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CS Lyases: Structure, Activity, and Applications in Analysis and the Treatment of Diseases

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I. Chondroitin Sulfate Glycosaminoglycans

Glycosaminoglycans (GAGs) are a family of highly sulfated, complex mixture of linear polysaccharides that display a wide array of biological activities (Boneu, 1996; Jackson *et al.*, 1991). GAGs can be classified into four basic types—hyaluronan, chondroitin/dermatan sulfates (CS/DS), heparin/heparan sulfate, and keratan sulfate (Capila and Linhardt, 2002; Esko and Selleck, 2002; Iozzo, 1998; Linhardt and Toida, 2004). Chondroitin/dermatan sulfates are the focus of this chapter. Chondroitin/dermatan sulfates are linear, polydisperse GAGs with a repeating core of disaccharide structure composed of a D-glucopyranosyl uronic (GlcAp) acid or L-idopyranosyl uronic (IdoAp) acid glycosidically linked to 2-deoxy, 2-acetamido-D-galactopyranose (GalpNAc) residue (Fig. 1). The major classes of the chondroitin family of GAGs are: chondroitin; chondroitin-4-sulfate (CS-A); dermatan sulfate (CS-B or DS), and chondroitin-6-sulfate (CS-C).

GAGs are the sulfated polysaccharide side chains of proteoglycans (PGs). These PGs are ubiquitous in animals and found localized on the external cell membrane and extracellular matrix (ECM) in all tissues (Iozzo, 1998). Despite intensive studies on this class of biopolymers, their precise chemical structures and biological functions are still not well understood. The major families of GAGs differ based on their disaccharide repeating unit, their linkage chemistry, and their sulfation pattern. Our current understanding of GAG structure and the biosynthetic pathway of GAG synthesis suggest the presence of defined sequences that specifically interact with an array of GAG-binding proteins (Esko and Selleck, 2002). Among these proteins are several families of growth factors, chemokines, enzymes, and adhesion proteins (Capila and Linhardt, 2002). The GAG chains of PGs act as receptors in signal transduction, controlling cell growth, differentiation, migration, adhesion, and other important physiological and pathophysiological events (Linhardt and Toida, 2004). CS is the predominant GAG present in aggrecan, the major PG of cartilage (Kresse and Schonherr, 2001). Due to the sulfo and carboxyl moieties of their GAG components, PGs concentrate large amounts of negative charge in the ECM. This has direct osmotic

effects on these tissues in which the GAGs are under hydrated due to constraints imposed by the collagen fiber network, giving cartilage its shock-absorbing function (Kempson, 1980). GalNpAc *O*-sulfonation of chondroitin can occur at the 4- and/or 6-positions (CS-A and CS-C, respectively), and it is not known how the sulfated units are distributed throughout the PG molecules or whether particular regions have different biological functions. CS-A, CS-C, and DS are found within the ECM or on cell membranes attached to a variety of proteins, including decorin, biglycan, and aggrecan (Kresse and Schonherr, 2001).

II. Enzymes Mediating GAG Synthesis

GAG presence is associated with all animals, ranging from *C. elegans* to man (Esko and Selleck, 2002). These polysaccharide chains are synthesized by a set of specialized enzymes that assemble an initiation tetrasaccharide on specific serine residues of the core protein, followed by successive addition of repeating disaccharide units to the nonreducing end by synthases (Spicer and McDonald, 1998; Yada *et al.*, 2003). GAGs, however, are not unique to eukaryotes. Several specialized microorganisms also produce simpler forms of these polymers. The enzymes mediating GAG synthesis have been characterized, and the ability to express them in large quantities would greatly facilitate the production of defined GAG components (Spicer and McDonald, 1998; Yada *et al.*, 2003).

Glycosaminoglycans are synthesized by the serial addition of UDP-sugars through the action of dedicated membrane anchored glycosyl transferases followed by several sulfotransferases, deacetylases, and epimerases (Esko and Selleck, 2002; Hannesson *et al.*, 1996; Silbert and Sugumaran, 2002; Sugahara and Kitagawa, 2002). Sugar transfer occurs in the Golgi, and the type of GAG added is believed to be dependent on as yet unclear signals present on the core proteins (Iozzo, 1998; Rosenberg *et al.*, 1997). In general, GAGs are added to a specific region of the core protein. In most cases a common tetrasaccharide linkage region is assembled by xylosylation of specific serine residues, followed by addition of two galactose units and glucuronic acid. The next step, addition of *N*-acetyl hexosamine determines whether the resulting chain will be CS/DS or heparan sulfate (HS). The three-dimensional structures of two of these biosynthetic enzymes have been determined (Negishi *et al.*, 2003). The final glycosyl transferases that add repeating disaccharides to extend the growing GAG chain are of greatest interest. These are bifunctional enzymes that alternatively add GlcUAp and GalNpAc or GlcNpAc. Families of these enzymes have been identified for human CS (Kitagawa *et al.*, 2001, 2003; Uyama *et al.*, 2003; Yada *et al.*, 2003) and HS (Esko and Selleck, 2002) synthesis. A gene coding for chondroitin synthase was identified in the K4 strain of *E. coli* (Ninomiya *et al.*, 2002). While the mammalian enzymes can only be expressed in tiny amounts, bacterial chondroitin synthase has been produced as a soluble recombinant protein (Yada *et al.*, 2003).

Since no protein core is associated with hyaluronic acid, a different biosynthetic mechanism is in place for this macromolecule. Two classes of hyaluronan synthases (HAS) have been characterized (DeAngelis, 1999). The class I enzymes are integral membrane proteins whereas the class II members are membrane associated through a C-terminal membrane spanning segment. Mammalian HAS belong to class I and appear to be bifunctional enzymes that add alternating UDP sugars to the growing hyaluronan chain, which is

extruded onto the cell surface and into the ECM. Three mammalian genes coding for HAS have been identified, and their gene structures are evolutionarily well conserved (Monslow *et al.*, 2003). These isoforms show high-amino acid sequence identity between themselves and to some bacterial enzymes, for example, *Streptococcus pyogenes* HA synthase. They contain seven putative membrane-spanning regions with a long cytoplasmic loop containing the putative UDP binding and glycosyltransferase catalytic sites (Itano and Kimata, 2002). A soluble and active fragment of human HAS2 was expressed in *E. coli* (Hoshi *et al.*, 2004), opening the door to functional and structural studies of this enzyme.

Assembly of heparan and chondroitin chains involves two additional modifications—sulfation of specific positions of the *N*-acetyl hexosamine and the uronic acid units and epimerization of glucuronic acid residues. C5-epimerases responsible for this step in heparan biosynthesis have been characterized (Li *et al.*, 1997), but those responsible for chondroitin to dermatan conversion remain unclear (Seidler *et al.*, 2002). Finally, a series of sulfotransferases acts to modify the CS/DS and HS/heparin families of PGs. The three-dimensional structures of two of these biosynthetic enzymes have been determined (Moon *et al.*, 2004; Thorp *et al.*, 2004).

III. CS Degrading Enzymes

Two chemically distinct enzymatic mechanisms have evolved for the degradation of GAGs, and the enzymes are accordingly classified as either hydrolases or lyases. Henrissat has divided these enzymes into families based on sequence similarity (http://afmb.cnrs-mrs.fr/CAZY/). Cleavage of the hexuronic acid!hexosamine bond always involves a standard glycosidase mechanism of either inverting or retaining type in which the glycosidic bond is hydrolyzed by addition of a water molecule (Fig. 2) (Zechel and Withers, 2000). In contrast, cleavage of the hexosamine!hexuronic acid bond can occur through either a hydrolytic, catalyzed by hydrolases, or an eliminative mechanism, catalyzed by lyases (Fig. 2) (Ernst *et al.*, 1995; Linhardt *et al.*, 1986; Michaud *et al.*, 2003). While polysaccharide hydrolases are found in virtually all organisms, polysaccharide lyases are not found in vertebrates.

In many tissues GAGs undergo rapid turnover. Hyaluronan degradation plays a major part in the release of PGs from cartilage that occurs in normal development and in arthritis (Sztrolovics *et al.*, 2002). It has been estimated that in the dermis, which contains more than half of the HA in the body, 50–75% of this GAG is turned over every 24 h (Frost *et al.*, 1996; McCourt, 1999). Similarly, cell surface HSPGs have a half-life of 3–8 h (Stringer and Gallagher, 1997), indicating the efficiency with which these molecules can be broken down. Following endocytosis of the substrates into the cell, complete GAG disassembly proceeds by an ordered desulfation/exolytic cleavage to yield monosaccharide products. The consequences of defects in enzymes mediating intracellular GAG degradation are evidenced by the different pathologies of the mucopolysaccharidoses (Leroy and Wiesmann, 1993). Extracellular GAG degradation, although of equal importance, is an incompletely understood process. Extracellular endolytic GAG-degrading enzymes, the hyaluronidases (Frost *et al.*, 1996), and heparanase (Vlodavsky *et al.*, 1999) have been characterized, and they play major roles in normal and pathological turnover of the ECM, and specific inhibitors of these enzymes would have important therapeutic benefits.

Testicular hyaluronidase has been known for many years (Kreil, 1995). This membrane-bound hydrolase cleaves hyaluronate but can also degrade CS and plays an important role in fertilization. It has been shown that this GAG hydrolase is the prototype of a six-member human gene family. Five functional hyaluronidases and an expressed pseudogene have been characterized. *Hyal-1*, *-2*, and *-3* occur as a cluster on chromosome 3 at position 3p21, while the well-characterized testicular hyaluronidase (also termed *PH20*) as well as *hyal-4* and the pseudogene (*hyalP1*) are found on chromosome 7q31 (Csóka *et al.*, 1999). While "hyaluronidase" has been generally considered to be a lysosomal enzyme, there is strong evidence for extracellular hyaluronidase activity. Various reports have associated elevated hyaluronidase levels with increased tumorogenicity (Madan *et al.*, 1999a,b; Novak *et al.*, 1999), and it has been demonstrated that hyal-2 is a cell surface receptor for a retrovirus in sheep (Miller, 2003).

A. Microbial CS-Degrading Enzymes (Polysaccharide Lyases)

In contrast to the vertebrates, microorganisms utilize an eliminative mechanism to breakdown GAGs, which involves abstraction of the proton at C-5 of the hexuronic acid by a general base and β-elimination of the 4-O-glycosidic bond with concomitant formation of an unsaturated C4–C5 bond within the hexuronic acid located at the nonreducing end (Fig. 2). The leaving group must be protonated, either by a side chain acting as a general acid or by proton abstraction from a water molecule. Proton abstraction and β-elimination are expected to proceed in a stepwise as opposed to concerted manner (Godavarti and Sasisekharan, 1998; Guthrie and Kluger, 1993). There is an extensive variation in specificity among lyases for different GAG types. Thus, chondroitinase B is specific for cleavage of DS, accepting only an iduronic acid, whereas chondroitinase ABC will accept either glucuronic acid or iduronic acid. Extensive biochemical and mutagenesis studies have been carried out on enzymes obtained from *Flavobacterium heparinum* (*Pedobacter heparinus*) that produces two chondroitinases (FlavoAC and FlavoB) (Gu *et al.*, 1995) and on two general specificity chondroitinases from *Proteus vulgaris* (PvulABCI and PvulABCII) (Hamai *et al.*, 1997). In addition, several hyaluronate lyases contributing to virulence have been characterized from different bacteria and bacteriophage (Hynes and Walton, 2000; Li *et al.*, 2000; Rigden and Jedrzejas, 2003). Of particular interest is the genomic sequence of the commensal bacterium, *Bacteroides thetaiotaomicron* (Xu *et al.*, 2003). *B. thetaiotaomicron* and *B. stercoris* (Ahn *et al.*, 1998; Kim *et al.*, 2000) are dominant members of the intestinal microbiota of humans and other mammals. Most notably the genome of *B. thetaiotaomicron* shows a markedly expanded repertoire of genes involved in polysaccharide uptake and degradation, specifically for utilizing a large variety of complex polysaccharides as a source of carbon and energy (Xu *et al.*, 2003). Among these are several chondroitinases (BthetABC and BsterABC) and heparinases, which contribute to the nutrition of the host (Ahn *et al.*, 1998; Kim *et al.*, 2000; Xu *et al.*, 2003).

While glycoside hydrolases display an extraordinary variety of folds (Bourne and Henrissat, 2001), only three folds have been identified for GAG lyases, the $(\alpha/\alpha)_5$-toroid, the right-handed β-helix, and a β-sandwich. The intriguing observation of both eliminative and hydrolytic enzymes within the first two-fold families and similarity of the β-sandwich to the N-terminal domain of PvulABCI suggest that binding of linear uronic acid-containing

polysaccharide substrates demands special structural features. Of the 15 classified lyase families and over 25 unclassified sequences, the fold has been established for 9 families encompassing several pectate/pectin lyase families, alginate, chondroitin, and rhamnogalacturonan lyases.

B. Purification and Characterization of Chondroitinases

Chondroitin AC and B lyases from *Flavobacterium heparinum* (FlavoAC and FlavoB) were first purified to homogeneity, and their physical and kinetic constants were determined in the Linhardt laboratory in 1995 (Gu *et al.*, 1995). From the N-terminal sequences that we determined, these enzymes were subsequently cloned and expressed in *E. coli* (Pojasek *et al.*, 2001). Chondroitinase ABC (BsterABC) was first isolated from B. stercoris by the Kim laboratory in 1998 (Ahn *et al.*, 1998), and the *B. thetaiotaomicron* chondroitinase ABC (BthetABC) has been cloned and expressed in the Kim laboratory (in preparation). The BsterABC and BthetABC enzymes appear to be structurally and catalytically similar to one another. The *P. vulgaris* chondroitinase ABC (a mixture of PvulABCI and -II) is the only commercially available chondroitinase ABC preparation (Seikagaku, Tokyo, Japan). The endolytic chondroitinase ABC (PvulABCI) was cloned and expressed in *E. coli* (Prabhakar *et al.*, 2005). Some of the well-characterized polysaccharide lyases acting on chondroitins are listed in Table I.

C. Chondroitin Lyase Structures and Mechanism

The Cygler laboratory has determined the three-dimensional structures of representatives of chondroitinases B, AC, and ABC (Fig. 3).

1. Chondroitin AC Lyase—The three-dimensional structures and the enzymatic mechanisms of two chondroitin AC lyases from *F. heparinum* (FlavoAC) and *Arthrobacter aurescens* (ArthroAC) were investigated. Both FlavoAC and ArthroAC act on CS-A and CS-C as well as on hyaluronan (Linhardt, 1994). Neither enzyme acts on pure DS, containing only repeating units of →3)GalNpAc4S (1→4)IdoAp(1→, and both AC lyases are inhibited by this GAG (Gu *et al.*, 1993). Studies in the Linhardt laboratory confirmed that both FlavoAC and ArthroAC act on the →3)GalNpAc(4S or 6S)(1→4)GlcAp(1→4) sequences found within CS and in many DS (Gu *et al.*, 1993). FlavoAC is an endolytic, and ArthroAC is an exolytic chondroitin lyase (Jandik *et al.*, 1994). The substrate specificities of both AC lyases have been extensively investigated on natural (Hernáiz and Linhardt, 2001; Linhardt, 1994; Yang *et al.*, 2000) as well as unnatural (Avci *et al.*, 2003) substrates. The structure of FlavoAC was determined at 1.9 Å resolution and revealed a two-domain molecule with the N-terminal α-helical domain and the C-terminal β-sheet domain (Fig. 3) (Féthière *et al.*, 1999). The N-terminal domain is folded into an incomplete double-layered $(\alpha/\alpha)_5$ toroid. This domain contains the catalytic machinery and provides a major part of the substrate-binding site. The C-terminal domain is composed of four antiparallel β-sheets. Since chondroitin AC lyase is inhibited by DS, we investigated complexes of FlavoAC with DS oligosaccharides. The tetrasaccharide binding site [subsites −2, −1, +1, +2 with the cleavage site between −1 and +1 using nomenclature according to Davies and coworkers (Davies *et al.*, 1997)] and four putative catalytic residues—His225, Tyr234, Arg288, and Glu371—have also been identified (Huang *et al.*, 2001). Expression of His225Ala,

Tyr234Phe, and Arg288Ala mutants in *F. heparinum*, by integration of the DNA containing the mutated gene into the genomic DNA of the bacterium, rendered the enzyme inactive (Blain *et al.*, 2002). Candidates for the general base, abstracting the glucuronic acid C-5 proton, were Tyr234 (transiently deprotonated during catalysis) or His225. The Tyr234 was deemed to be the best candidate to protonate the leaving group. Arg288 likely contributes to charge neutralization and stabilization of the enolate anion intermediate during catalysis.

Subsequently, the crystal structure of Tyr234Phe mutant with a CS-A tetrasaccharide was determined, confirming the general features of substrate binding, but this structure was inconclusive in the assignment of the role of general acid to either His225 or Tyr234 due to an enzymatically noncompetent conformation of the substrate (Fig. 4) (Huang *et al.*, 2001).

A breakthrough that allowed the assignment of the catalytic general base came from the investigation of chondroitin AC lyase from *A. aurescens* (ArthroAC). Although the amino acid sequence of this protein was not known at the onset of our investigations, it was likely that it shared homology with FlavoAC. We were fortunate in obtaining crystals diffracting to near atomic (1.25 Å) resolution (Lunin *et al.*, 2004). The resulting electron density maps allowed us to determine the amino acid sequence (Fig. 5), which was confirmed subsequently by mass spectrometry (MS) of tryptic peptides. This sequence showed 24% identity to FlavoAC. Using a series of short soaks of the crystals with CS-A tetrasaccharide, their immediate freezing in liquid nitrogen, and data collection at the synchrotron, we showed that the enzyme acted slowly in the crystal, allowing us to capture the enzymatically active conformation. This data resolved that Tyr242 acts as the general base that abstracts the proton and His233 helps in deprotonation of Tyr242 and in the proper orientation of the glucuronate acidic group. The glucuronate assumes a distorted boat conformation, much like that observed in lysozyme (Fig. 6).

Chondroitin *O*-methyl ester (C-OMe) is also depolymerized by chondroitin AC lyase from *F. heparinum* (Avci *et al.*, 2003). The major product isolated from the depolymerization reaction was found to be chondroitin Δdi-*O*-methyl ester (Fig. 7). Although, in chemical terms, abstraction of an acidic proton at the *α*-position of methyl ester group is expected, the esterification of the carboxylate group might alter the interaction of anion-stabilizing elements in an enzymatic reaction, adversely impacting catalysis (Fig. 2). Kinetic studies show that the K_M on C-OMe (12.0 mM) is comparable to CS-A (7.0 mM) and lower than that observed on chondroitin (63.0 mM). In contrast, the V_{max} on C-OMe (0.3 mmol min^{-1} mg^{-1}) is significantly lower than on CS-A (2.0 mmol min^{-1} mg^{-1}) or chondroitin (3.3 mmol min^{-1} mg^{-1}) suggesting that the binding step is less adversely impacted than catalytic step by methylation of the carboxyl group. The low K_M observed for C-OMe (comparable to CS-A) might be ascribed to the contribution of hydrophobic interactions between the methyl ester and the enzyme, replacing ionic interactions lost through the desulfonation and methyl esterification of the substrate. Both chondroitinase AC I from *F. heparinum* and chondroitinase AC II from *A. aurescens* were demonstrated by the Toida laboratory to be capable of depolymerizing hyaluronan *O*-methyl ester (Hirano *et al.*, 2005).

2. Chondroitin B Lyase—The Cygler laboratory has determined the structure of native *F. heparinum* chondroitin B lyase (FlavoB) and its complex with the reaction disaccharide

product (Huang *et al.*, 1999). The fold of this lyase is completely different to that of FlavoAC and belongs to the *β*-helix family with 13 coils. The soaking of chondroitin B lyase crystals with a DS tetrasaccharide resulted in a complex with two DS disaccharide reaction products occupying (−2, −1) and (+1, +2) subsites in the substrate-binding site. Unexpectedly, this structure showed the presence of a Ca^{2+} ion coordinated by conserved acidic residues and by the carboxyl group of the L-iduronic acid at the +1 subsite (Fig. 8).

Chondroitin B lyase was subsequently shown to absolutely require calcium for its activity, indicating that the protein-Ca^{2+}-oligosaccharide complex is functionally relevant. We proposed that the Ca^{2+} ion neutralizes the carboxyl moiety of the L-iduronic acid at the cleavage site, while the conserved Lys250 and Arg271 act as general base and acid, respectively. Model building showed that a DS substrate would bind in a bent conformation and that this sugar ring adopts a distorted conformation. The requirement of Ca^{2+} for catalysis was further investigated in collaboration with Dr Sasisekharan by measuring K_{cat} and K_M as a function of calcium concentration (Michel *et al.*, 2004).

3. Chondroitin ABC I (endo) Lyase—The Cygler laboratory crystallized (Huang *et al.*, 2000) and determined the structure (Huang *et al.*, 2003) of chondroitin ABC I (endo) lyase from *P.* vulgaris (PvulABCI). This 110 kD protein consists of three domains. The amino acid sequence comparison indicated only that the C-terminal domain is homologous to the C-terminal, noncatalytic domain of FlavoAC, and this was confirmed by the structure. The N-terminal domain has a similar fold to carbohydrate-binding domains of xylanases and some lectins while the middle domain showed, unexpectedly, structural similarity to the catalytic domain of FlavoAC and to hyaluronan lyases. The superposition of these two domains showed the conservation of residues forming the active site tetrad (Fig. 9).

The Asn175 of FlavoAC, which plays an essential role in binding the acidic group of glucuronic acid, is not conserved in PvulABCI. Instead, the side chain of Arg500 may perform this function. We speculated that the charged guanidinium group at the end of a long arm provides the flexibility essential for adapting the enzyme's catalytic machinery to two possible configurations of the acidic group at the C-5 position of the uronic acid ring. The substrate binding area in this structure is wide open, and we have not yet been able to obtain complexes with oligosaccharides (Fig. 10). It has not yet been possible to unequivocally deduce from this structure residues that contribute to substrate binding and the key protein–substrate interactions. Further efforts toward obtaining PvulABCI complexes with substrate or inhibitors are essential for the understanding of the mechanistic properties of these enzymes and their ability to break down CS and DS.

While the PvulABCI is an endolytic lyase, this bacterium also produces a closely homologous enzyme chondroitin ABC II (exo) lyase (PvulABCII) with similar spectrum of substrate specificities while being an exolytic lyase. We have obtained crystals of this protein and would like to determine its structure to understand the mechanism underlying exolytic vs. endolytic selectivity and to detail the catalytic mechanism.

IV. Analytical Applications

Determination of CS/DS oligosaccharide structure is a formidable analytical problem that has limited structure–activity relationship studies, and the development of improved methods is necessary for further progress. Current approaches involve the preparation of CS/DS oligosaccharides using chondroitin lyases followed by separation techniques including gel permeation chromatography (GPC) (Yang *et al.*, 2000), strong anion exhange-high performance liquid chromatography (SAX-HPLC) (Linhardt *et al.*, 1994; Yang *et al.*, 2000), polyacrylamide gel electrophoresis (PAGE) (Linhardt *et al.*, 1991, 1994), and capillary electrophoresis (CE) (Al-Hakim and Linhardt, 1991; Pervin *et al.*, 1993, 1994). These provide important data on composition and domain structure but generally yield indirect and incomplete sequence information. MS has also been applied to the analysis of CS/DS oligosaccharides (Kitagawa *et al.*, 1997; Lamb *et al.*, 1992; Yang *et al.*, 2000). Fast-atom bombardment (FAB-MS), electrospray ionization (ESI-MS), and matrix-assisted laser desorption ionization (MALDI-MS) are capable of determining the molecular weight of oligosaccharides. Nuclear magnetic resonance (NMR) spectroscopy provides for the accurate determination of the chemical fine structure of small CS/DS oligosaccharides (containing 2–14 saccharide units) (Linhardt *et al.*, 1992; Yang *et al.*, 2000).

A. Oligosaccharide Structure Analysis

GAG lyases are used for the structural analysis of GAGs. In a general oligosaccharide structure analysis procedure first, a small quantity of the polysaccharide is exhaustively depolymerized in the reaction using the proper lyase enzyme (Table II) (Linhardt, 1994). Next, a controlled, partial depolymerization is performed to obtain the UV absorbance value at 232 nm (the nonreducing end uronic acid residue absorbs at 232 nm) corresponding to the maximum number of oligosaccharides that can be detected. The partial depolymerization reaction is next scaled up, and the resulting oligosaccharide mixture is next size fractionated by GPC. This separation affords size-uniform oligosaccharide mixtures corresponding to oligosaccharides ranging in size from disaccharide to octadecasaccharide. PAGE analysis of these size-fractionated oligosaccharides demonstrates the degree of size separation. The size fractions are next purified by semipreparative SAX-HPLC, desalted and lyophilized. Analytical SAX-HPLC is used to assess whether a second semipreparative SAX-HPLC separation step is necessary. Finally, mass spectral analysis and 1D/2D NMR analysis are acquired for their structural elucidation.

Using chondroitin ABC lyase, we prepared eight oligosaccharides from DS and elucidated their structures (Fig. 11) (Yang *et al.*, 2000). Treatment of tetrasaccharide and hexasaccharide fragments with mercuric acetate afforded trisaccharide and pentasaccharide products, respectively. The purity of the oligosaccharides obtained was confirmed by analytical SAX-HPLC and CE. The molecular mass and degree of sulfation of the eight purified oligosaccharides were elucidated using ESI-MS, and their structures were established with NMR. These DS oligosaccharides are being used to study interaction of the DS with biologically important proteins.

B. Oligosaccharide Mapping

Quantitative saccharide compositional analysis of GAGs can be conveniently performed using high resolution, high sensitivity CE. CS and DS can be depolymerized using chondroitin lyases (or hyaluronate lyases) into eight to nine different disaccharides that can be easily resolved by CE (Al-Hakim and Linhardt, 1991). Sensitive (picomole range) UV detection is possible due to the presence of an unsaturated residue formed in each disaccharide through the eliminase action of the polysaccharide lyases. Oligosaccharide mapping of GAGs is comparable in principle to the peptide mapping of protein and has been widely used for the comparison of GAGs from different tissues or species (Linhardt *et al.*, 1991, 1992; Loganathan *et al.*, 1990). A GAG is first treated, either partially or completely, with various depolymerizing chemicals or enzymes, and PAGE maps are prepared using PAGE and SAX-HPLC (Linhardt et al., 1988). Using this approach we have studied chondroitinase ABC depolymerized mixtures of DS sample obtained from different species and tissue origins. High molecular weight and low-molecular weight DS as well as charge-fractionated DS, having substantially different heparin cofactor-II (HC-II) mediated antithrombin activities were examined. Gradient PAGE was used to study the DS oligosaccharide mixtures prepared by chondroitinase ABC treatment. Commercially available disaccharide standards have been examined by SAX-HPLC, and their retention times compared with the oligosaccharide mixture obtained from chondroitinase ABC treated DS. On the basis of these studies, certain structural features associated with DS have been established (Linhardt *et al.*, 1988).

C. Disaccharide Analysis

CS is widely used as a neutraceutical and pharmaceutical raw materials. As the number of products containing CS increases, stricter and more accurate evaluation should be required for the manufacture of high-quality products. Disaccharide analysis using HPLC should be useful for evaluation of the quality of CS as a pharmaceutical and nutraceutical ingredient. The pretreatment method followed by enzymatic digestion makes it possible to quantify CS content in soft capsules and liquid preparations and should be applicable for the quality control of CS. The present methods can be applied to confirm the purity and label claim of CS in raw materials, pharmaceuticals, and neutraceuticals.

The Kim laboratory performed the quantitative analysis of CS obtained from raw materials and various pharmaceutical preparations (Sim *et al.*, 2005). To quantify CS content in raw materials and in an ophthalmic solution, each test sample and the authentic CS were first digested by chondroitinase ABC. The CS disaccharides produced were analyzed by HPLC, and CS content was quantified by calculating the total peak areas of the disaccharides derived from a CS calibration curve. In the case of soft capsules, CS was first extracted with hexane followed by phenol-chloroform to remove oil and protein ingredients. The extracted CS samples were depolymerized by chondroitinase ABC, and CS content was determined. Quantitative analysis of the disaccharides derived from raw materials and an ophthalmic solution showed the CS contents (%) were 39.5–105.6 and 103.3, respectively. In case of CS analysis in soft capsules and liquid preparations, the overall recovery (%) of the spiked CS was 96.79–103.54 and 97.10–103.17, respectively (Table III). In conclusion, the quantitative

analysis of the disaccharides produced by enzymatic digestion can be used in the direct quantitation of CS containing pharmaceutical formulations.

V. Synthetic Applications

A. Oligosaccharide Preparation

Polysaccharide lyases have been used to produce Δ^4-uronate disaccharides and higher oligosaccharides from heparin, HS, CS, DS, hyaluronan, and chemically modified GAGs (Linhardt and Al-Hakim, 1991; Pervin *et al.*, 1995; Weiler *et al.*, 1992). Because both the polysaccharide substrates and enzymes are relatively inexpensive, these oligosaccharides can be prepared in large quantities at a low cost. The discovery of new GAGs, such as acharan sulfate (Kim *et al.*, 1996) as well as the mild acid hydrolyzate *E. coli* polysaccharide K5 (Razi *et al.*, 1995), can afford novel structures in large quantities and at low cost.

B. Chemoenzymatic Synthesis

We proposed that the lyase-derived oligosaccharides could be chemically linked together to form larger oligosaccharides with the requisite structure for a wide variety of biological activities. The first objective would be to differentially protect enzymatically prepared desulfated disaccharides and to use these neutral disaccharides to prepare larger target oligosaccharides (Fig. 12) (Islam *et al.*, 2003). The advantages of this approach are (1) disaccharides can be assembled into oligosaccharides with a reduced number of glycosylation reactions and (2) a high level of structural complexity (i.e., stereochemistry, sulfation pattern) is already built into these disaccharides. In addition, we investigated the use of 2,2,2-trifluorodiazoethane as a reagent for sulfo group protection in enzymatically prepared CS disaccharides (Fig. 12) (Avci *et al.*, 2004). This approach was first used for sulfate ester protection in carbohydrates by Flitsch and coworkers (Proud *et al.*, 1997). Once the sulfo groups have been protected, the free hydroxyl and carboxyl groups could be protected in organic solvents used in standard carbohydrate synthesis. This chemistry has been successfully used to selectively protect primary and secondary *O*- and *N*- sulfo groups in unprotected sulfated mono- and disaccharides in high yields (Karst *et al.*, 2003, 2004).

VI. Therapeutic Applications

Bacterial GAG-degrading enzymes also have direct medical applications. Heparinase is an important reagent that can be used to remove anticoagulant heparin from blood in the prevention of excessive bleeding following coronary artery bypass surgery (Langer *et al.*, 1982). *In vitro* experiments have revealed that chondroitinases inhibit melanoma invasion, proliferation, and angiogenesis (Denholm *et al.*, 2001). Subretinal injection of chondroitinase ABC (PvulABCI and PvulABCII) promotes retinal reattachment in rabbits (Yao *et al.*, 1992). Chondroitinase ABC (PvulABCI and -II) has also been applied therapeutically to treat invertebral disc protrusion (Kato *et al.*, 1990).

It has been shown that chondroitinase ABC (PvulABCI and -II) promotes the regeneration of central nervous system axons (Bradbury *et al.*, 2002; Pizzorusso *et al.*, 2002). Permanent paralysis can result in adult mammals following spinal cord injuries due to the inability of axons to regenerate (Fawcett and Asher, 1999). A glial scar develops at the site of the

central nervous system injury (Fitch and Silver, 1999). This scar is composed of ECM molecules and is particularly rich in CSPGs (Fawcett and Asher, 1999; Fitch and Silver, 1999). *In vitro* CSPGs inhibit axonal growth (McKeon *et al.*, 1991; Niederost *et al.*, 1999; Smith-Thomas *et al.*, 1994), and *in vivo* regions rich in CSPGs stop regenerating axons (Davies *et al.*, 1999). Chondroitinase ABC catalyzed removal of CS chains *in vitro* can reverse this inhibitory activity (Fidler *et al.*, 1999; McKeon *et al.*, 1995; Moon *et al.*, 2001; Zuo *et al.*, 1998). In a recent *in vivo* study in an adult rat spinal cord injury model, the delivery of chondroitinase ABC directly to the injury site promoted functional recovery (Bradbury *et al.*, 2002). In this study, chondroitinase ABC was delivered intrathecally to lessoned dorsal columns of adult rats. This treatment upregulated regeneration-associated protein in injured neurons, promoting regeneration of both ascended sensory projections and descending corticospinal tract axons. This treatment restored postsynaptic activity below the lesion after electrical stimulation of corticospinal neurons. Chondroitinase ABC also promoted functional recovery of locomotor and proprioceptive behaviors.

VII. Conclusions

Modern analytical methods, including NMR and MS, are widely used for the determination of CS structure. While modern spectroscopic techniques provide limited information on the structure of the intact CS polysaccharide, it is often useful to utilize chondroitin lyases to prepare oligosaccharides for more detailed structural determination. The structure, activity, and specificity of these enzymes were the focus of this chapter. These lyases can be combined with separation methods, such as chromatography and electrophoresis, for the preparation of CS oligosaccharides for biological evaluations as well as for disaccharide analysis, oligosaccharide mapping, and polysaccharide sequencing. These enzymes have also been shown to have direct therapeutic value. This chapter examined the various applications for this important class of enzymes.

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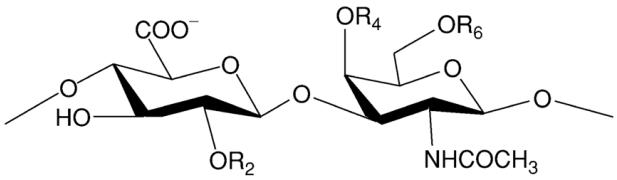
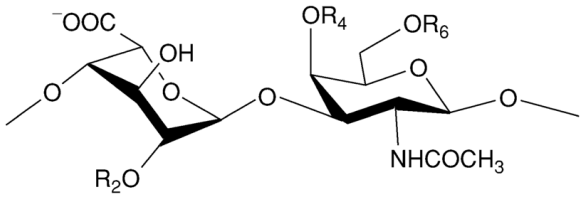


Chondroitin sulfate

CS-A: $R_4=SO_3^-$ R_2, R_6=H
CS-C: $R_6=SO_3^-$ R_2, R_4=H
Chondroitin: R_2, R_4, R_6=H
Oversulfated CS: 2 or 3 sulfo groups at R_2, R_4, R_6



Dermatan sulfate

DS (CS-B): $R_4=SO_3^-$ R_2, R_6=H
Oversulfated DS: 2 or 3 sulfo groups at R_2, R_4, R_6

FIGURE 1.

CS, oversulfated CS and chondroitin: the molecular weight ranges from 5000–50,000 (average 25 kDa). DS and oversulfated DS: the molecular weight ranges from 5000–50,000 (average 25 kDa).

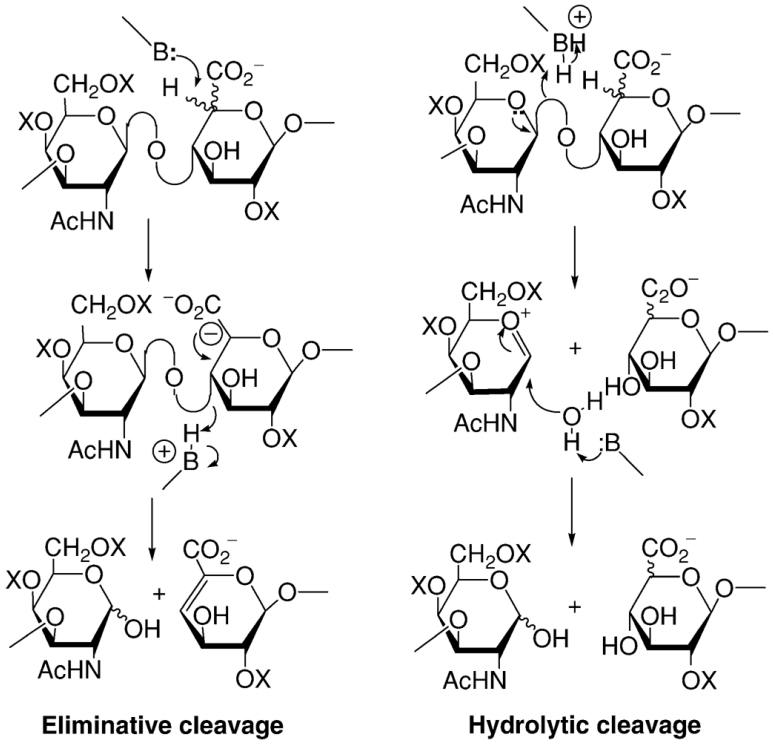


FIGURE 2.

Mechanism for the enzymatic breakdown of GAGs. Lyases catalyze eliminative cleavage and hydrolases catalyze hydrolytic cleavage leading to different oligosaccharide products.

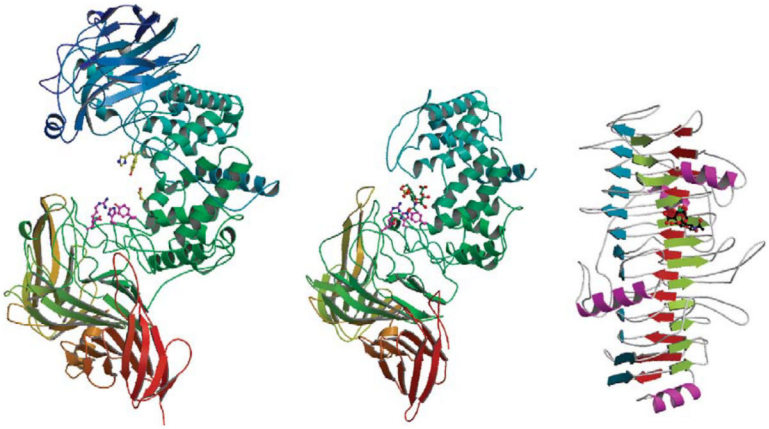


FIGURE 3.

Comparison of crystal structures of chondroitinases PvulABCI (left), FlavoAC (center), and FlavoB (right).

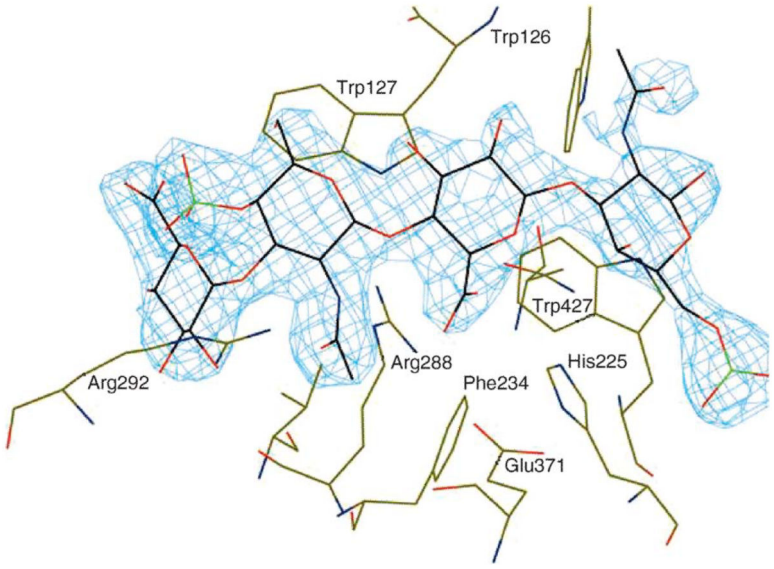


FIGURE 4.

Chondroitin-4,6-sulfate tetrasaccharide in the active site of FlavoAC Tyr234Phe mutant.

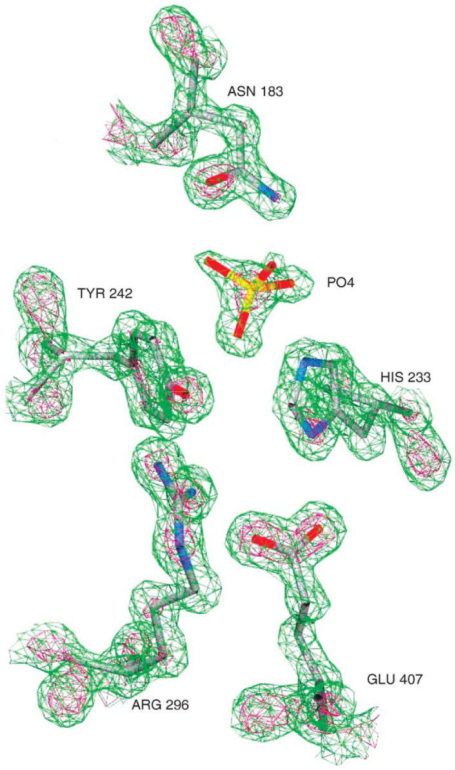


FIGURE 5.

Experimental electron density map of the active site region of ArthroAC. Green contours are drawn at 3σ level, red contours at 5σ level. In the native structure, there is a phosphate ion in the active site. Nitrogen atoms are blue, oxygens are red, carbons are gray, and phosphorus is yellow.

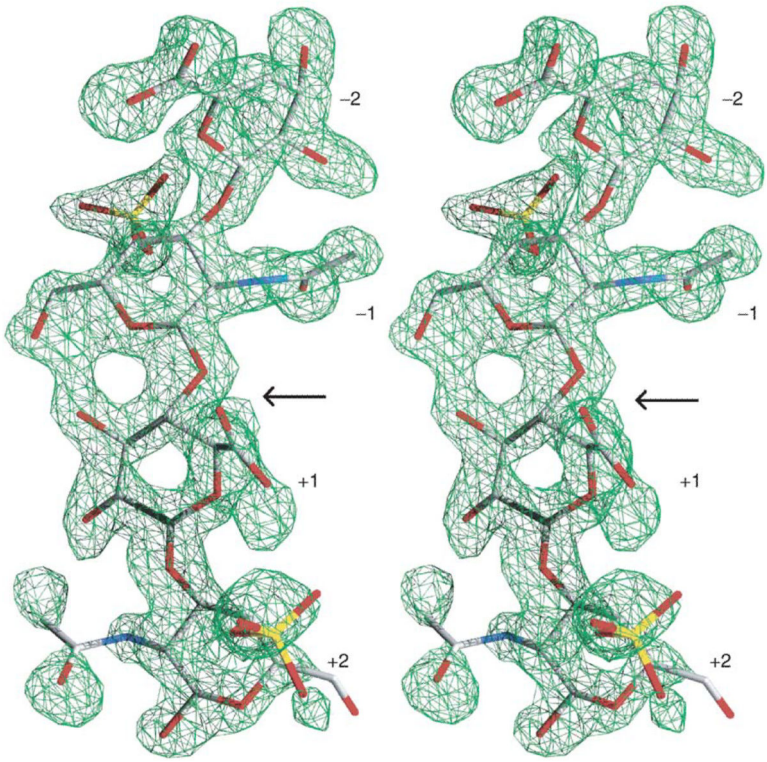


FIGURE 6.

Conformation of the chondroitin-4-sulfate tetrasaccharide substrate bound in the active site of ArthroAC. Omit electron density map is drawn at the 3σ level.

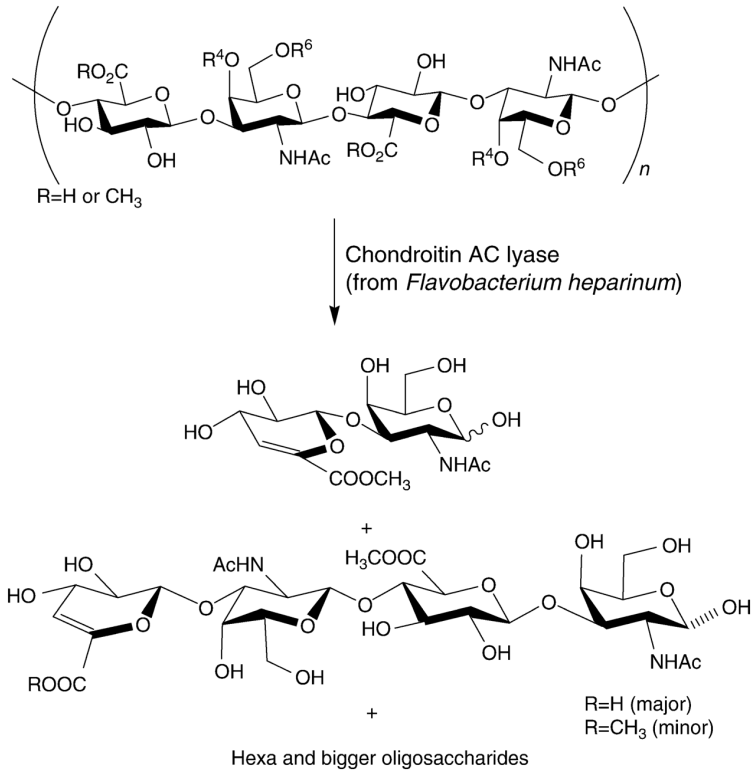


FIGURE 7.
Enzymatic depolymerization of chondroitin *O*-methyl ester.

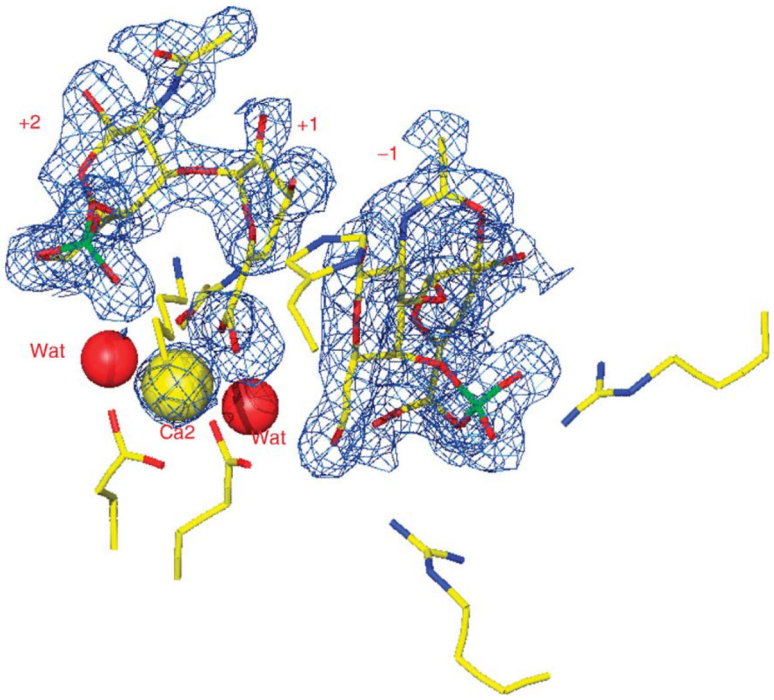


FIGURE 8.

Three disaccharide products bound in the active site of chondroitinase B. Bound calcium atom is shown as a yellow sphere, two water molecules as red spheres.

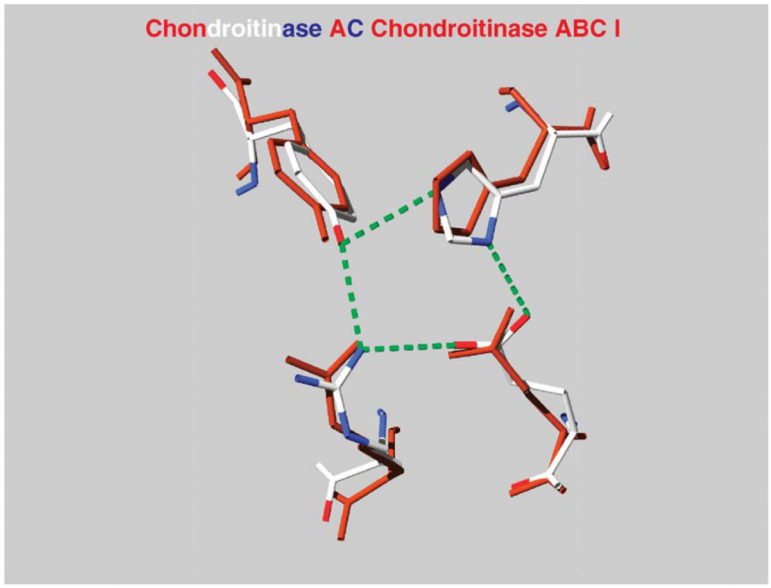


FIGURE 9.

The superposition of the active-site tetrad of FlavoAC and PvulABCI. The Asn175 of FlavoAC and Arg500 of PvulABCI are also shown.

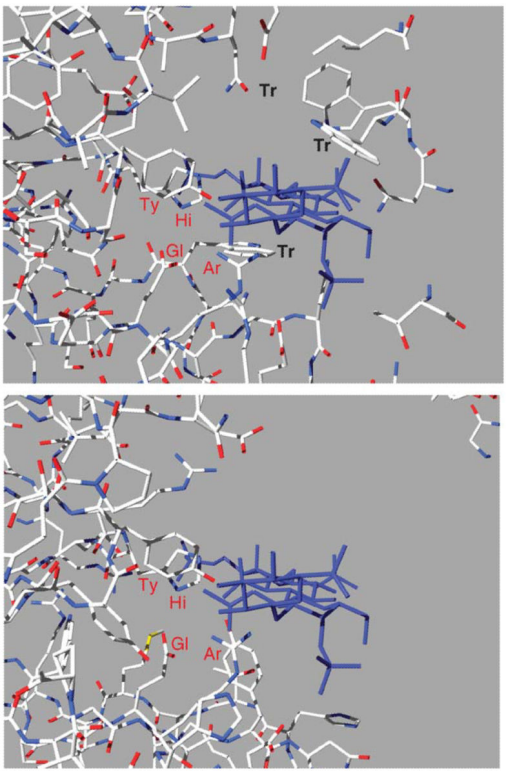


FIGURE 10.

The disposition of the substrate in FlavoAC (left) was transferred to PvulABCI (right) based on the superposition of the active site tetrad. In this open form of PvulABCI are very few contacts between the enzyme and its substrate.

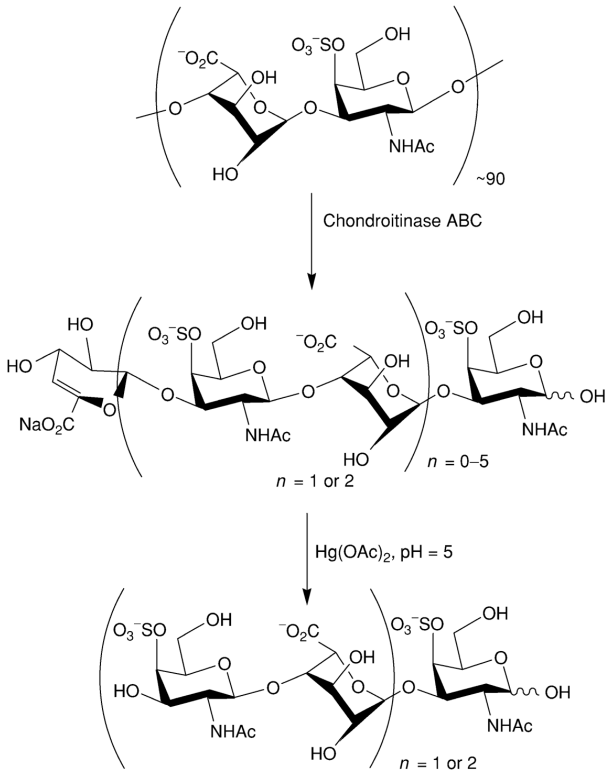


FIGURE 11.

Controlled enzymatic depolymerization of DS by chondroitinase ABC and mercuric acetate treatment to remove the unsaturated nonreducing end residue.

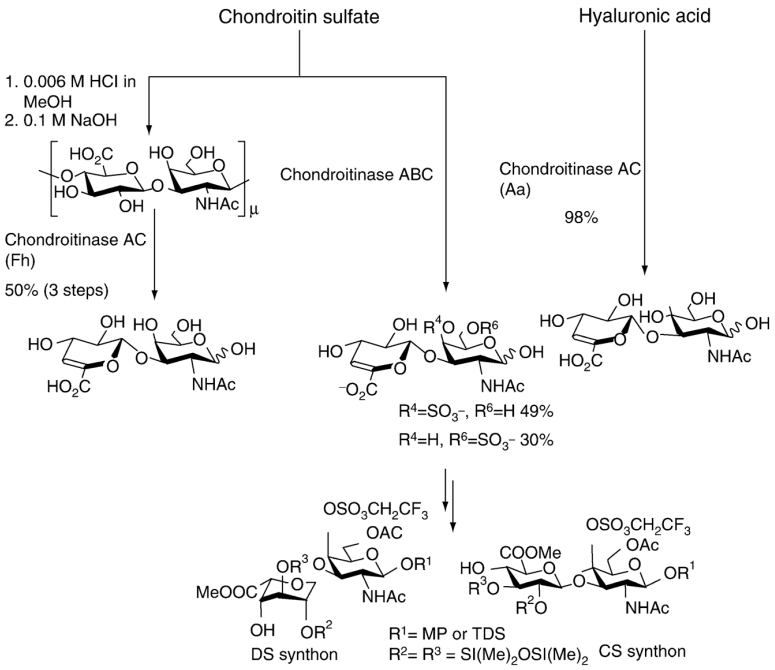


FIGURE 12.

Preparation of desulfated and sulfoprotected disaccharide starting materials for the synthesis of CS/DS/HA oligosaccharides.

TABLE I

Properties of Polysaccharide Lyases Acting on Chondroitins

Name[a]	Substrates	Linkage specificity[b]	Action pattern	Mr (Da)	K_M[c,d]	V_{max}[c,e]
Chondroitinase AC (Fh) (Gu *et al.*, 1995)	CS-A (4S) CS-C (6S) HA	→3)GalNAc(or GlcNAc) 4X,6X(1→4) GlcA(1→	Endo	74,000	9.3	121
Chondroitinase AC (Aa) (Linhardt, 1994; Lunin *et al*., 2004)	CS-A (4S) CS-C (6S) HA	→3)GalNAc(or GlcNAc) 4X,6X(1→4) GlcA(1→	Exo	79,840	0.01	
Chondroitinase B (Fh) (Gu *et al.*, 1995)	DS	→3)GalNAc 4X,6X(1→4)IdoA2X(l→	Endo	55,200	7.4	209
Chondroitinase ABC (Bs) (Hong *et al*., 2002)	CS-A (4S) CS-C (6S) DS	→3)GalNAc 4X,6X(1→4)UA2X(1→	Endo	116,000		45.7
Chondroitinase ABC I (Pv) (Hamai *et al*., 1997)	CS-A (4S) CS-C (6S) DS	→3)GalNAc 4X,6X(1→4)UA2X(1→	Endo	100,000	66	310
Chondroitinase ABC II (Pv) (Hamai *et al*., 1997)	CS-A (4S) CS-C (6S) DS	→3)GalNAc 4X,6X(1→4)UA2X(1→	Exo	105,000	80	34
Hyaluronate lyase (Pa) (Ingham *et al*., 1979)	HA CS-A (4S) CS-C (6S)	→3)GalNAc(or GlcNAc) 4X,6X(1→4) GlcA(1→		85,110		7.75

[a] Fh: *Flavobacterium heparinum*; Aa: *Arthrobacter aurescens;* Pv: *Proteus vulgaris;* Bs: *Bacteroides stercoris.*

[b] The primary sites of action are shown. X = SO_3^- or H, UA = glucuronic or iduronic acid.

[c] Kinetic parameters are given for the primary substrates.

[d] Apparent K_m in μM.

[e] V_{max} in μmol min^{-1} mg^{-1}.

TABLE II

Reaction Conditions for Polysaccharide Lyases Acting on Chondroitins with Optimum Buffers and Reaction Conditions

Lyase	Buffer	Optimum temperature (°C)
Chondroitinase ABC	Tris.Cl/sodium acetate, pH 8	37
Chondroitinase AC	Tris.Cl/sodium acetate, pH 8	37
Chondroitinase B	Ethylenediamine/acetic acid/NaCl, pH 8	25
Hyaluronate lyase	Sodium acetate/NaCl, pH 5.2	30

TABLE III

Quantitation of CS from Pharmaceuticals[a]

Sample	Labeled amount (mg)	Determined amount (mg)	Label claim (%)
Ophthalmic solution	2	2.07	103.33
Liquid preparation	30	29.25	97.48
Soft capsule	120	114.06	95.05

[a]The CS content was quantified by calculating the total peak areas of the disaccharides derived from a CS calibration curve.