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Structural and Functional Comparison of Polysaccharide-Degrading Enzymes

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Abbreviations: **DD**, double displacement; **ECM**, extracellular matrix of tissues; **GAG**, glucosaminoglycan; **FheCACL**, *Flavo bacterium heparinum* chondroitin **AC** lyase; **FheCBL**, *Flavobacterium heparinum* chondroitinase B; **HA**, hyaluronan; **HL**, hyaluronate lyase; **PAD**, proton acceptance and donation; **PDB**, Brookhaven Protein Data Bank; **PG**, proteoglycan; **RHAMM**, receptor for hyaluronic acid-mediated motility; **SagHL**, *Streptococcus agalactiae* hyaluronate lyase; **SpnHL**, *Streptococcus pneumoniae* hyaluronate lyase; **SspAL**, *Sphingomonas* species alginate lyase A1-III.

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Abstract: Sugar molecules as well as enzymes degrading them are ubiquitously present in physiological systems, especially for vertebrates. Polysaccharides have at least two aspects to their function, one due to their mechanical properties and the second one involves multiple regulatory processes or interactions between molecules, cells, or extracellular space. Various bacteria exert exogenous pressures on their host organism to diversity glycans and their structures in order for the host organism to evade the destructive function of such microbes. Many bacterial organism produce glycan-degrading enzymes in order to facilitate their invasion of host tissues. Such polysaccharide degrading enzymes utilize mainly two modes of polysaccharide-degradation, a hydrolysis and a β-elimination process. The three-dimensional structures of several of these enzymes have been elucidated recently using X-ray crystallography. There are many common structural motifs among these enzymes, mainly the presence of an elongated cleft transversing these molecules which functions as a polysaccharide substrate binding site as well as the catalytic site for these enzymes. The detailed structural information obtained about these enzymes allowed formulation of proposed mechanisms of their action. The polysaccharide lyases utilize a proton acceptance and donation mechanism (PAD), whereas polysaccharide hydrolases use a direct double displacement (DD) mechanism to degrade their substrates.

KEY WORDS: glycans, polysaccharide lyases, polysaccharide hydrolases, three-dimensional structure, mechanism of action

I. INTRODUCTION

Oligosaccharide chains are ubiquitously present on cell, protein, and lipid surfaces. They perform a variety of important biological functions and play an important role in, for example, microbe-host interactions. For higher organisms such as vertebrates, the fine regulation of synthesis and degradation of sugars is essential not only for their primary functions but also for many regulatory processes. Their primary functions often are related to mechanical properties of polysaccharides and their complexes in environments such as the extracellular matrix of tissues (ECM). Their regulatory processes involve lymphocyte homing or intercellular interactions and proliferation and locomotion (Fraser and Laurent, 1989; Laurent and Fraser, 1992). These physiological regulatory processes are often facilitated by rapid turnover of sugar molecules and their complexes or assemblies.

Sugar (glycan) molecules have a universal presence, and the enzymes that degrade them appear to be ubiquitous. Examples of such enzymes are polysaccharide hydrolases and lyases. Many of these glycan-degrading enzymes are of bacterial origin. Bacteria, in particular bacterial pathogens, often utilize glycan-degrading enzymes to overcome the host defense mechanism to advance bacterial invasion of the host and to reach desired host sites that are essential for continuing bacterial invasion. These exogenous pressures force the host to greatly diversify its glycan structures in order to avoid degradation by these exogenous elements. The exact mechanism for glycan degradation is largely speculative due in part to the lack of structural information about glycans and glycan-degrading enzymes. Recently, however, a substantial amount of three-dimensional structural information has become available for polysaccharide-degrading enzymes. At lease 10 new structures have been reported since 1996. This structural information often has precluded the proposal of a more precise catalytic mechanism of action for these enzymes. These enzymes can be grouped into two groups, hydrolases and lyases. Hydrolases degrade glycans via hydrolysis of glycosidic bonds between sugars, and lyases degrade glycans by using a β-elimination process. In this review we discuss predominantly the polysaccharide lyases, their structural motifs, and their mechanism of catalysis. The structure and mechanism of action of polysaccharide lyases are also compared with polysaccharide hydrolases.

II. GLYCANS

Extracellular proteins and lipids often have oligosaccharide chains (glycans) attached to them. Glycans often mediate structural and/or regulative biological processes such as recognition by endogenous or exogenous glycan receptors (Rademacher et al., 1988; Paulson, 1989; Esko, 1991; Hart, 1992; Kobata, 1992; Lis and Sharon, 1993, Varki, 1992, Varki and Marth, 1995; Gahmberg and Tolvanen, 1996; Drickamer and Taylor, 1998; Gagneux and Varki, 1999). The specific function of many cell-type or molecule-specific glycan types and their complex structures have not yet been determined (Varki and Marth, 1995). Diversification of glycans is found everywhere, between or within species, and among different molecules or cell types of the same organism (Gagneux and

Varki, 1999). Glycans also change during different stages of cellular or molecular development (Maly et al., 1996; Tsuji, 1996). Complexity of functional and regulatory properties of glycans is still largely unknown. The focus of this review is the plant and bacterial polysaccharides and polysaccharides of the extracellular matrix of tissues, including glucoseaminoglycans, with an emphasis on hyaluronan and hyaluronan-degrading enzymes.

A. Plant and Bacterial Polysaccharides

An example of a plant and bacterial glycan is alginate. Alginate is a polymer that causes gel and viscosity formation on brown seaweed and bacteria (Preiss and Ashwell, 1962). Alginate is a polymeric sugar built from blocks of β-D-mannuronate and the C5 epimer α-L-glucuronate (Figure 1) (Murata

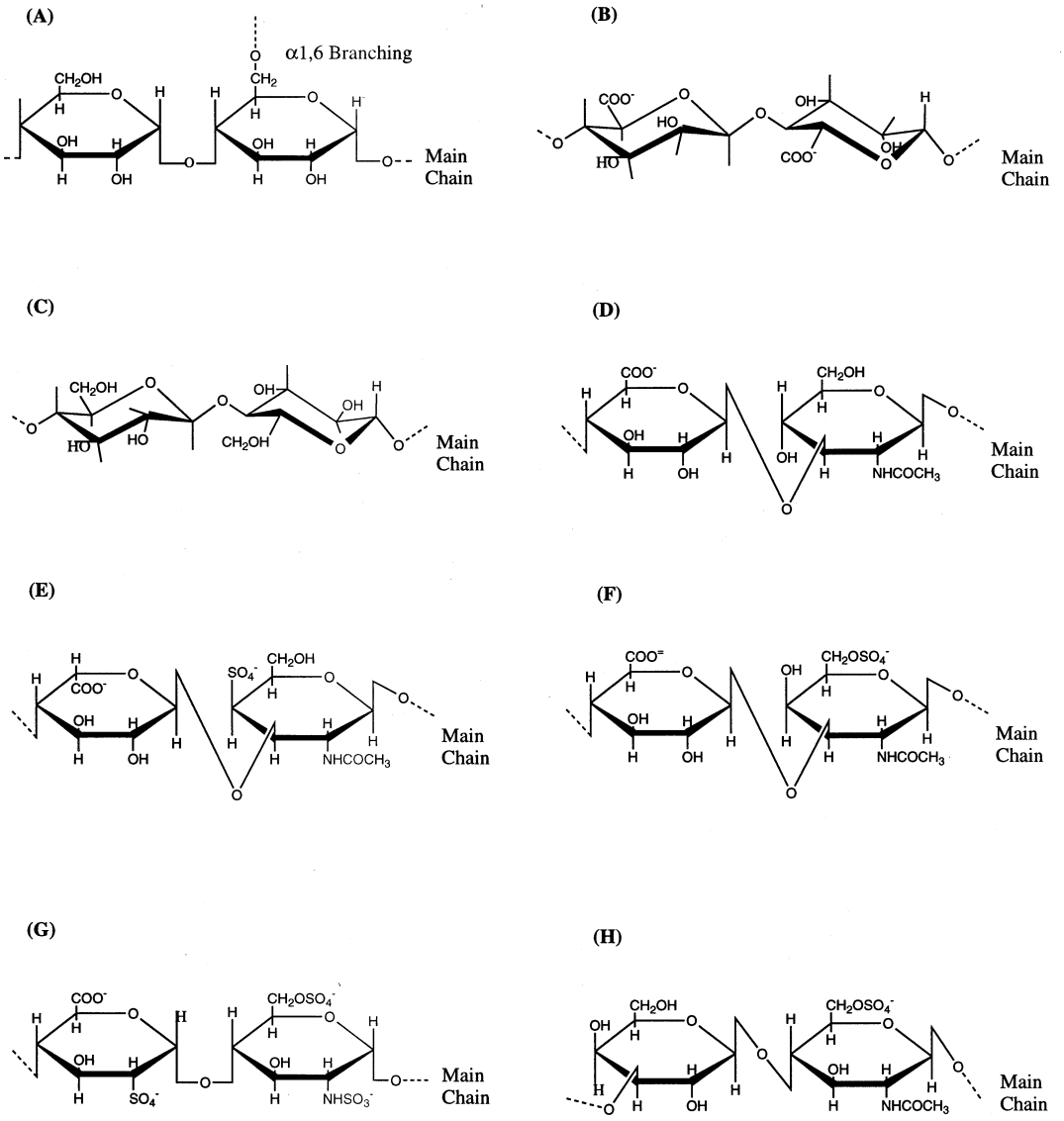


FIGURE 1. Primary structures of building blocks of selected polymeric polysaccharides. **(A)** amylopectin; **(B)** alginate (poly β-D-mannuronate); **(C)** cellulose; **(D)** hyaluronan; **(E)** dermatan sulfate; **(F)** chondroitin-6-sulfate; **(G)** heparin; **(H)** keratan sulfate.

et al., 1993). Alginate is degraded by alginate lyases that cleave the β-1,4 glycosidic bond in the alginate polymer through the β-elimination process, resulting in 4-deoxy-α-L-erythro-hex-4-ene pyranosyluronate-containing saccharides (Preiss and Ashwell, 1962; Lindhardt et al., 1986; Brown and Preston, 1991). These degrading enzymes have been identified in bacteria, especially marine bacteria (Davidson et al., 1976; Matsubara et al., 1998) and others such as *Bacillus circulans* (Hansen et al., 1984), *Sphingomonas* species (Yonemoto et al., 1991), *Klebsiella* species (Baron et al., 1994; Caswell et al., 1989; Lange et al., 1989), and *Pseudonomas* species (Sutherland and Keen, 1981; Monday et al., 1996; Linker and Evans, 1984; Dunne and Buckmire, 1985). Mucoid strains of *P. aeruginosa* have the ability to synthesize the alginate biofilm that protects this bacterial pathogen from the host immune system and protects it from medicinal antibacterial agents (Boyd and Chakrabarty, 1995).

Higher plants synthesize pectic substances, an example of another glycan that is composed mainly of α-1,4 linked galacturonsyl residues. These residues are found in the plant cell wall and middle lamella (Figure 1) (Barling et al., 1995; Keen and Tamaki, 1986). Plant microbes, on the other hand, synthesize a variety of enzymes that cleave these pectic polymers. These enzymes include multiple isoforms of pectate and pectin lyases, polygalacturonases, and pectin methylesterases. For example, *Erwinia chrysanthemi* secrets five pectate lyase isoforms, PelA, PelB, PelC, PelD, and PelE. The sequence homology between these enzymes is relatively high, ranging from 27 to 90% (Keen and Tamaki, 1986; Tamaki et al., 1988).

B. Extracellular Matrix of Tissues

In higher multicellular eukaryotic organisms the space between cells is composed of the extracellular matrix of tissues. The ECM consists of insoluble collagen fibrils and soluble polymeric structures that together are responsible for the tissue adjustment related to the stress of movement and the maintenance of the organism's shape (Scott, 1975 and 1992). The fibrils resist tensile stress, whereas the soluble polymers resist compressive forces applied to the matrix. Examples of this type of tissue include skin, blood vessels, and tendons. However, in addition to the mechanical functions, these tissues evolved to play an essential part in the multiple regulatory functions. Collagen fibrils form an orthogonal matrix filled with proteoglycans (PGs) (Scott, 1975 and 1992). These PGs usually have a globular protein part (head) to which glucoseaminoglycans (GAGs) are attached (tails) (Scott et al., 1986). Connective tissue GAGs are built of long, unbranched chains of repeating disaccharides such as sulfated chondroitins, dermatans, keratans, and unsulfated hyaluronan (HA) (Figure 1). Related heparans are also composed of chains of disaccharide units but are predominantly associated with cell and basement membranes instead of PGs.

C. Glucoseaminoglycans

Glucoseaminoglycans (GAGs), oligosaccharide chains of the extracellular matrix of tissues, are a specific type of glycan. Evolutionary selection pressures and exogenous selection pressures mediated by microbes interacting with GAGs contributed to the diversification of GAGs (Gagneux and Varki, 1999). These polymers differ in the type of disaccharides they utilize as building blocks, and in the linkage between the building blocks (Scott and Heatley, 1999). Such diversification led to their division into three structural groups:

(1) cellbiose (e.g., hyaluronan), (2) polylactoseamines (e.g., dermatan, chondroitin, and keratan sulfate), and (3) polymaltose (heparin, heparan sulfate). Chondroitin, keratan sulfate, and hyaluronan (HA) have very similar polymer backbone structures (Glcβ1→3Glcβ1→4) (Figure 1). Dermatan contains iduronate groups that can assume multiple ring conformations, causing potentially more diverse polymer conformations. Structural studies of GAG polymers showed that they assume helical conformations with 2 to 8 disaccharides per complete helix turn, depending on experimental conditions such as pH and the presence of certain cations (Scott and Tigwell, 1978; Atkins et al., 1980).

One of the more studied GAG polymers, in terms of structure, is hyaluronan. Structural studies of hyaluronan's secondary structures in water-based medium showed that it assumes a two disaccharide unit helix (twofold helix) (Scott and Tigwell, 1978; Scott et al., 1991). Structural investigations of other GAGs suggest that chondroitin and keratan sulfates also assume a twofold helical conformation in aqueous solutions (Hounsell, 1989). The structure of dermatan is somewhat different in that 2-, 3-, and 8-fold helices were observed (Mitra et al., 1983).

D. Hyaluronan

Hyaluronan is one of the components of ECM and GAG structures. HA is synthesized at the cellular membrane (Prehm, 1983) and is distributed essentially in the extracellular space. It is built from repeating disaccharide units of the chemical structure $[\rightarrow 3)GlcNac(\beta 1\rightarrow 4)GlcUA(\beta 1\rightarrow]_n$, where GlcNAc is *N*-acetyl-D-glucosamine and GlcUA is D-glucoronic acid (Figure 1). HA that is present in natural sources and has an enormous size, up to 25,000 disaccharide units or 10^7 Da. Due to its chemical structure, it has a large capacity to attract and hold water. The molecular tertiary structure of HA contributes to its visco-elastic properties even at relatively low concentrations (Delpech et al., 1997). HA is present in nearly all tissues throughout the vertebrates and lower marine organisms and is even present in some bacteria such as *Streptococcus zooepidemicus*. In addition to its mechanical function related to its tensile properties, HA is involved in the complex carbohydrate metabolism related to very basic biological processes such as cell migration (see below). Fine regulation of the synthesis and degradation of HA is essential to such processes. This regulation influences various other processes through HA's interactions with many macromolecules. HA is known to interact with the aggregating large proteoglycan of cartilage (aggrecan), particularly with the G1 domain of aggrecan and the link proteins that stabilize this interaction (Keiser et al., 1972; Gregory, 1973; Hardingham, et al., 1986; Hassel et al., 1986; Neame and Barry, 1993; Perkins et al., 1992). Another example of a large proteoglycan interacting with HA is a versican (Zimmermann et al., 1989; LeBaron et al., 1992; Bertrand et al., 1992). Hyaluronectin glycoprotein, ranging from 45 to 100 kDa in size (Delpech et al., 1985), is yet another extracellular protein that binds to HA. Fibronectin, another component of the extracellular matrix of tissues (ECM), was also found to interact with HA, although the interaction is weaker (Ruoslahti et al., 1981). In addition to ECM based interactions with HA, there are numerous cell surface receptors for HA, including the receptor for hyaluronic acid-mediated motility (RHAMM) (Turley and Auersperg, 1989; Turley, 1989) and CD44, which is present on lymphocytes (Murakami et al., 1991; Miyake et al., 1990). RHAMM has been

associated with cell motility, which declines when the binding of HA to cells is lost. CD44 has somewhat different affinities for HA depending on its origin (Stamenkovic et al., 1891; Thomas et al., 1992), and it is involved in lymphocyte homing and intercellular adhesion.

Structural studies of hyaluronan on a tertiary level revealed that when the polyanionic charge in the HA solution is low (low pH) it forms dimers (Staskus et al., 1988) and elastic gels (Gibbs et al., 1968), but at physiological conditions it assumes a duplex formation (Turner et al., 1988). Later studies showed that HA can form a meshwork dependent on the chain length of the HA polymer (Scott et al., 1991). The tertiary structures of other GAGs are similar to HA structures. Additionally, the similar composition and shape of GAGs makes them amenable to hetero-aggregation (Scott, 1989). Due to different patterns of sulfation, different carbonyl group positions, and the attachment to different protein components like proteoglycans (PGs) (many of which are tissue specific), the aggregation of GAGs is also specific to different tissues such as soft or hard tissues (Scott, 1989 and 1992).

III. POLYSACCHARIDE-DEGRADING ENZYMES

A. Polysaccharide Lyases

Microorganisms often degrade glycans in order to, for example, facilitate their invasion of tissues of host organisms, especially tissues essential for their further development and progress of infection (Sutherland, 1995). The group of enzymes that cleave glycan structures are polysaccharide lyases and hydrolases. The lyases act as elimenases for certain glycosidic bonds (Linker et al., 1956; Linhardt et al., 1986). During the catalytic process an unsaturated, chromophoric bond that absorbs energy in the UV range is formed at a Δ4,5 site in the uronic acid residue, likely at the nonreducing end of the product. These glycan-degrading enzymes include heparin lyases I, II, and III, chondroitin sulfate lyases ABC, AC, and B, and hyaluronate lyases (Yamagata et al., 1968). These enzymes have selection mechanisms for the cleaved glycosidic bonds that range from very specific to random selection of the cleavable bond (Linhardt et al., 1982b and 1992). The exact mechanism of this catalytic process was still largely speculative partly due to the lack of sufficient three-dimensional structural information about these enzymes and the limited number of biochemical and molecular biology studies. Examples of structures known to date include *Flavobacterium heparinum* chondroitin AC lyase (Fethiere et al., 1999), chondroitinase B (Huang et al., 1999), *Sphingomonas* species alginate lyase A1-III (Yoon et al., 1999), *Aspergillus awamori* and *Saccharomycopsis fibuligera* glucomylase (Aleshin et al., 1992; Sevcik et al., 1998), *Clostridium thermosellum* endoglucanase CelD (Juy et al., 1992), *Clostridium thermocellum* endoglucanase CelA (Alzari et al., 1996), *Thermomonospora fusca* endo/exocellulase (Sakon et al., 1997), *Aspergillus niger* pectin lyase (Mayans et al., 1997; Vitali et al., 1998), *Erwinia chrysanthemi* (Yoder et al., 1993a and b; Scavetta et al., 1999), and *Bacillus subtilis* (Pickersgill et al., 1994) pectate lyases (Figure 2). Another group of enzymes that has the ability to degrade glycans is polysaccharide hydrolases, which use the hydrolysis of the glycosidic bond between the glycan building blocks to accomplish the degradation. An example of such enzyme is hyaluronidase.

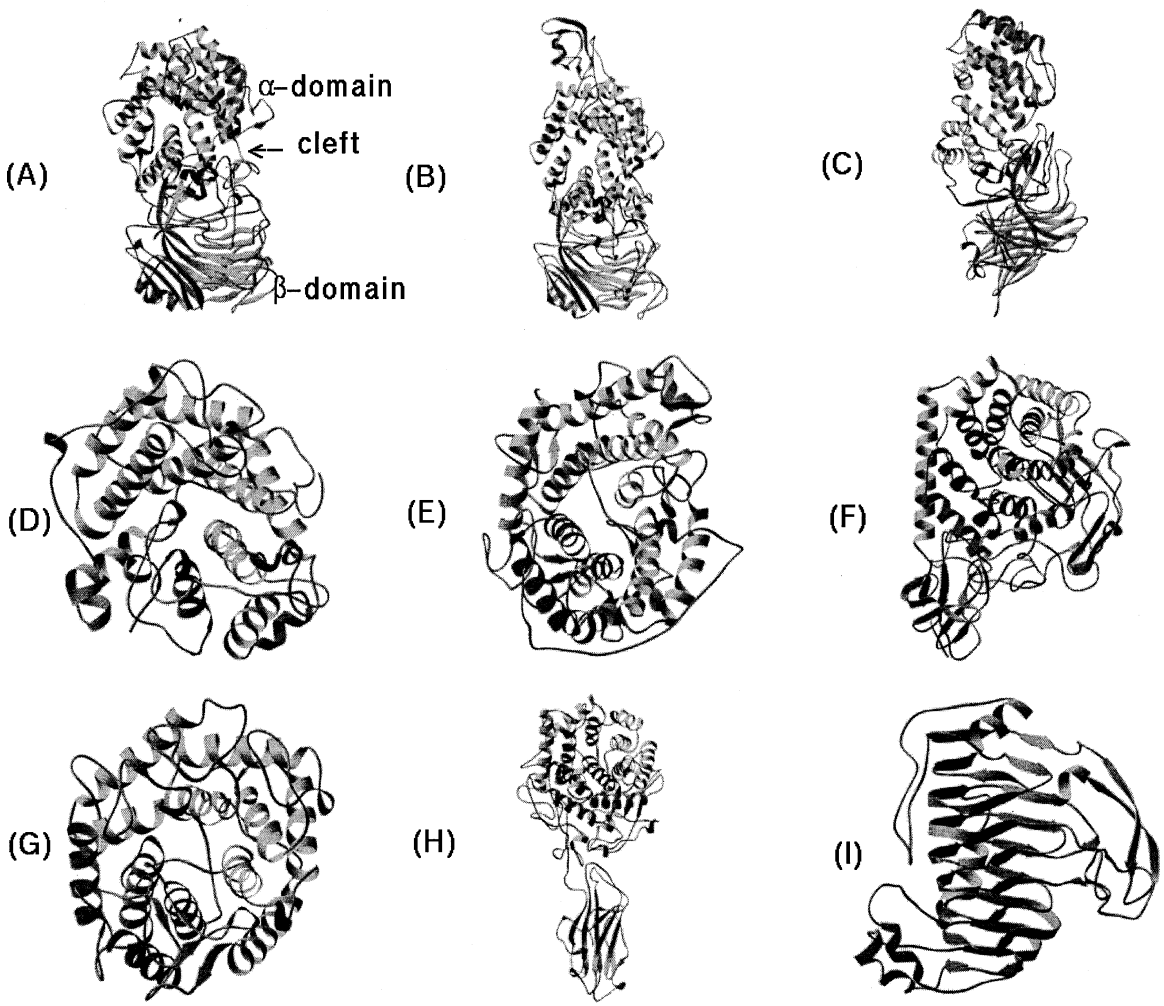


FIGURE 2. Three-dimensional structures of selected polysaccharide-degrading enzymes. For (A-H) the catalytic cleft transverses horizontally, the α-helical domains at the wider opening of the barrel structure with a/a topology. For (I) the cleft transverses vertically the β-sheet helix of the molecule. For all molecule orientations the cleft faces the reader. Figures C-I were made using the structure coordinates deposited to the Brookhaven Protein Data Bank (pdb). **(A)** *S. pneumoniae* hyaluronate lyase (Li et al., 2000; pdb: 1egu)); **(B)** *S. agalactiae* hyaluronate lyase (Li and Jedrzejas, 2000; pdb: 1f1s)); **(C)** *Flavobacterium heparinum* chondroitin AC lyase (pdb: 1cb8); **(D)** *Sphingomonas* species alginate lyase A1-III (pdb: 1qaz); **(E)** *Aspergillus awamori* glucomylase (pdb: 1dog); **(F)** *Clostridium thermosellum* endoglucanase CelD (pdb: 1clc); **(G)** *Clostridium thermocellum* endoglucanase CelA (pdb: 1cem); **(H)** *Thermomonospora fusca* endo/exocellulase (pdb: 1tf4); **(I)** *Aspergilus niger* pectin lyase (pdb: 1idj).

B. Hyaluronidases

Hyaluronidase enzymes degrade hyaluronan, yielding various lengths of oligosaccharide units as end products. There are three main classes of hyaluronidases: (1) hyaluronate 4-glycanohydrolase (hyaluronoglucosaminidase), e.g., testicular hyaluronidase; (2) hyaluronate glycano-hydrolase, e.g., leech hyaluronidase; and (3) hyaluronate lyase, e.g., bacterial hyaluronidase (Mentzel and Farr, 1998). Hyaluronate 4-glycanohydrolases cleave *β-N*-acetyl-hexosamine-(1→4) glycosidic bonds in HA, and chondroitin and chon-

droitin sulfates yield even-numbered oligosaccharide units of HA and *N*-acetylglusosamine at the reducing end. Hyaluronate glycano-hydrolases, such as leech hyaluronidase, are specific for the glucuronic linkage in HA and are inert toward other polysaccharides. Finally, a bacterial hyaluronidase, hyaluronate lyase (HL), cleaves HA β-GlcNAc-(1→4) glycosidic bonds using a β-elimination process. This is in contrast to other hyaluronidases that are hydrolases and yield 4,5-unsaturated oligosaccharides of various lengths, sometimes as small as disaccharides of HA (Figure 1). *Streptococcus hyalurolyticus* HL yields, for example, tetra- and hexa-saccharides as final degradation products of HA (Shimada and Matsumura, 1980). Some of the bacterial HLs, such as *Streptococcus agalactiae* HL, also cleave chondroitin sulfates at every unsulfated disaccharide repeat (Baker et al., 1997).

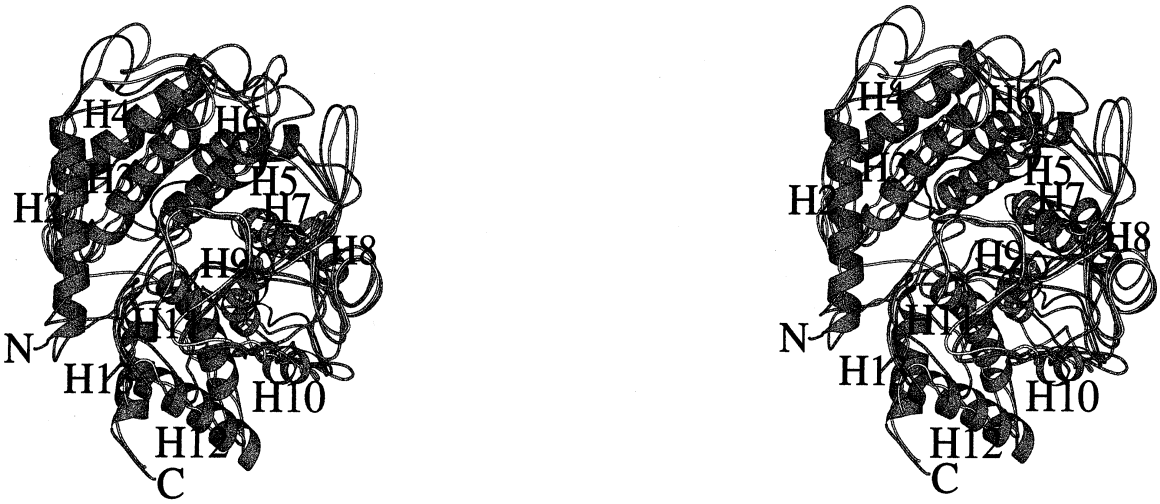
IV. STRUCTURAL COMPARISON OF SELECTED ENZYMES DEGRADING POLYMERIC SACCHARIDES

Three-dimensional structures of several enzymes that degrade polysaccharide chains have recently been elucidated primarily by X-ray crystallography (see section III.A). These structures include pneumococcal (Jedrzejas et al., 1998a and b; Li et al., 2000; Ponnuraj and Jedrzejas, 2000) and *Streptococcus agalactiae* hyaluronate lyases (Li and Jedrzejas, 2000; Jedrzejas and Chantalat, 2000), chondroitin AC lyase (Fethiere et al., 1999), alginate lyase (Yoon et al., 1999), glucomylase (Aleshin et al., 1992; Sevcik et al., 1998), endoglucanase CelA (Alzair et al., 1996), CelD (Juy et al., 1992), and endo/exocellulase (Sakon et al., 1997) (Figure 2). They all share one common structural motif, a barrel-like fold with α_6/α_6, α_6/α_5 or α_5/α_5 topology, but differ mostly in the number of helices and the details of the barrel-like fold. All contain a cleft transversing the molecule where the substrate, a polysaccharide chain, can bind and is degraded through hydrolysis or β-elimination involving selected residues from the substrate binding and catalytic cleft.

A. Structural Implications of the Barrel-Like Fold

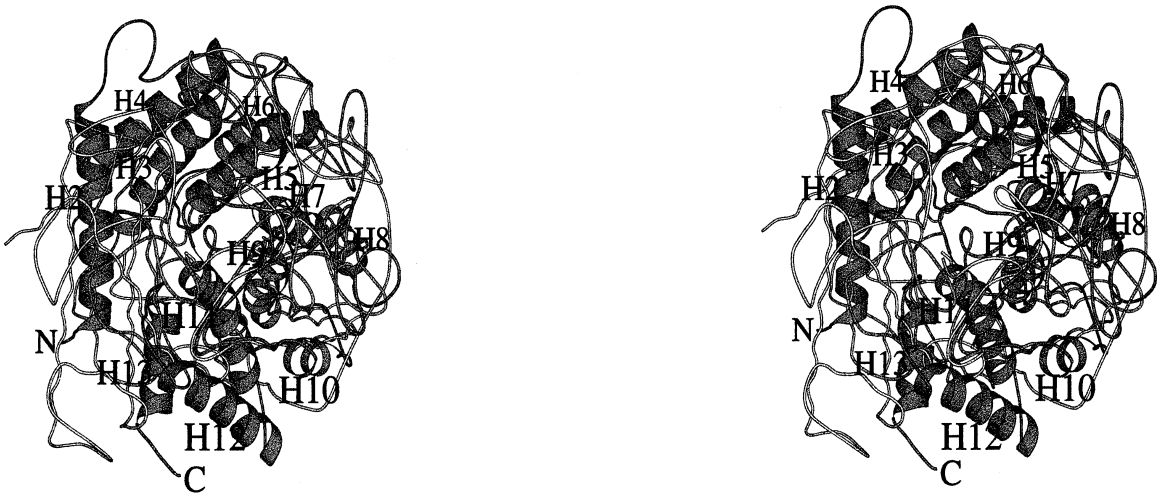
The structures of the enzymes discussed above have at least one commonality, an $\alpha_{5\text{-}6}/\alpha_{5\text{-}6}$ type of the fold present in at least one of the domains (Figures 2 and 3). The helices comprising this motif create a barrel-like structure, with one end of the barrel being wider than the other, usually. At this wider end of the barrel structure an elongated cleft is formed that is narrower or wider depending on the enzyme and probably on the substrate. In this cleft the substrate, that is, hyaluronan, chondroitin, alginate, maltodextrin, or cellulose, binds and is degraded into smaller polysaccharide units. Some of these enzymes are hydrolases and some are lyases. The enzymes that are most closely related to pneumococcal hyaluronate lyase (SpnHL) in terms of structure (the focal enzyme of this review) include *S. agalactiae* hyaluronate lyase (SagHL), *Flavobacterium heparinum* chondroitin AC lyase (FheCACL), and to a lesser extent the *Sphingomonas* species alginate lyase A1-III (SspAL) (Figure 3). Both hyaluronate lyases, SpnHL and SagHL, and the chondroitin lyase, FheCACL, are the most closely related in terms of fold and structure (Figure 3). SpnHL, SagHL, and FheCACL are composed of two domains, a catalytic domain that is a highly α-helical α/α barrel domain, and a neighboring domain, rich in

A



B

FIGURE 3. Stereo view of structural alignments of overall fold of selected polysaccharide chain-degrading lyases. **(A)** The enzymes shown are *S. pneumoniae* (thicker tracing) and *S. agalactiae* hyaluronate lyases, *Flavobacterium heparinum* chondroitin AC lyase (pdb: 1cb8), and *Sphingomonas* species alginate lyase A1-III (pdb: 1qaz). The enzymes are rotated 90° around the vertical axis relative to the orientation shown in Figure 2. The catalytic cleft is perpendicular to the plane of the figure. **(B)** The alignment of the α-domains of the enzymes listed above. For the *S. pneumoniae* hyaluronate lyase α-helices are shown as ribbons and are numbered as in Li et al., 2000. **(C)** Structural alignment of the *S. pneumoniae* hyaluronate lyase α-domain (the same orientation as in Figure 3b) with two most similar hydrolases: *C. thermocellum* endoglucanase CelA (pdb: 1cem) and *A. awamori* glucomylase (pdb: 1dog). The hydrolase enzymes are composed only of the α-helical domain.



C

β-sheets, that interacts with the catalytic domain. A comparison of both hyaluronate lyase structures revealed the likely allosteric character of both enzymes induced by substrate binding (Li and Jedrzejas, 2000). Although the chondroitin AC lyase structure does not reveal this allosteric character, it cannot be excluded at this time. For all three enzymes the α-helical and the β-sheet domains are connected by only one flexible linker peptide (Li et al., 2000), composed of several residues (Figures 2 and 3). Therefore, it is feasible that both domains have a significant degree of movement with respect to one another, which could account for at least two properties for these enzymes: (1) widening and narrowing of the catalytic cleft to accommodate the substrate binding prior to catalysis, (2) attenuation of activity by regulating access to the active site cleft via blocking its entrance.

A comparison of the SpnHL and SagHL suggests that the dimensions of the catalytic cleft can be altered. The clefts of these two enzymes differ primarily in their width, with the SgaHL cleft being wider (Figure 4). Although both domains of these enzymes are very similar in structure, the structure of the whole enzyme has less similarity due to the different positioning of the two domains relative to one another. Both enzymes, however, degrade the same substrate, hyaluronan, and they utilize the β-elimination process that produces disaccharides of HA as the final degradation product (Baker et al., 1997; Jedrzejas et al., 1998a and b; Li et al., 2000; Li and Jedrzejas, 2000). The exact mechanism at this time has been suggested only for SpnHL (Li et al., 2000) and for SagHL (Li and Jedrzejas, 2000). The proposed mechanism primarily involves three catalytic residues, His399, Asn349, and Tyr408 (pneumococcal hyaluronate lyase enzyme numbering scheme) (Figure 5). These three residues are conserved in sequence and in structure among SpnHL, SgaHL, and FheCACL (Figures 5 and 6). In alginate lyase the His399 residue is not present, the Asn349 residue is replaced by a histidine, and the remaining Tyr408 residue is conserved (Figure 5). The mechanism of catalysis of these lyases has been proposed based on the native structure of SpnHL and its complex with the disaccharide product and based on mutation studies of the enzyme. The mechanism has been termed proton acceptance and donation (PAD) (Figure 7) (Li et al., 2000; Li and Jedrzejas, 2000; Ponnuraj and Jedrzejas, 2000) and includes five proposed steps. First, the sub-

FIGURE 4. Structural alignment of the pneumococcal hyaluronate lyase with *S. agalactiae hyaluronate* lyase.The differences in the width of the catalytic cleft for both enzymes, suggesting an allosteric property, and the presence of an additional N-terminal β-sheet domain for the *S. agalactiae* enzyme are shown. The catalytic residues of both enzymes present in the cleft are highlighted. The pneumococcal enzyme and the *S. agalactiae* enzyme are shown.

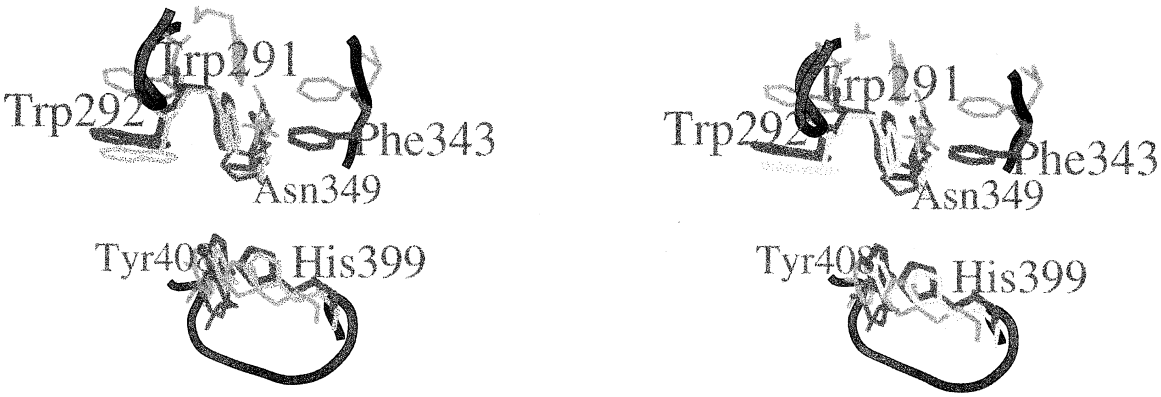
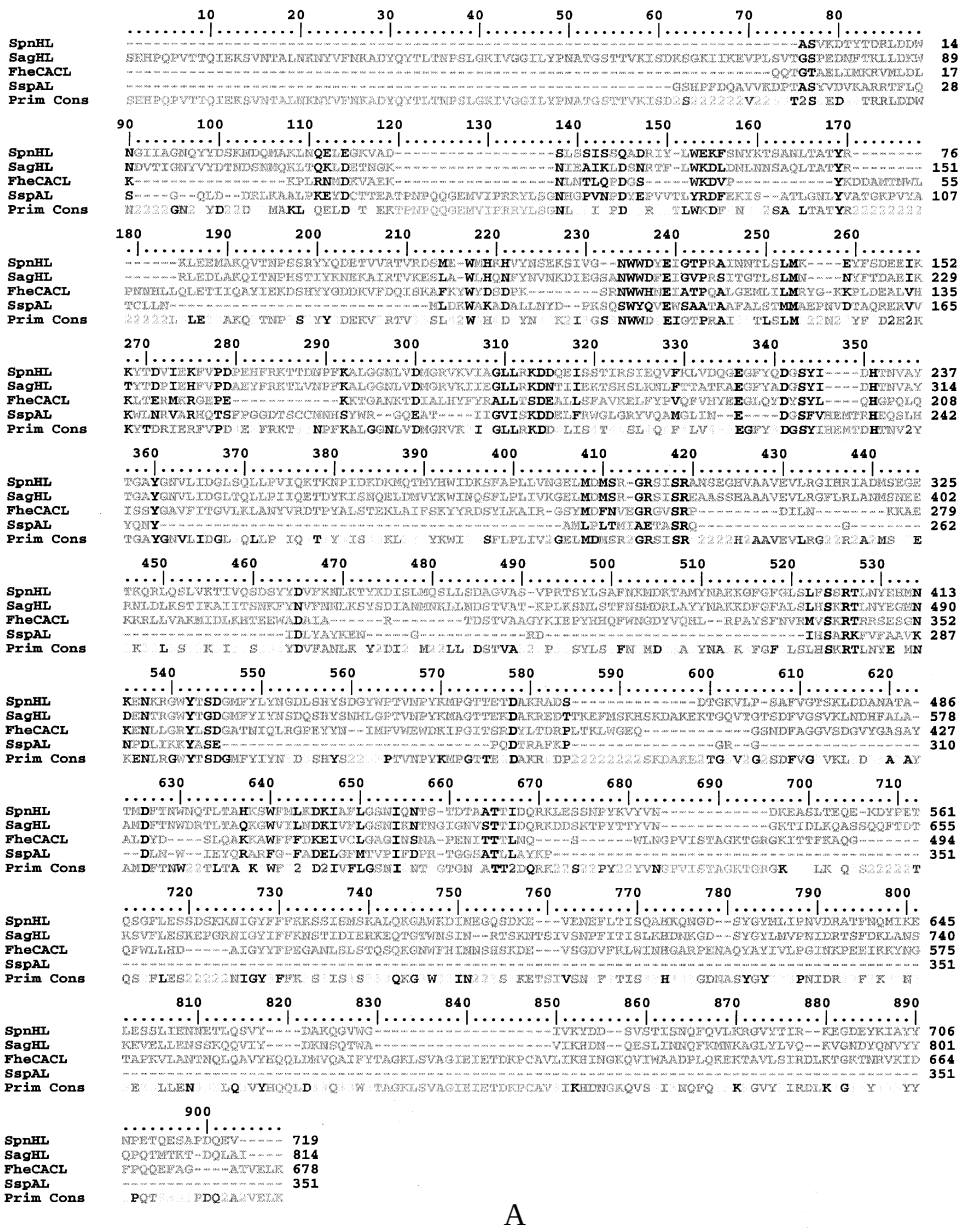


FIGURE 5. Structural alignment of the catalytic cleft residues of selected lyases. The catalytic residues Asn349, His399, and Tyr408 of *S. pneumoniae* and *S. agalactiae* hyaluronate lyases, *F. heparinum* chondroitin AC lyase (pdb: 1cb8), and *Sphingomonas* species alginate lyase A1-III (pdb: 1qaz) are shown. In addition, the aromatic patch composed of Trp291, Trp292, and Phe343 residues is indicated (SpnHL numbering scheme).



A

FIGURE 6. Sequence alignment of selected polysaccharide degrading enzymes. **(A)** Alignment of polysaccharide lyases utilizing β-elimination and a proton acceptance and donation mechanism to degrade substrates: pneumococcal (SpnHL) and *S. agalactiae* (SagHL) hyaluronate lyases, *F. heparinum* chondroitin AC lyase (FheCACL), *Sphingomonas* species alginate lyase A1-III (SspAL). The sequence data were edited and analyzed using the Multiple Protein Sequence Analysis (MPSA) program (Blanchet et al.,1999). The multiple sequence alignment with hierarchical clustering was performed to align multiple sequences with Multalin program version 5.3.2 (Corpet, 1988). Even though the structures are very similar, the overall sequence homology is only 10%. However, the catalytic residues His399 and Tyr408 are conserved for all four enzymes in this sequence alignment. The Ans349 residue is not conserved in the SspAL sequence, likely due to a slightly modified mechanism of action for this enzyme that might not require Asn349 or an equivalent residue. **(B)** Alignment of polysaccharide hydrolases utilizing a double displacement mechanism to degrade the substrates: *C. thermosellum* endoglucanase CelD (1clc) and CelA (1cem), *T. fusca* endo/exocellulase, and *A. awamori* glucomylase (1dog). The overall sequence homology is only 6%. Due to very low homology, the catalytic residues, such as Glu179 and Glu400 for the *A. awamori* glucomylase, are not conserved in the sequence alignment of these enzymes. Structural alignment of these enzymes is necessary to identify residues that are similar in function. (Blanchet et al., 1999).

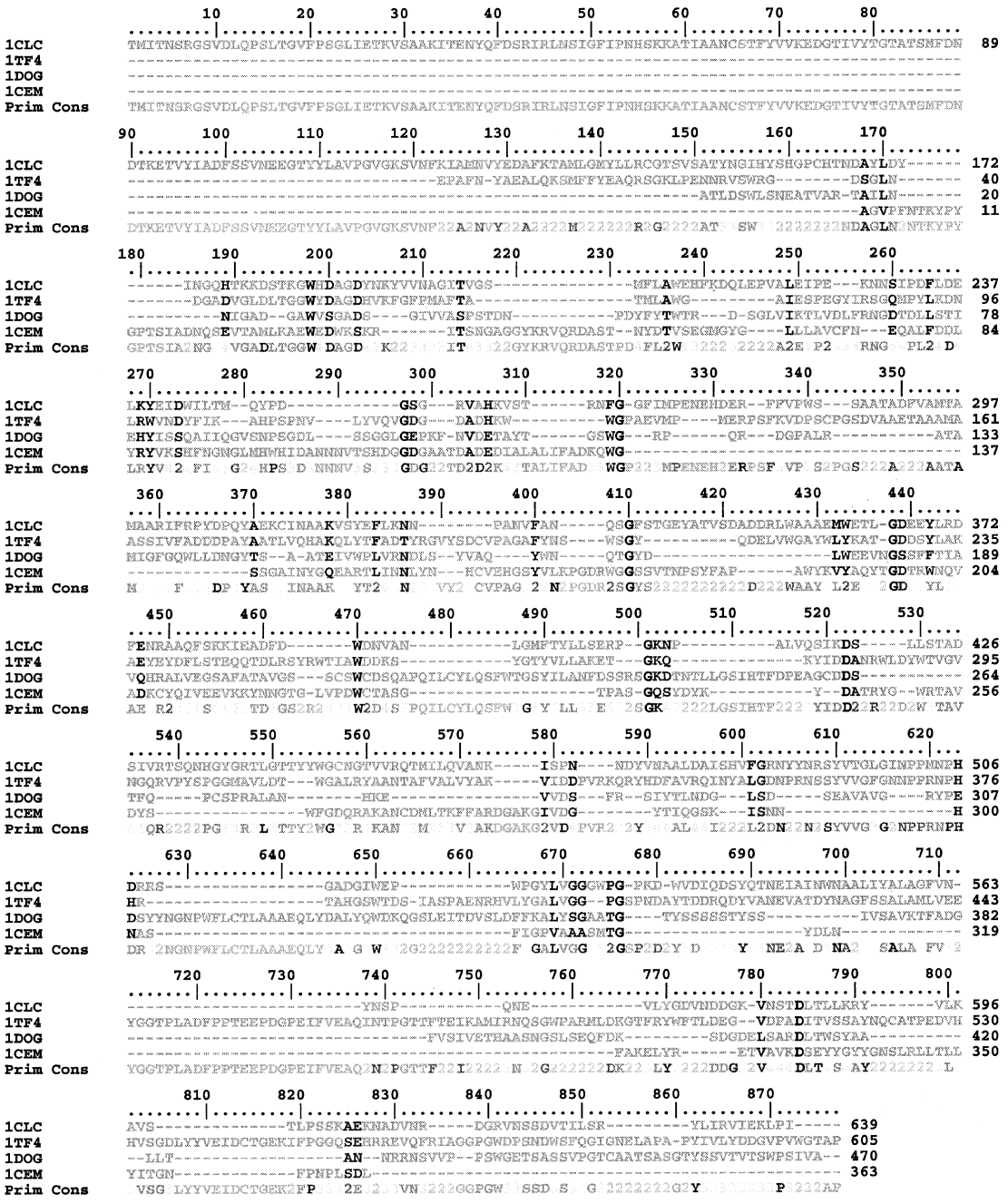


FIGURE 6B

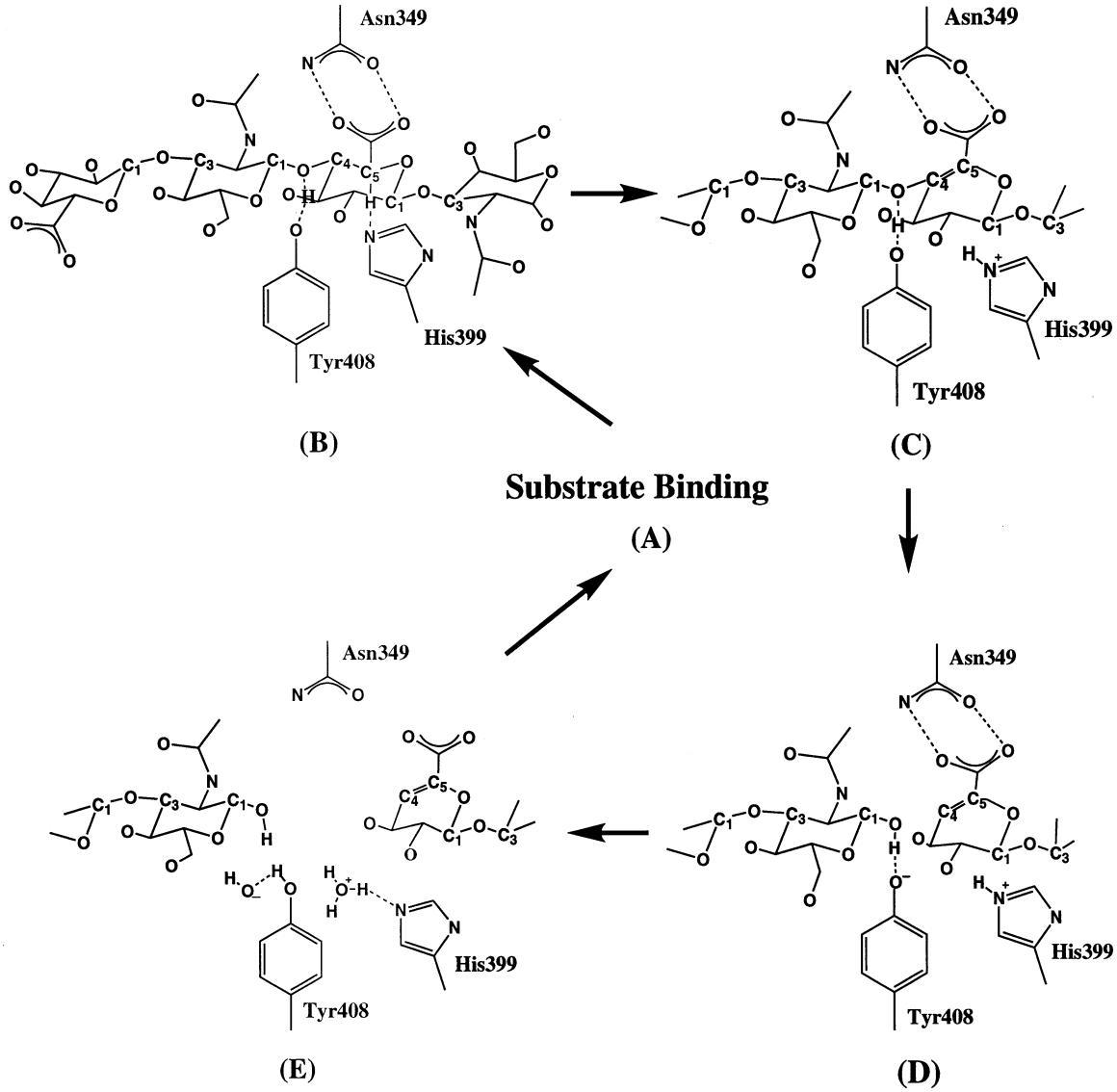


FIGURE 7. Proton acceptance and donation (PAD) mechanism. The mechanism is characteristic of polysaccharide lyases utilizing the β-elimination reaction to degrade polysaccharides. It consists of five catalytic steps A-E, as described in the text.

strate binds to the enzyme's catalytic cleft. Next, the carboxyl group of the glucuronic moiety of HA (Figures 1 and 7) on the C5 carbon atom is neutralized by Asn349. Then, the C5 proton is extracted by His399 to form a double bond between carbon atoms C4 and C5. Following this, the β1,4 glycosidic bond is broken after a proton is donated by Tyr408 (SpnHL numbering scheme). Finally, the cleaved disaccharide substrate exits the active site, while the catalytic residues His399 and Tyr408 balance the protons by their exchange with water. Following the exit of the product, the enzyme is then ready for the next step of catalysis. Catalytic residues His399 and Asn349 are situated at opposite sides of the cleft and the Tyr408 residue is located at the floor of the cleft (Figure 5). For the catalysis to happen these three residues must be a suitable distance from the substrate, as is the case for the SpnHL and FheCACL structures. In the SagHL structure, the residues equivalent to His399 and Asn349 (Figures

3a, 4, and 8) are separated by a distance too long for catalysis to occur. This observation supports the notion of domain movement to accomplish the catalysis. Alternatively, the opening to the active site cleft could become more narrow during catalysis, which likely occurs after substrate binding (Figure 4). This relative movement of the domains likely positions the catalytic residues in a proper arrangement for catalysis to occur, suggestive of an allosteric property.

As mentioned above, the β-domain of the enzyme might regulate access to the catalytic cleft at the edge of the α-domain simply by blocking the entrance to the cleft. However, the second possibility for functionality of the β-sheet domain is that the long, extended loops might change conformation due to events such as binding a Ca^{2+} activator or binding a substrate. For the hyaluronate lyases introduced above, Ca^{2+} ions are essential for activity (Jedrzejas et al., 1998a and b; Li et al., 2000). The precise mechanism of how Ca^{2+} influences the enzyme activity is not known at present, but the crystal structures suggest that it might involve regulation of substrate access to the cleft. This access to the cleft could be regulated by changing the conformation of loops, probably originating in the β-sheet domain. Once Ca^{2+} binds to the enzyme and interacts with loops, the loops are probably in an open conformation and allow for catalysis. However, when Ca^{2+} is not present, the conformation of the loops may change and block access to the cleft, preventing the substrate from binding and rendering the enzyme inactive. The structural and functional properties of the calcium binding sites of the *Clostridium thermocellum* endonuclease CelD (Figure 2) (Lamed et al., 1983), an enzyme-degrading cellulose that also has a barrel structure, were investigated by Chauvaux et al. (1995). Among three Ca^{2+} sites identified, one had low affinity and was close to the active site cleft. This study was consistent with the binding site stabilizing the active conformation of CelD. The other two Ca^{2+} binding sites have high affinity and seem to be required for Ca^{2+} binding to the low-affinity site. Even though no detailed structural changes upon Ca^{2+} binding to CelD were described, all sites were located in the loop areas of the enzyme and involved Ca^{2+} coordinating to the side chains of the enzyme and to ordered water molecules. It is likely that when Ca^{2+} is not present in these sites, the

```
                          349
SpnHL          341 NPFKALGGNLVDMGRVKVIAGLLRKDDQEISSTIRSIEQVFKLVDQGEGF 390
SagHL          421 NPFKALGGNLVDMGRVKIIEGLLRKDNTIIEKTSHSLKNLFTTATKAEGF 470
FheCACL        170    KKTGANKTDIALHYFYRALLTSDEALLSFAVKELFYPVQFVHYEEGLQYD 219
SspAL          188     CCNNHSYWRGQEATIIGVISKDDELFRWGLGRYVQAMGL 226

                             399      408
SpnHL          391 YQDGSYIDHTNVAYTGAYGNVLIDGLSQLLPVIQKTKNPIDKDKMQTMYH 440
SagHL          471 YADGSYIDHTNVAYTGAYGNVLIDGLTQLLPIIQETDYKISNQELDMVYK 226
FheCACL        220    YSYLQHGPQLQISSYGAVFITGVLKLANYV 249
SspAL          238         MTRHEQSLHYQNYAMLPLTMIAETA 261
```

FIGURE 8. Structure-based sequence alignment of catalytic residues of selected lyases. The sequences of *S. agalactiae* hyaluronate lyase (SagHL), *F. heparinum* chondroitin AC lyase (FheCACL), and *Sphingomonas* species alginate lyase A1-III (SspAL) were aligned against the *S. pneumoniae* hyaluronate lyase (SpnHL). The alignment is based on the three-dimensional structural alignment of these enzymes as shown in Figure 3a. The catalytic residues are highlighted and numbered using the SpnHL numbering scheme.

loop conformations change. Due to the close proximity of the low-affinity site to the active center, this conformational change might affect either substrate binding or the proper placement of active site residues for catalysis. For the endonuclease CelD the loops are part of the barrel-like α-helical domain. For other enzymes such as hyaluronate lyases or chondroitin AC lyase, it is likely that these loops originate from the neighboring β-sheet domain that is very close to the ridge of the catalytic cleft, located at the wider end of the barrel-like α-helical structure. Based on an analysis of these structures, the role of the β-sheet domain that has a four-layered sandwich fold seems to be the modulation of substrate access to the active site cleft via changing the conformation of elongated loops, likely induced by Ca^{2+} binding.

As discussed earlier, charge neutralization of the C5 atom glucuronate moiety of HA is very important for its degradation by SpnHL. Interactions of Ca^{2+} ions with the HA glucuronate group could also facilitate such a process and provide another possible explanation for the absolute calcium requirement for SpnHL activity. Structural investigations of plant pectate lyase and its complex with galacturonopyranose pentasaccharide (Yoder et al., 1993b, Scavetta et al., 1999), requiring calcium ions for activity, revealed that Ca^{2+} interacts with the carboxlate group of galacturonopyranose. Such Ca^{2+} interaction involves charge neutralization of the carboxylate-containing group. In addition, structural studies of hyaluronan showed divalent cations like Ca^{2+} acting as a bridge, linking carbohydrate groups of adjacent strands of HA. This observation suggests yet another possible role for calcium in catalysis. It is possible that calcium might bridge the hyaluronan substrate and SpnHL to facilitate optimal binding and/or optimal conformation of HA substrate for catalysis. More studies are needed to fully explain the precise role of Ca^{2+} in the degradation of polysaccharides.

Some of the polysaccharide-degrading enzymes with the α/α barrel topology have only an α-helical domain (Figures 2 and 3). An example of this is *Sphingomonas* species alginate lyase, whose crystal structure has been determined recently at high resolution (Figure 2) (Yoon et al., 1999). This enzyme has an α_6/α_5-barrel topology with a cap-like long loop extending over the catalytic cleft located at the wider opening of the barrel-like domain. As in the other enzymes discussed above, this cleft is built predominantly from loops between α-helices of the α_6/α_5 barrel.

On the other hand, the hyaluronate lyase enzymes seem to have a multidomain architecture. Starting from the C-terminus, the domains include the following: attachment domain to the peptidoglycan structures of the microorganism, the predominantly β-sheet domain likely involved in Ca^{2+} induced modulation of activity of the enzyme, the catalytic α_5/α_5 barrel domain with the catalytic cleft, and finally one or more domains at the amino-terminus that also seem to have a high β-sheet content (Li and Jedrzejas, 2000). The amino terminal domain(s) also are likely involved in the modulation of the substrate access to the active site cleft. Although there is no three-dimensional structural information about the amino terminal region, part of the N-terminal domain(s) were visible in the recent crystal structure of the truncated but active form of the *S. agalactiae* hyaluronate lyase (Figure 2) (Li and Jedrzejas, 2000). Part of this domain also has a β-sheet structure with β-strands arranged in a two-layered sandwich-like fold. This domain seems to extend itself toward another ridge of the active site cleft, as does the other b-sheet domain, and also may modulate access to the active site cleft (Figures 2 and 3). These two β-sheet domains, the C- and N-terminal do-

mains, flank the active site cleft on opposite sides. More studies are needed, however, to fully delineate the functional properties of the β-sheet domains. All domains are connected by single peptide linkers of various lengths located on only one side of the whole enzyme.

B. Barrel-Like Structure and Mechanism of Action of Polysaccharide-Degrading Enzymes

The elongated cleft that transverses the enzyme-degrading polysaccharides containing barrel-like structures is characteristic of all these enzymes. The cleft is always located at the wider opening of the barrel and contains a significant number of positively charged residues (Figures 5 and 9) (Li et al., 2000; Fethiere et al., 1999; Yoon et al., 1999). The catalytic activity has been assigned to the residues in this cleft at least for the SpnHL, SagHL, FheCACL, and SspAL enzymes (Figure 5). Unlike alginate lyase, the first three enzymes, SpnHL, SagHL, and FheCACL, have in addition to the barrel domain a β-sheet domain that likely modulates the substrate access to the catalytic cleft. The polysaccharide substrates are highly negative in charge (Laurent, 1970) and are likely to complement the predominantly positively charged cleft (Figure 9). The interactions of the substrates with the lyases are probably based in a large part on charge-charge interactions, including salt bridges (Yang et al., 1993 and 1994a and b). Such interactions are necessary not only for substrate binding but also for precise substrate positioning for catalysis. In addition, the polysaccharide substrates, like hyaluronan, have significant hydrophobic properties due to a localized accumulation of C-H carbohydrate and hydrophobic groups (Scott, 1992). These hydrophobic groups form hydrophobic patches on the surface of the polysaccharides (Scott and Heatley, 1999). The hydrophobic character of at least some polysaccharides was implicated in their ability to interact with lipids as well as with proteins, including the polysaccharide lyases (Park et al., 1997). Both hyaluronate lyases, SpnHL and SagHL, and the FheCACL enzyme have an aromatic/hydrophobic patch in the area of the active site center (Figure 4). This patch consists of three aromatic/hydrophobic residues, Trp291, Tro292, and Phe343 (SpnHL numbering scheme). The side chains of two of these residues, Trp292 and Phe343, are oriented parallel to one another and are also parallel to the cleft side wall exposed to interactions with substrate bound in the cleft. In the FheCACL enzyme the residue corresponding to Phe343 is not present, making the aromatic patch significantly smaller. The aromatic patch is also not present in the alginate lyase structure. Li et al. (2000) suggested the importance of the enzymes' aromatic patch in the precise positioning of the polysaccharide substrates for catalysis. In addition, it was suggested that this patch likely plays a role in selection of the size of the degradation product. In the SpnHL and SagHL enzymes this patch interacts exactly with only two sugar units of hyaluronan (Li et al., 2000; Baker et al., 1997). For both enzymes the smallest degradation product is a disaccharide unit of HA, which is comparable in surface area to the aromatic patch. The residues constituting the patch are located on one side of the cleft, whereas the catalytic residues Asn349, His399, and Tyr408 are all either at the bottom of the cleft or opposite to the aromatic patch. The extent of the aromatic patch or its lack thereof might be responsible for the end degradation products of other polysaccharide lyases such as *Streptomyces hyalurolyticus* hyaluronate lyase that produces tetra- and hexa- but not disaccharides (Li et al., 2000; Shimada and Matsumura, 1980). The SspAL enzyme, on the other hand, appears not to have any aro-

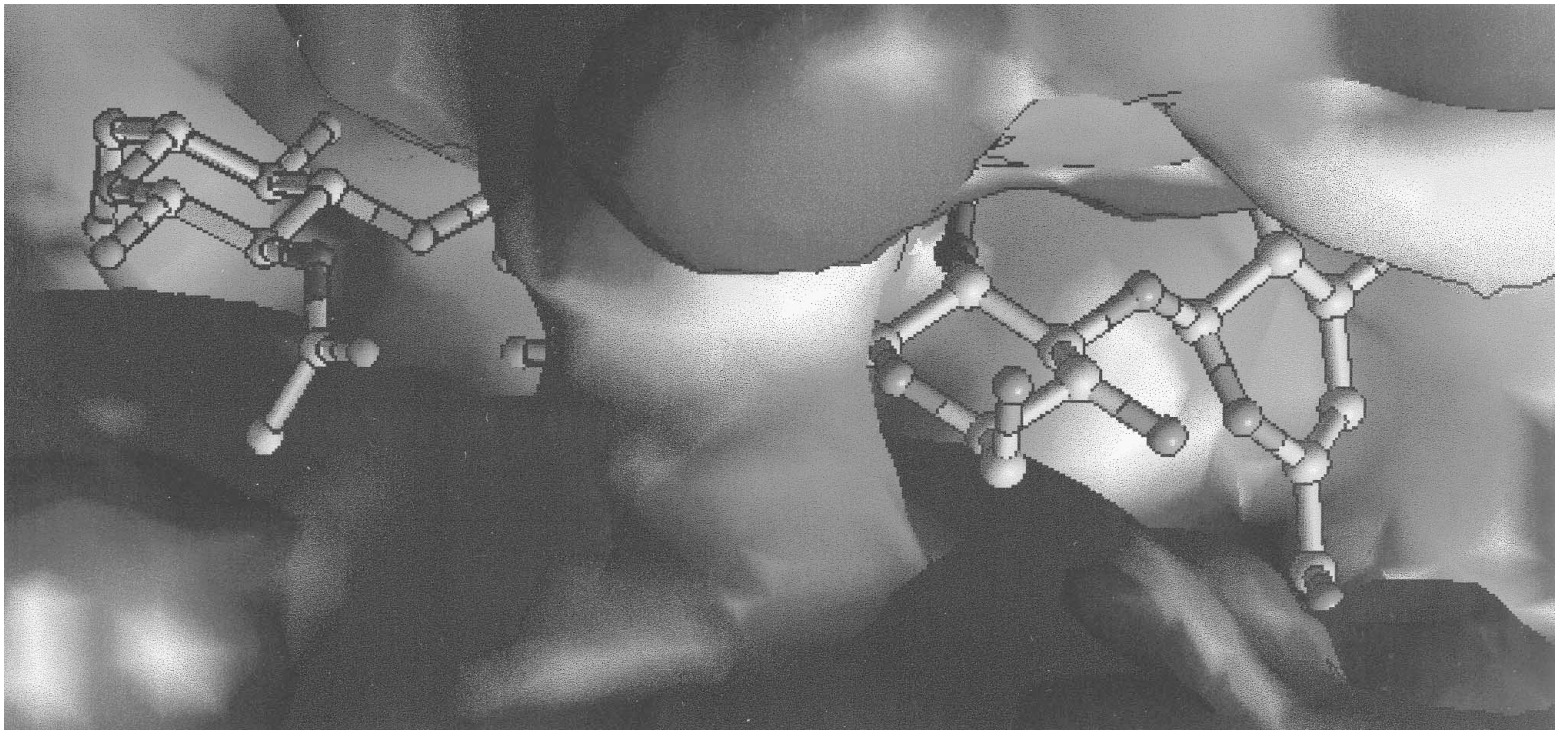


FIGURE 9. Surface of the catalytic cleft of *S. pneumoniae* hyaluronate lyase. The surface is color-coded by the magnitude of electrostatic potential. Two disaccharide units of HA product are also shown bound in the active site cleft. The catalytic residues (not shown) are in the central part of the figure in the region exactly between the two disaccharides.

matic patch, and it acts on alginate tetrasaccharides as the minimal substrate and produces di- and tri-saccharide units as end degradation products (Yonemoto et al., 1993; Murata et al., 1993).

C. Diversity of Structural Folds for Polysaccharide-Degrading Enzymes

Even though the majority of the polysaccharide-degrading enzymes have at least a domain with a barrel-like fold, some of the enzymes have different structural folds even though the catalytic cleft always seems to be present. An example is *Flavobacterium heparinum* chondroitinase B (FheCBL) degrading dermatan sulfate (Figure 10) (Huang et al., 1999). This chondroitinase B adopts a right-handed parallel β-helix fold. This fold is similar to folds present in other polysaccharide-degrading enzymes such as pectin lyases (Mayans et al., 1997; Vitali et al., 1998) and pectate lyases (Pickersgill et al., 1994; Yoder et al., 1993a and b, Scavetta et al., 1999). In these structures polysac-

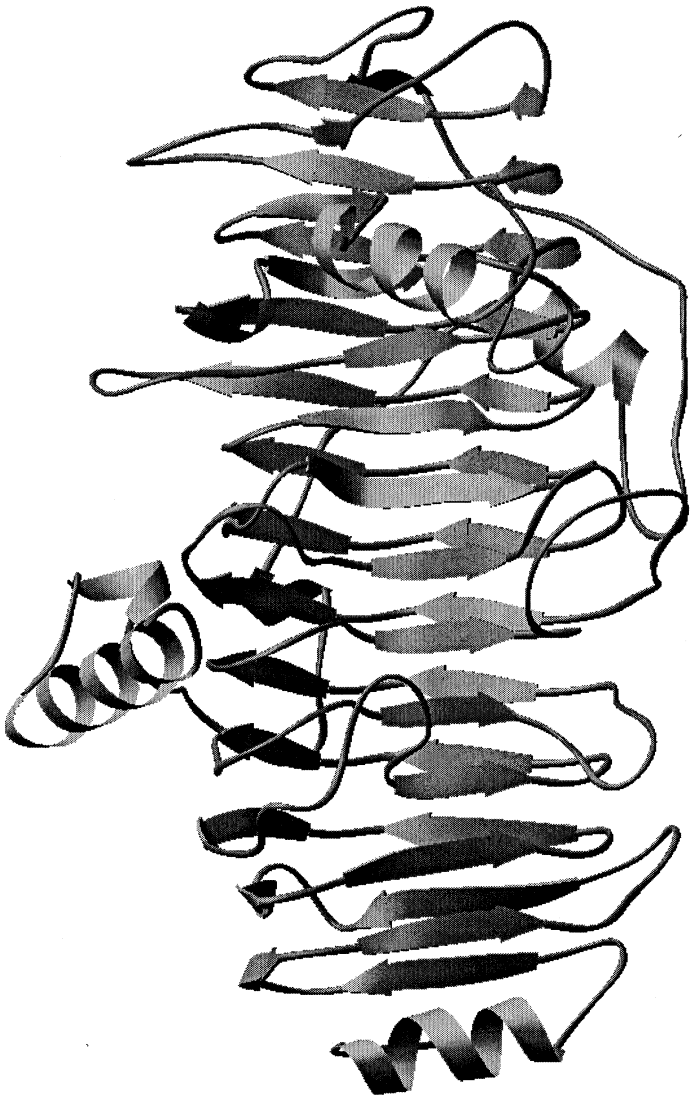


FIGURE 10. Structure of *F. heparinum* chondroitinase B. The enzyme adopts a right-handed parallel β-helix fold. A catalytic cleft spans vertically alongside of the central part of the enzyme (pdb: 1dbg).

charide binds in a cleft, as is the case for barrel fold lyase structures, but the cleft is built from loops extending from the surface of the β-helix structure. This cleft also has a predominantly electro-positive character, as is the case for clefts in barrel-like folds described earlier. The predominantly electronegative dermatan sulfate substrate binds in the cleft primarily based on electrostatic interactions (Huang et al., 1999). The major final degradation products of the enzyme are unsaturated di-saccharides obtained in the process of β-elimination by cleaving the β1,4 glycosidic bond of dermatan sulfate (Gu et al., 1995; Jandik et al., 1994; Michelacci and Dietrich, 1975).

The right-handed parallel β-helix fold has also been identified in several polysaccharide hydrolases such as rhamnogalacturonase A (Petersen et al., 1997), polygalacturonase A (Pickersgill et al., 1998), and the tailspike protein of phage P22 (polysaccharide hydrolase) (Steinbacher et al., 1994 and 1996). Such similarity implies that the cleft present in the right handed β-helix as well as in the barrel-like folded structures is not specific for the lyases (β-elimination process to break down polysaccharides) or hydrolases (hydrolysis of polysaccharides) but more likely for binding the elongated, negatively charged polysaccharide substrates. The mechanism responsible for the catalytic process to break down the substrates can be accomplished by the residues in the cleft. For the β-elimination reaction, these residues were clearly identified to be Asn349, His399, and Tyr408 for the SpnHL enzyme or similar, corresponding residues in other enzymes (Figures 5 and 7).

The precise mechanism for the hydrolysis type reaction is still under investigation, but it is suggested to involve a double-displacement mechanism at the C1 carbon atom of the polysaccharide substrates (Koshland, 1953), involving a covalent glycosyl-enzyme intermediate and a net retention of the anomeric configuration of the substrate (Figure 11) (Davies et al., 1998; Divne et al., 1998). For the process of creating the intermediate, an oxocarbenium ion-like transition state has been proposed (Sinnott, 1990). Inherently, two glutamic acid residues are involved in the process: Glu212 and Glu217 for the cellbiohydrolase I from *Trichoderma reesei* (Figure 12) (Divne et al., 1998), and Glu 139 and Glu228 for the *Bacillus agaradherans* family 5 endoglucanase (Figure 13) (Davies et al., 1998). The two hydrolase enzymes, cellbiohydrolase and endoglucanase, have different folds that allow for the formation of a long cleft capable of substrate binding. Cellbiohydrolase contains a large β-sandwich (Figure 12), whereas endoglucanase $(\beta/\alpha)_8$ barrel (Figure 13). In some enzymes the cleft may be covered to form a tunnel that is specific for cellbiohydrolases (Kleywegt et al., 1997; Rouvinen et al., 1990). Endoglucanases, however, have a more open active site (Spezio et al., 1993). Some hydrolytic enzymes that degrade polysaccharides instead use a direct-displacement mechanism where the leaving group of the substrate is replaced by water. In this case, the anomeric configuration of the C1 carbon is inverted (McCarter and Withers, 1994; Davies and Henrissat, 1995).

V. CONCLUSIONS AND GENERAL COMPARISON OF MECHANISMS OF POLYSACCHARIDE DEGRADATION USED BY LYASES AND HYDROLASES

The mechanism of all lyase enzymes that degrade polysaccharides is based on the β-elimination process. For at least some polysaccharide lyases, this process is based

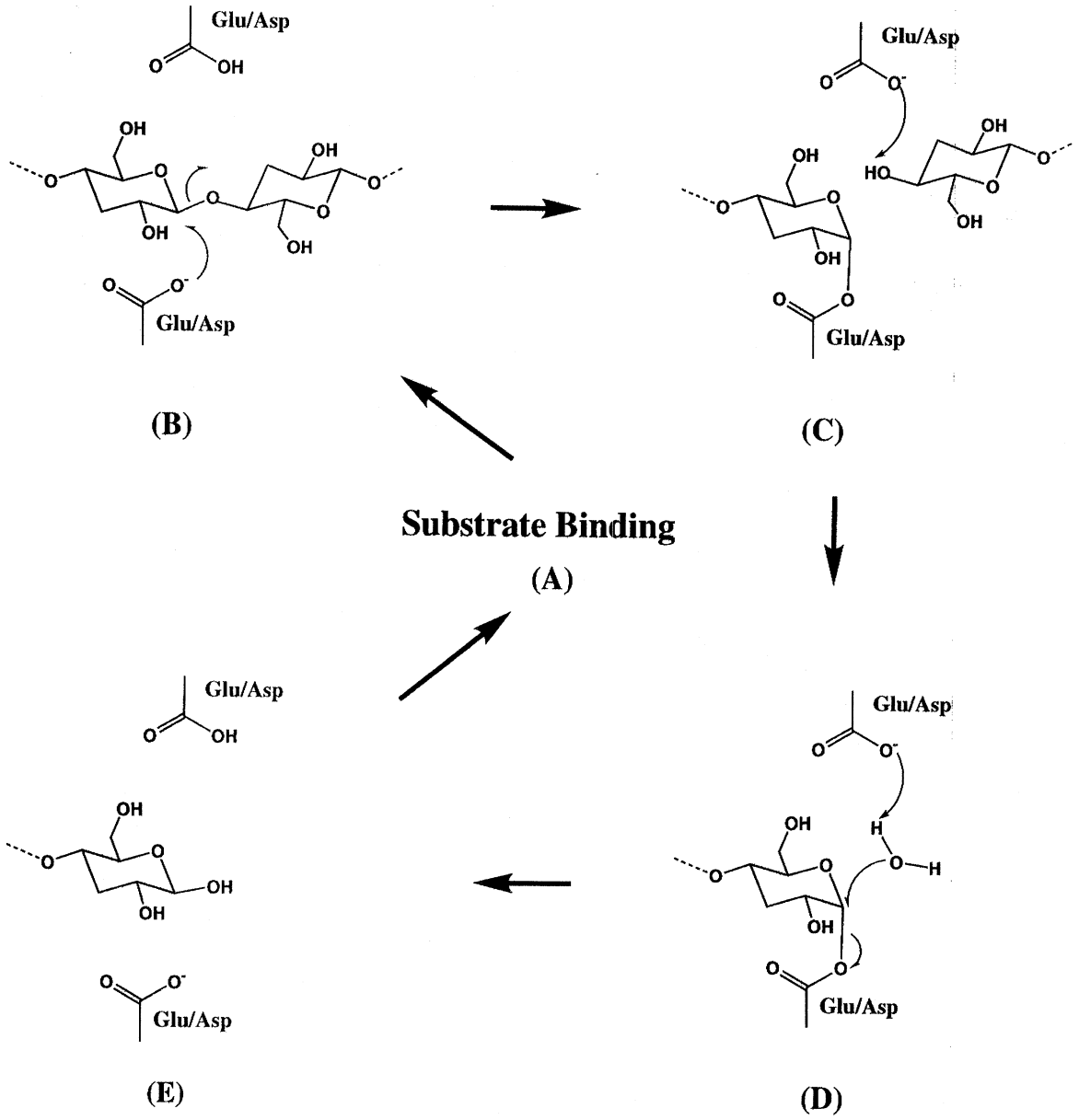


FIGURE 11. Double displacement (DD) mechanism. This mechanism is characteristic of polysaccharide hydrolases that degrade polysaccharides. The catalytic process proceeds through an intermediate reaction step that forms an enzyme-saccharide covalent bond.

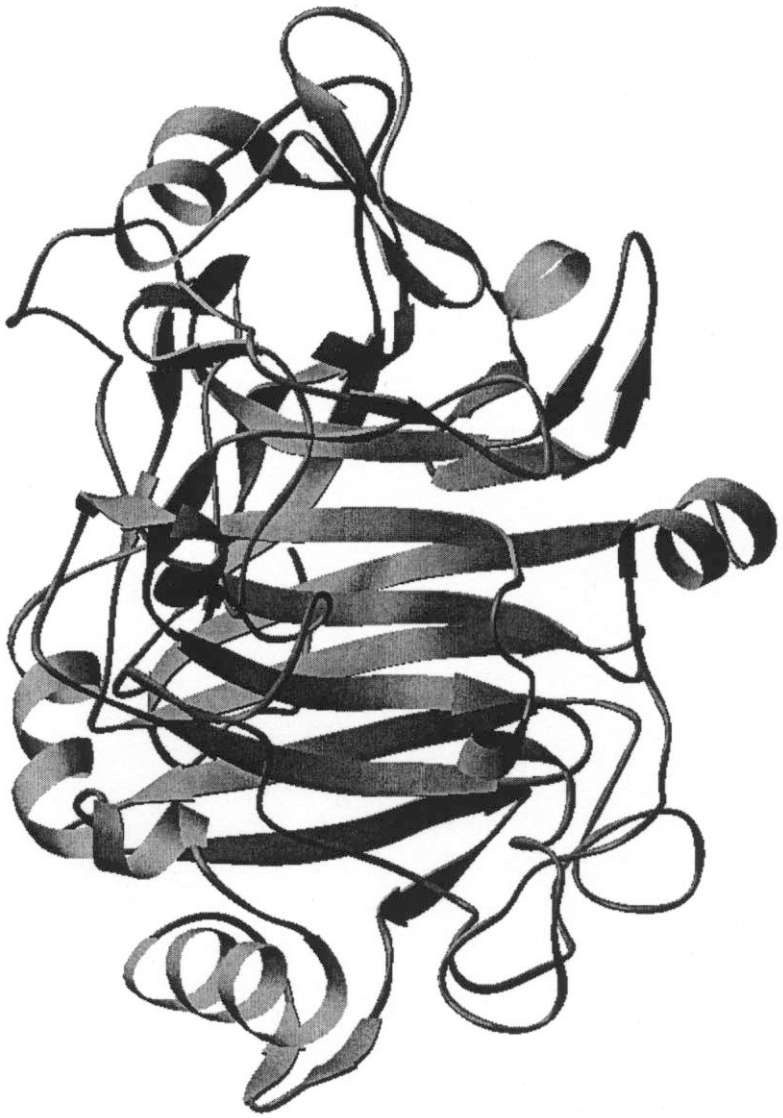


FIGURE 12. Structure of *T. reesei* cellbiohydrolase I. The enzyme assumes a large β-sandwich motif that facilitates the formation of a catalytic cleft oriented vertically on the side of the molecule facing the reader (pdb: 8cel).

FIGURE 13. Structure of *B. agaradherans* family 5 endoglucanase. The endoglucanase enzyme assumes yet another type of fold, a $(\beta/\alpha)_8$ fold. This fold is similar to the α/α barrel except that the inner helices are replaced by β-strands (pdb: 1a3h). The catalytic cleft transverses the molecule horizontally in the region facing the reader.

on the proton acceptance and donation (PAD) mechanism described by Li et al. (2000) (Figure 7). The catalysis through this mechanism involves several general steps: (1) the polysaccharide substrate binds in the cleft, (2) the C5 carbon atom is acidified, (3) a proton from the C5 carbon is extracted and an unsaturated bond is formed between C4 and C5 of the leaving polysaccharide group, (4) the glycosidic bond is broken after a proton is donated from the protein, and, finally (5) the product leaves the active site of the lyase and protons are balanced by exchange with the water environment (Li et al., 2000; Ponnuraj and Jedrzejas, 2000; Li and Jedrzejas, 2000). During the process the C5 carbon atom changes its hybridization from sp^3 to sp^2 with respective changes in the product conformation of the sugar ring (puckering of the sugar ring).

On the other hand, the hydrolysis of polysaccharides seems to follow different mechanisms, either a direct or a double displacement process (Figure 11). The double-displacement mechanism involves two glutamic acid or aspartic acid residues separated by ~6 Å. The steps involved in this mechanism are as follows: (1) binding of the polysaccharide substrate, (2) cleaving the glycosidic bond in the substrate and forming a covalently linked glycosyl-enzyme intermediate with the inversion of the anomeric C1 atom configuration (glycosylation), and (3) cleaving the enzyme-glycosyl bond involving a water molecule with the assistance of the deprotonated carboxylate residue leading to the second inversion of the configuration of C1 (deglycosylation). The proton balance of Glu/Asp residues for this reaction is also likely to proceed through an exchange with the water microenvironment. The anomeric configuration of the C1 carbon atom of the substrate is retained after the conformation is inverted twice during the catalysis. The formation of an oxocarbenium-ion transition state has been implicated in this process. The optimal distance between the two carboxylic acid residues involved in catalysis should be ~6 Å. The direct-displacement involves water displacing the leaving product with an inversion of the C1 atom anomeric configuration (McCarter and Withers, 1994; Davies and Henrissat, 1995).

The common feature of both lyases and hydrolases is the presence of an elongated cleft for the binding of the polysaccharide substrate. There are several distinct folds that can sustain the formation of such cleft, with the barrel fold and the right-handed β-helix being most predominant. Other folds have also been observed that can sustain the formation of a cleft from the loops created between structural elements such as helices and β-sheets.

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