of action of chondroitinase B using defined substrates of varying length [154]. This chapter builds on the characterization (Chapter 4) of the role of specific amino acids in substrate binding and catalysis in chondroitinase B by exploring the mode of action of the enzyme on defined DS substrates.

Advances in the application of analytical techniques, such as capillary electrophoresis (CE) and matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS), have enabled the detection and characterization of minute amounts of GAGs [35]. Importantly, these sensitive analytical techniques were combined to demonstrate that heparinase I cleaves defined oligosaccharide substrates via an exolytic, processive mechanism [34] and that heparinase II acts through a non-random, non-processive, endolytic mode of action [39]. Further coupling CE and MALDI-MS with the heparinas and a bioinformatics-based, property-encoded nomenclature (PEN) to describe the various HSGAG disaccharide building blocks led to a sequencing strategy (PEN-MALDI) for HSGAG oligosaccharides [60]. Combining CE and MALDI-MS with the chondroitinases in a similar fashion will assist in elucidating structure-function relationships underlying a variety of emerging CS/DS-mediated biological events.

5.2 Enzymatic Generation and Isolation of Defined DS Oligosaccharides

The first step in characterizing the mechanism of action of chondroitinase B was the generation and isolation of defined DS-derived oligosaccharides. Porcine intestinal mucosa DS was partially digested using the R364A mutant chondroitinase B that was previously shown to have decreased reaction kinetics allowing for a greater control over the rate of the
digestion [151]. The reaction conditions were optimized to provide maximal yield of DS-derived oligosaccharides ranging from tetra- to dodecasaccharides. After the completion of the enzymatic digestion, the reaction products were separated on a Bio-gel P6 column yielding six defined peaks (Figure 5.1). Each fraction was further purified using anion exchange HPLC and the resulting peaks were desalted to yield pure oligosaccharides.

![Absorbance at 232 nm vs. Fraction #](image)

**Figure 5.1: Generation and purification of defined DS oligosaccharides.** DS was partially digested with the chondroitinase B mutant, R364A, and the products were separated on a Bio-gel P6 column. (A) Six distinct peaks with absorbance at 232 nm were pooled, lyophilized, and further separated using HPLC. Each peak was analyzed using capillary electrophoresis and MALDI-MS to assess their purity and to assign their identity.

Each oligosaccharide isolated from the six peaks in the P6 profile was analyzed using a tandem approach of CE and MALDI-MS to confirm its identity, purity, and composition (Table 5.1). As a representative of this analysis, Figure 5.2 is CE electropherogram of the major constituent of Peak 2 in the P6 profile. The single peak in the CE profile clearly indicated that the oligosaccharides had been purified to homogeneity (Figure 5.2). For MALDI-MS analysis, the oligosaccharide resulting from Peak 2 was complexed with a basic peptide (RG$_{15}$) and analyzed in the linear mode. The MALDI-MS profile revealed two defined peaks representing the uncomplexed RG$_{15}$ (3218.9 Da) and the oligosaccharide:peptide complex (5515.9 Da) (Figure 5.2). The difference between the masses of the two peaks (2297.0 Da) confirms that Peak 2 from the P6 profile is a
decasaccharide with 5 sulfates. The mass of the decasaccharide calculated from the MALDI-MS data agrees exactly with the expected mass (Table 5.1). Compositional analysis of the decasaccharide peak using chondroitinase ABC revealed that the 4-O sulfated disaccharide (Di) was the sole product confirming the structure in Figure 5.3.

**Table 5.1**: DS-derived oligosaccharides and their masses

<table>
<thead>
<tr>
<th>Oligosaccharide</th>
<th>Chemical Structure</th>
<th>Complex Mass (Da)</th>
<th>Calculated Mass (Da)</th>
<th>Expected Mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Di</td>
<td>ΔUA-H$_{\text{Nac,4S}}$</td>
<td>n.d.</td>
<td>n.d.</td>
<td>503.3</td>
</tr>
<tr>
<td>Di-sc</td>
<td>ΔUA-H$_{\text{Nac,4S-sc}}$</td>
<td>n.d.</td>
<td>n.d.</td>
<td>560.4</td>
</tr>
<tr>
<td>Tetra</td>
<td>ΔUA-H$<em>{\text{Nac,4S-I-H$</em>{\text{Nac,4S}}$}}$</td>
<td>4316.7</td>
<td>918.8</td>
<td>918.8</td>
</tr>
<tr>
<td>Tetra-sc</td>
<td>ΔUA-H$<em>{\text{Nac,4S-I-H$</em>{\text{Nac,4S}}$-sc}}$</td>
<td>4192.5</td>
<td>976.7</td>
<td>975.9</td>
</tr>
<tr>
<td>Hexa</td>
<td>ΔUA-H$<em>{\text{Nac,4S-(I-H$</em>{\text{Nac,4S}}$)2}}$</td>
<td>4690.9</td>
<td>1378.5</td>
<td>1378.2</td>
</tr>
<tr>
<td>Hexa-sc</td>
<td>ΔUA-H$<em>{\text{Nac,4S-(I-H$</em>{\text{Nac,4S}}$)2-sc}}$</td>
<td>4650.8</td>
<td>1435.2</td>
<td>1435.3</td>
</tr>
<tr>
<td>Octa</td>
<td>ΔUA-H$<em>{\text{Nac,4S-(I-H$</em>{\text{Nac,4S}}$)3}}$</td>
<td>5057.2</td>
<td>1837.5</td>
<td>1837.6</td>
</tr>
<tr>
<td>Octa-sc</td>
<td>ΔUA-H$<em>{\text{Nac,4S-(I-H$</em>{\text{Nac,4S}}$)3-sc}}$</td>
<td>5109.8</td>
<td>1895.0</td>
<td>1894.7</td>
</tr>
<tr>
<td>Deca</td>
<td>ΔUA-H$<em>{\text{Nac,4S-(I-H$</em>{\text{Nac,4S}}$)4}}$</td>
<td>5515.9</td>
<td>2297.0</td>
<td>2297.0</td>
</tr>
<tr>
<td>Deca-sc</td>
<td>ΔUA-H$<em>{\text{Nac,4S-(I-H$</em>{\text{Nac,4S}}$)4-sc}}$</td>
<td>5568.4</td>
<td>2354.1</td>
<td>2354.1</td>
</tr>
<tr>
<td>DoDeca</td>
<td>ΔUA-H$<em>{\text{Nac,4S-(I-H$</em>{\text{Nac,4S}}$)5}}$</td>
<td>5972.8</td>
<td>2755.8</td>
<td>2756.4</td>
</tr>
</tbody>
</table>

The same combination of CE, MALDI-MS, and compositional analysis was performed on all of the isolated oligosaccharides to confirm their identity and purity. Peak 1 from the P6 profile was a dodecasaccharide containing 6 sulfates with a mass of 2755.8 Da (Table 6.1). Peak 3 was an octasaccharide with 4 sulfates at an observed mass of 1837.5 Da. Peak 4 yielded a hexasaccharide containing 3 sulfates and a mass of 1378.5 Da. Peak 5 was a tetrasaccharide with 2 sulfates with a mass of 918.8 Da. Importantly, all of the masses for the oligosaccharides obtained by MALDI-MS deviated from the expected mass by ≤1 Da (Table 5.1). Finally, peak 6 was identified as the 4-sulfated disaccharide using CE and was not analyzed by MALDI-MS (Table 5.1). The use of MALDI-MS was critical for assigning the identity of each of the oligosaccharides isolated from the partial digest of DS. A computational exercise completed previously by our group revealed that from only the mass of a GAG oligosaccharide of up to a tetradecasaccharide in length, one could assign the oligosaccharide length and the number of sulfates that modify it [60]. Combining this MALDI-MS analysis with the CE-based compositional analysis, we were able to unambiguously assign a structure to each of the DS-derived oligosaccharides (Table 5.1).
Prior NMR analysis of DS-derived oligosaccharides identified all of the saturated uronic acids as IdoA [154]. Therefore, the logical assumption was made that the structures of the oligosaccharides in the current study contain IdoA. It is important to note that while one previous study used MALDI-MS to identify a single DS-derived hexasaccharide [155], the current study represents the first broad-range application of MALDI-MS for the characterization of a diversity of DS-derived oligosaccharides.

5.3 Molar Quantitation of CE Data

To develop a more quantitative technique for representing the amount of the different oligosaccharide products in a given CE profile, a set of standard curves were generated using the uronic acid plate assay [156]. Glucuronic acid and galacturonic acid (0 - 21 nmol) were used to generate standard curves to which each of the DS-derived oligosaccharides (Di – Deca) was compared, thereby enabling the determination the molar concentration of each of the oligosaccharides. The GlcA and GalA standard curves compared well with on another and the uronic acid assay was repeated at least six times for each oligosaccharide to insure a standard deviation of less than 10%. In parallel, a dilution series of each oligosaccharide was run on the CE and the peak areas were plotted as a function of oligosaccharide sample concentration. These experiments yielded a set of standard curves.
that enable the direct conversion of a CE peak area into a molar concentration of that oligosaccharide in a sample. Using these standard curves, the molar amount of each reaction product, as well as each substrate, was calculated for all of the enzymatic reactions described below.

![Structure of relevant DS oligosaccharides](image)

**Figure 5.3:** Structure of relevant DS oligosaccharides. (Top) A five-sulfated decasaccharide derived from the partial enzymatic digest of DS. The decasaccharide is characterized by sulfates at the 4-O position of each GalNAc, IdoA epimers of the uronic acids, and a $\Delta^{4,5}$ unsaturated double bond at the non-reducing end. (Middle) A three-sulfated hexasaccharide derived from the partial enzymatic digest of DS. (Bottom) The same hexasaccharide with a semicarbazide mass tag attached to its reducing end. The presence of the semicarbazide label enabled tracking of the reducing end disaccharide during the enzymatic degradation by capillary electrophoresis and MALDI-MS. The decasaccharide (Top) was also labeled in a similar fashion (not shown).

### 5.4 Mechanism of Action of Chondroitinase B

The 5-sulfated decasaccharide (Deca) was selected as the initial substrate for exploring the action pattern of chondroitinase B. Deca’s reasonable length and its two cleavable internal bonds as well as two external bonds make it an ideal substrate for these experiments (Figure 5.3). Enzymatic reactions conditions were optimized such that the product profile at a variety of time points could be analyzed using CE. Ultimately, 300 nM chondroitinase B was incubated with 220 $\mu$M Deca at 30°C. Aliquots were removed at varying time points ranging from 10 s to 120 min, heat inactivated, diluted, and analyzed by CE. The peak areas for the different reaction products were used to calculate a molar concentration for each oligosaccharide that, in turn, was plotted as a function of time (Figure 5.4). Each of the
oligosaccharide peaks were identified by co-migration with defined oligosaccharide standards and confirmed by MALDI-MS (data not shown).

![Graph showing degradation of Deca](image)

**Figure 5.4: Chondroitinase B degradation of Deca.** Chondroitinase B was incubated with the five-sulfated decasaccharide for defined period of times and the enzymatic products were analyzed by CE. The resulting peak areas in the electropherogram were converted to molar concentrations (see text) and plotted versus time. (Left) During the 120 min. digestion of Deca (○), there was an initial appearance of Hexa (■) and Tetra (▲) with very little Octa (×) and Di (●) products indicating that chondroitinase B is an endolytic enzyme. (Right) This observation was confirmed by examining the products of the enzymatic reaction during the first 60 s. Later in the reaction time course as Deca was depleted (Left), the concentration of Hexa decreased with a concomitant increase in Di and Tetra, implying that chondroitinase B prefers longer substrates (Deca) to shorter ones (Hexa).

Over the 120 min time course of the experiment, the major product produced is Tetra with significant yet diminishing amounts of Hexa also present (Figure 5.4). Examination of the products produced during the first 60 s of the reaction revealed that Tetra and Hexa are produced in increasing, nearly equal molar amounts with negligible amounts of Octa and Di produced during this early phase of the reaction (Figure 5.4). Taken together, these results clearly demonstrate that chondroitinase B is an endolytic enzyme. In fact, a comparison of the amount of Hexa (the product of endolytic cleavage) to Octa (the product of exolytic cleavage) produced during the first minute of the reaction yields a 91% endolytic mode of action for chondroitinase B. Additionally, the lack of Di products implies that chondroitinase B is a non-processive enzyme. Di would be an obvious reaction product if chondroitinase B continued to degrade a bound oligosaccharide, a pattern that is seen with both heparinase I and endogalacturonase I from *Aspergillus niger* with their respective substrates [34,157]. Chondroitinase B likely releases the cleavage products after each round of degradation with subsequent re-binding initiating the next round of catalysis in a similar fashion to heparinase II [39]. Importantly, the direct observation of the endolytic action pattern reported here is in
agreement with a previous study that relied on changes in sample viscosity and gel electrophoresis as indirect measures of the mechanism of action of chondroitinase B [6].

Another interesting observation is that the molar concentrations of Tetra and Hexa in the reaction become divergent once the Deca substrate has been depleted. In addition, a rise in the concentration of Di accompanies the rise in the concentration of Tetra (Figure 5.4). These observations suggest that chondroitinase B prefers longer substrates, such as Deca to shorter ones, such as Hexa. To confirm this observation, each of the oligosaccharides at a concentration of approximately 150 μM was digested independently with chondroitinase B and the rate of product appearance was measured using CE and corrected for enzyme concentration. Chondroitinase B shows a clear preference for longer oligosaccharides with the rate of cleavage for Deca being 18-fold higher than the rate of cleavage for Tetra. In addition, chondroitinase B cleaves Octa at a 7-fold higher rate than it cleaves Hexa. This preference for longer substrates is comparable to what was observed with both heparinase I and II [34,39], hyaluronan lyase from group B streptococci [158], and the endopeptate lyases from Erwinia chrysanthemi [159].

Figure 5.5 (Left) A schematic representation of the semicarbazide labeled hexasaccharide. The triangle represents the non-reducing end 4-sulfated disaccharide with the Δ4,5 double bond. Each circle is a 4-sulfated disaccharide and the star represents the semicarbazide label on the reducing end of the oligosaccharide. The arrows indicate potential cleavable bonds at site I and site II. (Right) A schematic representation of a semicarbazide labeled decasaccharide. The shapes are the same as described for the hexasaccharide. The decasaccharide has four cleavable bonds; two terminal, exolytic bonds (site I and IV) and two internal, endolytic bonds (site II and III).

5.5 Chondroitinase B Digestion of End-labeled Oligosaccharides

The DS oligosaccharides were labeled at the reducing end with semicarbazide thereby introducing a mass tag that could be tracked by mass spectrometry [39]. To ensure the reducing end had been labeled, the reactions were analyzed by both CE and MALDI-MS (data not shown). Interestingly, the end-labeled oligosaccharides had a noticeable increase in their migration time in the CE. The redistribution of the charge density that results from the semicarbazide label stabilizing the ring-opened form of the GalNac likely produces this
observed migration time shift (Figure 5.2). Capitalizing on this shift in migration time and the relative simplicity of the reaction products from DS, we were able to use the CE to track the formation of the reaction products that contained the reducing end GalNac labeled with semicarbazide and compare them to products generated from internal cleavage of the oligosaccharide substrate. The reaction products are expressed as the fraction of each respective the oligosaccharide species in the electropherogram [i.e. Hexa-sc/(Hexa-sc + Hexa)]. This enabled us to directly assign relative rates of cleavage for the different bonds in up to a decasaccharide by chondroitinase B (see below). In addition, the integration of each oligosaccharide peak in an electropherogram resulted in significantly more quantitative data than that produced using MALDI-MS or other MS-based techniques that are semi-quantitative at best [35]. Therefore, this CE-based technique represents a significant improvement on previous techniques used to explore the action pattern of other polysaccharide degrading enzymes [39].

![Absorbance at 232 nm](image)

**Figure 5.6: Digestion of Hexa-sc.** A hexasaccharide labeled at the reducing end was digested with chondroitinase B (Top) and the R364A mutant (Bottom) and analyzed using capillary electrophoresis. (Top) The initial reaction products resulting from the digestion of the Hexa-sc (H-sc) substrate by chondroitinase B are Tetra (T), Tetra-sc (T-sc), Di and Di-sc. (Bottom) There was a noticeable increase in the relative concentration of T-sc and Di produced when H-sc was degraded by R364A, suggesting that this mutant has an altered mode of action when compared to chondroitinase B. (* denotes the remaining unlabeled Hexa impurity from the semicarbazide labeling)

**Digestion of Hexa-sc:** A pure hexasaccharide with 3 sulfates was labeled with semicarbazide overnight at 40°C (Figure 5.3 and 5.5). The efficiency of the labeling reaction was 95% as determined by CE (data not shown). The mass observed mass of Hexa-sc was 1435.2 Da as measured by MALDI-MS indicating an increase of 57.0 Da (expected increase
of 57.1 Da) by the addition of the semicarbazide tag (Table 5.1). Compositional analysis of Hexa-sc yielded Di and Di-sc products at the expected ratio of 2:1 (data not shown).

Experiments were performed to determine suitable digestion conditions under which the products as well as the substrate were detectable using CE and MALDI-MS. Recombinant chondroitinase B was added to a concentration of 170 nM to the labeled hexasaccharide and incubated at 30°C for 3 min. The sample was heat inactivated at 85°C for 5 min. and then analyzed by CE. Under these reaction conditions, detectable amounts of Tetra and Tetra-sc as well as Di and Di-sc were observed (Figure 5.6 and Table 5.2). A significant amount of the Hexa-sc substrate remained indicating that the products observed in the CE were indicative of the initial rate of enzymatic cleavage (Figure 5.6). The products of cleavage at both Site I and Site II in the Hexa-sc are close to evenly distributed suggesting that chondroitinase B cleaves each bond with equal efficiency (Table 5.2). The slight disparity between the higher molar proportions of the unlabeled products compared to the labeled products likely results from the cleavage of the remaining unlabeled substrate material still present in the starting sample (Table 5.2 and Figure 5.6). Importantly, the activity of chondroitinase B did not seem to be altered by the presence of the semicarbazide group on Hexa-sc as, a priori, the enzyme would be expected to cleave both internal bonds of a hexasaccharide with equal efficiency given its endolytic nature.

**Table 5.2:** Cleavage of Hexa-sc with Chondroitinase B

<table>
<thead>
<tr>
<th>Cleavage Site</th>
<th>Reaction Product</th>
<th>[Oligosaccharide]</th>
<th>Fraction of Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site I</td>
<td>Tetra-sc Di</td>
<td>24.8 µM</td>
<td>0.46</td>
</tr>
<tr>
<td>Site II</td>
<td>Tetra Di-sc</td>
<td>29.3 µM</td>
<td>0.54</td>
</tr>
</tbody>
</table>

**Digestion of Deca-sc:** A decasaccharide with 5 sulfates was labeled at the reducing end with semicarbazide (Figure 5.3 and 5.5). The labeling reaction was 98% complete as indicated by CE (data not shown). The mass observed mass of Deca-sc

**Table 5.3:** Cleavage of Deca-sc with chondroitinase B

<table>
<thead>
<tr>
<th>Cleavage Site</th>
<th>Reaction Product</th>
<th>[Oligosaccharide]</th>
<th>Fraction of Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site II</td>
<td>Tetra Hexa-sc</td>
<td>13.3 µM</td>
<td>0.38</td>
</tr>
<tr>
<td>Site III</td>
<td>Tetra-sc Hexa</td>
<td>23.1 µM</td>
<td>0.62</td>
</tr>
</tbody>
</table>

24.8 µM      0.66
was 2354.1 Da as measured by MALDI-MS indicating that an increase of 57.1 Da (expected increase of 57.1 Da) by the addition of the semicarbazide tag (Table 5.1). Compositional analysis of Deca-sc yielded Di and Di-sc in the expected ratio of 4:1 (data not shown).

Chondroitinase B at a final concentration of 170 nM was incubated with Deca-sc at 30°C for 30 s, heat inactivated, and analyzed by CE. After the 30 s digestion, a significant amount of the Deca-sc substrate was still present, representing 36% of the total peak area in the electrophoretogram, indicating that the reaction was in its initial phase (Figure 5.7). In agreement with the endolytic mechanism of chondroitinase B, no Octa or Di products were formed during the initial cleavage of Deca-sc implying that cleavage occurred only at Site II and III (Figure 5.5). The lack of Di products also suggests that the enzyme is not processive. Interestingly, the product profile suggests that chondroitinase B prefers to cleave Deca-sc at Site III, the internal bond closest to the reducing end at a threefold higher rate than Site II, the internal bond closer to the non-reducing end (Figure 5.5 and Table 5.3). This unequal cleavage is in contrast to the Hexa-sc data where both bonds are cleaved with equal efficiency by chondroitinase B and implies that the enzyme is non-random in addition to endolytic (Table 5.2).

![Figure 5.7: Digestion of Deca-sc. A decasaccharide labeled at the reducing end with semicarbazide was digested with chondroitinase B (Left) and the R364A mutant (Right) and analyzed by capillary electrophoresis. (Left) The major products of the digestion of Deca-sc (D-sc) were Hexa-sc (H-sc), Hexa (H), Tetra-sc (T-sc), and Tetra (T). The higher relative amounts of T and H-sc indicate that chondroitinase B acts in a non-random fashion, preferring to cleave the internal bond proximal to the reducing end to the internal bond nearest the non-reducing end. (Right) Digestion of D-sc with the R364A mutant produces the same products as in the chondroitinase B digestion. However, the relative amount of each product is different implying that the R364A mutant has lost the non-random aspect of the mode of action, thus cleaving both internal bonds with near equal efficiency. (* denotes the remaining unlabeled Deca impurity from the semicarbazide labeling)
5.6 The R364A chondroitinase B mutant

A combination of crystal structure analysis [51] and modeling [151] previously implicated Arg364 in chondroitinase B in binding DS. Specifically, the basic side chain of this amino acid was positioned to make favorable contacts with the 4-O sulfate of the GalNac occupying the putative –1 subsite in chondroitinase B [51,151]. Given that 4-O sulfation is the hallmark modification present in DS, Arg364 was speculated to play a critical role in determining the substrate specificity of chondroitinase B. In fact, when this residue was mutated to alanine, the resulting chondroitinase B mutant displayed diminished catalytic efficiency and an altered product profile as analyzed by CE [151]. Therefore, we sought to further examine the effect of the R364A mutation on the action pattern of chondroitinase B by digesting Hexa-sc and Deca-sc with the mutant enzyme.

Hexa-sc was first digested with the R364A mutant. Since the R364A mutant has a significantly reduced catalytic efficiency compared to chondroitinase B, 370 nM of enzyme was used and the reaction was incubated for 20 min. at 30°C. The reaction was heat inactivated as before and analyzed using CE. After the 2 hr incubation with R364A, the reaction still contained 25% of the initial Hexa-sc substrate and the distribution of reaction product was noticeably different from the distribution produced with the recombinant chondroitinase B (Figure 5.6 and Table 5.4). Instead of degrading each bond with equal efficiency as seen with chondroitinase B (Table 5.2), the product profile suggests that the R364A mutant cleaves at Site I with a four-fold higher rate than at Site II as indicated by the 4:1 molar distribution ratio of the products (Table 5.4). Therefore, the R364A mutant, in addition to having reduced reaction kinetics, also has an altered action pattern on a hexasaccharide substrate.

Deca-sc was digested with R364A to examine if the differences in the action pattern seen with the Hexa-sc substrate were replicated with a decasaccharide. As was the case with the Hexa-sc reaction, 370 nM R364A was incubated with Deca-sc for 1 min to compensate
for the reduced catalytic efficiency of R364A compared to that chondroitinase B. Similarly to the CE profile produced by chondroitinase B, the R364A product profile shows no significant production of Octa or Di species (Figure 5.7). Therefore, the Arg to Ala mutation does not alter the endolytic mechanism or lack of processivity of chondroitinase B. However, in contrast to the chondroitinase B CE profile, the R364A product profile suggests that the mutant cleaves Site II and III at close to comparable rates as indicated by the nearly equivalent molar ratio of the reaction products (Table 6.5 and Figure 6.7). Therefore, the Arg364Ala mutation alters the preference of chondroitinase B from cleaving closer to the reducing end of the oligosaccharide at Site III to cleaving both of the internal bonds at a comparable rate.

The results above clearly demonstrate that Arg364 is important in the normal enzymatic processing of DS by chondroitinase B. Comparing the product profile of the degradation of Hexa-sc by chondroitinase B with the R364A mutant, clearly demonstrates that Arg364 contributes important contacts with the DS substrate in the −1 subsite that allow for its normal positioning in the active site [51,151]. Removal of these contacts leads to a three-fold increase in cleavage rate at Site II compared to Site I (Figure 5.5 and Table 5.5) suggesting that there is likely another residue(s) in the +1 or +2 subsite responsible for positioning the substrate for cleavage [151]. In fact, the R364A mutant is unable to cleave Tetra as a substrate (data not shown) further implying that a balance of contacts between the −1 and the +1/+2 subsites is required for the normal catalytic function of chondroitinase B. Furthermore, the altered product profile with Deca-sc confirms that Arg364 is required for normal substrate binding. In fact, removal of Arg364 leads to shift in the action pattern of chondroitinase B from non-random to random. Similarly altering a single amino acid in endopolygalacturonase I and II leads to shift from processive to a non-processive mode of action [157]. However, in this case the R364A mutant retains the non-processive, endolytic mechanism displayed by chondroitinase B.

**Table 5.5**: Cleavage of Deca-sc with R364A

<table>
<thead>
<tr>
<th>Cleavage Site</th>
<th>Reaction Product</th>
<th>[Oligosaccharide] μM</th>
<th>Fraction of Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site II</td>
<td>Tetra</td>
<td>22.6</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td>Hexa-sc</td>
<td>20.9</td>
<td>0.42</td>
</tr>
<tr>
<td>Site III</td>
<td>Tetra-sc</td>
<td>25.1</td>
<td>0.53</td>
</tr>
<tr>
<td></td>
<td>Hexa</td>
<td>28.7</td>
<td>0.58</td>
</tr>
</tbody>
</table>
5.7 Conclusions and Significance

We have applied the analytical techniques of CE and MALDI-MS to the quantitative analysis of the enzymatic degradation products from the depolymerization of defined DS-derived oligosaccharides by chondroitinase B. Chondroitinase B degrades polymeric DS substrates in a non-random, non-processive, endolytic mode of action and kinetically favors longer substrates to shorter ones. Labeling the reducing end of defined hexa- and decasaccharide with semicarbazide provided a convenient mass tag and altered the migration time of the oligosaccharides in the CE. Using these labeled oligosaccharides, we were able to demonstrate that chondroitinase B favors endolytic bonds closer to the reducing end of the substrate. In addition, examination of the product profile of the R364A mutant revealed that this residue plays a critical role in the binding of DS substrates for catalysis. Removal of Arg364 leads to a random action pattern without altering the endolytic, non-processive function of chondroitinase B.

The results of the current study build on previous work by our laboratory [148,151] and others [6,51] to provide further insight into the activity of chondroitinase B thereby increasing its utility as a tool to study the structure-function relationship of DS. Given the recent emergence of DS as a critical regulator of growth factor signaling in wound healing [146] and potentially in diseases such as cancer [139,145,160], the coupling of enzymatic tools with the sensitive analytical techniques of CE and MALDI-MS is critical for future examination of the saccharide:protein interactions underlying these biological events. In addition, the methodologies detailed within can be extended to study the mode of action of other CS/DS degrading enzymes, such as chondroitinase AC and ABC. As a whole, this ongoing research will develop the chondroitinases as critical components of sequencing methodology for bioactive CS/DS oligosaccharides akin to the PEN-MALDI strategy developed for the sequencing of HSGAGs [60].
THESIS SUMMARY AND FUTURE WORK

Glycosaminoglycans are complex, heterogeneous polysaccharides that actively regulate a diversity of biological and pathological events. The combination of their chemical heterogeneity and relative biological scarcity has hindered the characterization GAGs, especially when compared to the research progress made for other biopolymers, such as DNA and proteins. However, the recent coupling of the heparinase with the sensitive analytical tools of MALDI-MS, capillary electrophoresis, and NMR has spawned the creation of several advanced research schemes for determining the structure-function relationship for bioactive HSGAGs. Importantly, the increased knowledge of HSGAG function generated from these experiments has led to the production of improved clinical therapies for the regulation of coagulation and the treatment of cancer.

Chondroitin sulfate and dermatan sulfate are also emerging as important regulators of a wide-range of biological events, ranging from coagulation to cartilage integrity to cancer. But like the case with HSGAGs 10 years ago, enzymatic tools are lacking for adequately understanding structure-function relationships for this class of GAGs. Therefore, using the biochemical characterization of the role of histidine in heparinase III as a foundation, the research in this thesis focused on developing the chondroitinases as enzymatic tools for the analysis of CS/DS. The first step in this endeavor was the development of a recombinant expression system for the Flavobacterial enzymes, chondroitinase AC and B.

With a firm biochemical and kinetic understanding of the recombinant enzymes in place, the next step was exploring the roles of specific amino acids in the catalytic degradation of dermatan sulfate by chondroitinase B. Initial modeling studies of a tetrasaccharide into the active site of the enzyme revealed potential roles for several amino acids in substrate binding and catalysis. The biochemical and kinetic characterization of a variety of site-directed mutant enzymes helped confirm some of the predictions arrived at from the modeling study.

An additional crystallographic study of a dermatan sulfate hexasaccharide soaked into the chondroitinase B crystal revealed the presence of a calcium ion in the active site. Further biochemical experiments and site-directed mutagenesis established a critical catalytic role of the calcium ion and further defined the role of other active site amino acids in the activity of chondroitinase B. Taken together, this research provides a comprehensive picture of the
contribution of individual active site residues along with the calcium ion in the β-elimination mechanism by which chondroitinase B degrades dermatan sulfate.

Finally, using a combination of defined dermatan sulfate oligosaccharides and the sensitive analytical techniques of MALDI-MS and CE, the mode of action of chondroitinase B was explored. The enzyme degrades the defined DS oligosaccharides through an endolytic, non-random, non-processive mechanism, preferentially cleaving longer oligosaccharides over shorter ones. Finally, Arg364 is critical for the catalytic degradation of DS likely through mediating important substrate binding interactions. As a whole, the research in this thesis provides a comprehensive understanding of the enzymology of chondroitinase B, the only known enzyme that degrades DS as its sole substrate.

The development of the chondroitinase as enzymatic tools will greatly assist in the characterization of bioactive CS/DS oligosaccharides in a fashion analogous to the use of the heparinases for studying HSGAGs. Taken together the results outlined in this thesis represent a significant advancement in understanding the biochemistry of the chondroitinases. This body of work can be built upon by further combining the chondroitinases the analytical techniques of CE and MALDI-MS discussed within to develop a sequencing methodology for bioactive CS/DS oligosaccharides. The property encoded nomenclature (PEN) developed for HSGAGs can be extended to CS/DS oligosaccharides to provide the necessary bioinformatics framework for handling the chemical complexity of the biopolymers. Such advancement would likely result in a virtual explosion in the understanding of the structure-function relationships that underlie the diversity of biological and pathological events regulated by CS/DS oligosaccharides. In addition, NMR can be used to further explore the conformation DS oligosaccharides when bound to proteins and enzymes, such as KGF or chondroitinase B. This structural information will provide further insight into the chemical similarities and overlapping biological roles shared by DS and HSGAG oligosaccharides. Finally, the chondroitinases can be directly employed in in vitro and in vivo models to better understand the role of CS/DS oligosaccharides in biological systems. Taken together, these future experiments relying on the work presented in this thesis as a foundation will enable a revolution in understanding the biological roles of CS/DS oligosaccharides. This knowledge, in turn, will likely fuel the development of novel therapeutics for chronic diseases such as cancer and osteoarthritis.
Appendix I:

MATERIALS AND METHODS

Chapter 4, pt. II - Materials and Methods:

Materials. Porcine intestinal mucosa dermanan sulfate, chondroitin 4-sulfate, and chondroitin 6-sulfate were purchased from Sigma (St. Louis, MO). The disaccharide standards were from Seikagaku/Associates of Cape Cod (Falmouth, MA). Oligonucleotide primers for PCR mutagenesis were from Invitrogen (Carlsbad, CA). The sources of all other reagents used are from common sources or are as noted in the Methods section.

PCR Site-Directed Mutagenesis of Chondroitinase B. Arg271 was mutated to lysine and glutamate, and Glu243 and Glu245 were mutated to alanine using overlap extension PCR for 15 cycles [151]. The primer sequences for each of the mutants are listed below. The R271K mutant primers have the sequences 5’-ATGAACCTTTAAACACGTTGAT-3’ and 5’-ATCACCCTGGTTAAAGTTTCAT -3’. The R271E mutant primers have the sequences 5’-ATGAACCTTTGAACACGTTGAT-3’ and 5’-ATCACCCTGGAAAGTTTCAT-3’. The E243A mutant primers have the sequences 5’-GCGTCAGGATTCGGCAGCAGGATCATCACCCG-3’ and 5’-GGTGATGATCTCTGTGCTGCGAATACGCCGACGC-3’. The E245A mutant primers have the sequences 5’-TCGGAAGCGAGCGATCATCACCC-3’ and 5’-GGTGATGATCGCTGCTCCG-3’. The N-terminal and C-terminal primer sequences are as previously described [148]. The PCR reaction products were separated on an agarose gel and the band corresponding to the proper length was excised. The DNA was extracted from the gel using a Gel Purification Kit (Qiagen, Valencia, CA), the insert was subcloned into pCRT7/NT (Invitrogen, Carlsbad, CA), and the plasmid was prepared using a Miniprep kit (Qiagen). Each of the clones was sequenced to verify the presence of the individual alanine point mutations. Each chondroitinase B mutant was excised from pCRT7/NT using Nde I and
BamH I (New England Biolabs, Beverly, MA) enzyme cocktail and subcloned into a pET15b expression vector (Novagen, Madison, WI) that had been digested previously with these same enzymes. Recombinant chondroitinase B that had been cloned in a similar fashion was also expressed and compared to each of the alanine mutants.

**Protein Expression and Purification.** Recombinant chondroitinase B and the site-directed mutants were expressed and purified as previously described [148,151]. Purity of recombinant chondroitinase B and the site-directed mutants were assessed by SDS-polyacrylamide gel electrophoresis analysis using precast 12% gels, the Mini-Protean II apparatus, and the Silver Stain Plus kit (Bio-Rad, Hercules, CA). A relative protein concentration was calculated using the Bradford Assay (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard.

**Kinetic Analysis.** The activity of chondroitinase B and the various site-directed mutants was determined by adding 10-50 μl of the sample to a 1 ml cuvette containing 1 mg/ml of DS in 50 mM Tris-HCl, pH 8.0 at 30°C with or without the addition of exogenous calcium as noted. Product formation was monitored as an increase in absorbance at 232 nm as a function of time [148,151]. The kinetic parameters, K_m and k_cat, were calculated for chondroitinase B and the site-directed mutants by obtaining the initial reaction rate (v_0) as a function of substrate concentration at various calcium concentrations. Approximately 1 μg (13 pmol) of enzyme was added to a 1 ml of DS at concentrations ranging from 0.010 μg/ml to 2 mg/ml. The initial rate was measured for 4-10s at 30°C in the same Tris-HCl buffer used for the activity assay. The slope of the resulting line, assuming zero order kinetics, was plotted versus the substrate concentration using SigmaPlot (SSPS, Inc., Chicago, IL). The K_m (μM) and V_max (μM/s) were calculated using the Michaelis-Menten equation: v_0 = (V_max * [S])/(K_m + [S]). The k_cat (s⁻¹) was calculated by dividing the V_max by the concentration of enzyme in the reaction.

**Dermatan Sulfate Digestion and Capillary Electrophoresis.** To examine changes in product profile of each site-directed mutant when compared to recombinant chondroitinase B (20μg), digests of 1 mg/ml DS 50 mM Tris-HCl, pH 8.0 with 5 mM calcium chloride were performed
for 12-14 h. at 30°C. The digests were analyzed using capillary electrophoresis as previously described [151]. Briefly, the chondroitinase B and site-directed mutant digestes were diluted twofold and analyzed with an extended path-length cell and a voltage of 30 kV applied using reverse polarity. The running buffer consisted of 50 mM Tris, 10 μM dextran sulfate that had been brought to a pH of 2.5 using phosphoric acid and the saccharide products were detected by monitoring at 232 nm. The total peak area for the recombinant chondroitinase B and mutant digest profiles was calculated by totaling the areas of the ΔUA2S-GalNAc4S; ΔUA-GalNAc4S,6S; and ΔUA-GalNAc4S peaks. The total peak area for the R364A mutant also included the sum of the area of the three additional oligosaccharide peaks. The ratio of the ΔUA-GalNAc4S peak area to the total peak area was then calculated for the recombinant chondroitinase B and each mutant for a comparison of overall enzymatic activity.

Circular Dichroism. Recombinantly expressed chondroitinase B, R271E, R271K, E243A, and E245A were concentrated and buffer-exchanged into 50 mM sodium phosphate, pH 7.0 using a Centricon 10 Filter (Millipore, Watertown, Massachusetts). CD spectra were collected on an Aviv 62DS spectropolarimeter equipped with a thermostatic temperature controller and interfaced to an IBM microcomputer. Measurements were performed in a quartz cell with a 1 mm path length. Spectra were recorded at 25°C, in an average of 10 scans between 205 and 270 nm, with a 1.0 nm bandwidth and a scan rate of 3 nm/min. CD band intensities are expressed as molar ellipticities, θ_M, in degrees·cm²·dmol⁻¹.

Chapter 5 - Materials and Methods:

Materials. Dermatan sulfate from porcine intestinal mucosa, glucuronic acid, and galacturonic acid were purchased from Sigma. Caffeic acid and sodium tetraborate were purchased from Fluka. Chondroitinase ABC was purchased from Seikagaku/Associates of Cape Cod (Falmouth, MA). Chondroitinase B and the R364A mutant were recombinantly expressed in E. coli and purified as described previously [148,151]. Protein concentrations were calculated using the Bradford assay (Bio-Rad) with bovine serum albumin as a standard. All other reagents used are from common sources or are as noted under “Materials and Methods.”
Isolation of defined DS oligosaccharides. Dermatan sulfate was suspended in 50 mM Tris-HCl, pH 8.0 at a concentration of 10 mg/ml. To complete the partial digestion of the DS, 150 μg of the R364A recombinant chondroitinase B mutant was added to 10 ml of the DS solution. The reaction was incubated at 30°C for 16 hr. The amount of R364A added to the reaction mixture was optimized using CE (see below) to ensure a maximal range of partially digested DS reaction products. Upon completion of the reaction, the DS products were separated on a 2.5 x 120 cm Bio-gel P6 column (Bio-Rad) with 500 mM ammonium bicarbonate as the mobile phase. Fractions with an absorbance at 232 nm, the λ_{max} for the Δ^{4,5} double bond formed in the DS product by chondroitinase B, were pooled corresponding to the peaks containing various length DS oligosaccharides and lyophilized to dryness. The oligosaccharide pools were re-suspended in water and further fractionated by HPLC using an amine column with a gradient of 0.1 M to 1.0 M sodium phosphate, pH 4.5 over 30 min. Peaks were collected and desalted on a 2.5 x 55 cm Bio-gel P2 column (Bio-Rad) with a mobile phase of 500 mM ammonium bicarbonate. Fractions with absorbance at 232 nm were pooled, lyophilized to dryness, and re-suspended in water.

Semicarbazide derivitization. The reducing end of the DS oligosaccharides was specifically derivatized with semicarbazide to provide a mass tag for MALDI-MS and to produce an altered migration time in the CE. Oligosaccharide solutions were mixed 1:1 (v/v) with 50 mM semicarbazide in 60 mM Tris/acetic acid, pH 7.0 [39]. Reactions were heated at 40°C for 16 hr and then analyzed using CE. The percent completion of each reaction was calculated using the ratio of the peak areas for the product and the unlabeled substrate in the CE.

Enzymatic Digests. Enzymatic digests were completed by adding 1 μl of varying dilutions of chondroitinase B (10-100 nM) or R364A (370 nM) to 15 μl reaction. Reactions were performed in 50 mM Tris-HCl, pH 8.0 with substrate concentrations ranging from 100-200 μM. The reactions were incubated at 30°C for defined periods of time and heat inactivated at 85°C for 5 min. The reaction products and substrate were analyzed using CE and MALDI-MS as described below without any further sample preparation.
Uronic acid plate assay. A 96 well plate assay was used for determining the relative amount of uronic acid in a DS oligosaccharide sample [156]. Standards of galacturonic acid (GalA) and glucuronic acid (GlcA) ranging from 0-10 μg in a total volume of 40 μl water were added to the standard wells. Varying volumes of each of the DS oligosaccharide samples were diluted into 40 μl for comparison to the GalA and GlcA standards. 200 μl of sodium tetraborate in concentrated sulfuric acid was added to each well and mixed by pipetting. The plate was incubated at 80°C for 1 hr. After the incubation, the plate was cooled to room temperature and 40 μl of a 1:100 dilution of 100 mg/ml 3-phenylphenol in DMSO with 80% sulfuric acid (v/v) in water was added to each well. The plate was incubated at room temperature for 15 min. and the color change was analyzed in a UV plate reader at λ_{abs} of 540 nm. The absorbance of three different amounts of each oligosaccharide was compared to the standard curves to determine the molar concentration of uronic acid in each sample. The appropriate conversion factor for each length DS oligosaccharide (i.e. 5 moles GlcA/1 mole Deca) was used to calculate the molar concentration of each oligosaccharide sample.

MALDI-mass spectrometry. MALDI-MS experiments were completed conditions similar to those developed for the analysis of heparin/heparan sulfate oligosaccharides [35]. Briefly, a fresh saturated caffeic acid solution (~ 12 mg/ml) in 70% acetonitrile was mixed with a molar excess of basic peptide (Arg-Gly)$_{15}$ prior to the 1:10 dilution of the oligosaccharide. Spots were pre-seeded on a stainless steel MALDI plate as previously described [35]. A 1 μl aliquot of the sample/matrix solution was added to a pre-seeded spot and allowed to dry. MALDI-MS spectra were acquired in the linear mode on a PerSeptive Biosystems (Framingham, MA) Voyager Elite time-of-flight instrument. Delayed extraction was used to increase resolution as previously described [35]. Spectra were externally calibrated using the signals for the RG$_{15}$ and the RG$_{15}$:Deca complex.

Capillary electrophoresis. Capillary electrophoresis was performed using similar conditions to those developed for the separation of heparin/heparan sulfate disaccharides [35]. Briefly, uncoated fused silica capillaries (i.d. of 75 μm and l_{tot} of 80.5 cm) coupled with an extended path detection cell were used on a Hewlett-Packard 3DCE unit. Oligosaccharides were detected at 232 nm using an electrolyte solution of 50 mM Tris/phosphoric acid, pH 2.5.
Dextran sulfate was added to the buffer to suppress nonspecific interactions with fused silica wall of the capillaries. Electrophoretic separation was performed using reverse polarity at a voltage of $-30$ kV. Peak identities were confirmed by co-migration with known standards. A dilution series of each oligosaccharide was run on the CE to generate a set of standard curves for determining the molar amount of each species in a electropherogram.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔUA</td>
<td>uronic acid with a unsaturated 4,5 double bond</td>
</tr>
<tr>
<td>ATIII</td>
<td>antithrombin III</td>
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<tr>
<td>CB</td>
<td>calcium binding site</td>
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<tr>
<td>CE</td>
<td>capillary electrophoresis</td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalo virus</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CS</td>
<td>chondroitin sulfate</td>
</tr>
<tr>
<td>CS&lt;sub&gt;tetra&lt;/sub&gt;</td>
<td>structure of chondroitinase B with a chondroitin-4SO₄ tetrasaccharide</td>
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<tr>
<td>Deca</td>
<td>dermatan sulfate decasaccharide with 5 sulfates</td>
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<tr>
<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
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<tr>
<td>Di</td>
<td>dermatan sulfate disaccharide with 1 sulfate</td>
</tr>
<tr>
<td>DS</td>
<td>dermatan sulfate</td>
</tr>
<tr>
<td>DS&lt;sub&gt;hexa&lt;/sub&gt;</td>
<td>structure of chondroitinase B with a dermatan sulfate hexasaccharide</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>ESI</td>
<td>electrospay ionization</td>
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<tr>
<td>FAB</td>
<td>fast atom bombardment</td>
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<tr>
<td>FACE</td>
<td>fluorophore-assisted carbohydrate electrophoresis</td>
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<td>FGF</td>
<td>fibroblast growth factor</td>
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<tr>
<td>FGFR</td>
<td>fibroblast growth factor receptor</td>
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<td>GAGs</td>
<td>glycosaminoglycans</td>
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<td>Gal</td>
<td>galactose</td>
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<td>GalNac</td>
<td>N-acetyl galactosamine</td>
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<tr>
<td>GlcA or G</td>
<td>glucuronic acid</td>
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<td>N-acetyl glucosamine</td>
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<td>H</td>
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<td>Hexa</td>
<td>dermatan sulfate hexasaccharide with 3 sulfates</td>
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<tr>
<td>HGF/SF</td>
<td>hepatocyte growth factor/scatter factor</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>------------</td>
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<td>HIT</td>
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<td>HPLC</td>
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<td>IdoA or I</td>
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<td>MALDI-MS</td>
<td>matrix-assisted laser desorption ionization mass spectrometry</td>
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<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
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<td>PG</td>
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<td>RG$<em>{19}$$R$ &amp; RG$</em>{15}$</td>
<td>basic peptides composed of different Arg-Gly repeats</td>
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<td>SAX</td>
<td>strong anion exchange</td>
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<tr>
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<td>semicarbazide reducing end label</td>
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<td>surface noncovalent association mass spectrometry</td>
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<td>Tetra</td>
<td>dermatan sulfate tetrasaccharide with 2 sulfates</td>
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<td>tissue factor pathway inhibitor</td>
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<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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<tr>
<td>$V_{max}$</td>
<td>maximal velocity of an enzymatic reaction</td>
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<tr>
<td>vWF</td>
<td>von Willebrand Factor</td>
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REFERENCES


