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Enzymatic deconstruction of xylan for biofuel production

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Abstract

The combustion of fossil-derived fuels has a significant impact on atmospheric carbon dioxide (CO₂) levels and correspondingly is an important contributor to anthropogenic global climate change. Plants have evolved photosynthetic mechanisms in which solar energy is used to fix CO₂ into carbohydrates. Thus, combustion of biofuels, derived from plant biomass, can be considered a potentially carbon neutral process. One of the major limitations for efficient conversion of plant biomass to biofuels is the recalcitrant nature of the plant cell wall, which is composed mostly of lignocellulosic materials (lignin, cellulose, and hemicellulose). The heteropolymer xylan represents the most abundant hemicellulosic polysaccharide and is composed primarily of xylose, arabinose, and glucuronic acid. Microbes have evolved a plethora of enzymatic strategies for hydrolyzing xylan into its constituent sugars for subsequent fermentation to biofuels. Therefore, microorganisms are considered an important source of biocatalysts in the emerging biofuel industry. To produce an optimized enzymatic cocktail for xylan deconstruction, it will be valuable to gain insight at the molecular level of the chemical linkages and the mechanisms by which these enzymes recognize their substrates and catalyze their reactions. Recent advances in genomics, proteomics, and structural biology have revolutionized our understanding of the microbial xylanolytic enzymes. This review focuses on current understanding of the molecular basis for substrate specificity and catalysis by enzymes involved in xylan deconstruction.

Keywords

acetyl xylan esterase; arabinofuranosidase; biofuels; ferulic acid esterase; glucuronidase; xylan; xylanase; xylosidase

Introduction

Recent concerns in regards to global climate change have led to an emphasis on decreasing fossil fuel as liquid fuel for transportation and a shift to renewable liquid fuels, such as cellulosic ethanol. While the term ‘cellulosic ethanol’ creates the impression that such biofuels will be derived from cellulose, it is important to note that the term indeed alludes to ethanol originating from both cellulose and hemicellulose as substrates. Thus, it is perhaps more informative to use the expression biomass derived fuels or, in short, the very appropriate term ‘biofuels’. Corn residues and sugarcane bagasse are the current foci as potential sources of cellulosic biofuels, but future sources are likely to include the perennial grasses, Switchgrass (McLaughlin & Kszos, 2005; Parrish & Fike, 2005), and Miscanthus

(*Miscanthus × giganteus*) due to their high yield, minimal requirement for nutrient and water input, and the fact that they can be grown in locations which would not compete with current food crops (Heaton *et al.*, 2008a, b). The degradable components, mostly cellulose and hemicellulose, derived from these perennial grasses are anticipated to become significant substrates in the future for bioconversion to ethanol or other higher molecular weight alcohols and hydrocarbons (Somerville, 2007). The composition of dry plant biomass harvested from these perennial grasses consists primarily of the plant cell wall polymers, cellulose (31%), xylan (20%), and lignin (18%) (Fig. 1). Cellulose is a highly homogeneous polymer that consists of β -1,4-linked glucose units, which make interstrand hydrogen bonds to form a highly stable crystalline lattice (Laureano-Perez *et al.*, 2005). Xylan, on the other hand, is a heteropolymeric hemicellulosic polymer that consists predominantly of the pentose sugars arabinose and xylose, although it may also contain glucuronyl, feruloyl, and acetyl groups (Ebringerova & Heinze, 2000). Complete hydrolysis of the two major components, cellulose and xylan, of perennial grasses, therefore, releases glucose, xylose, and arabinose, which can then be fermented or bioconverted to biofuels. The third major component, lignin, is a phenolic polymer that associates with plant cell wall polysaccharides, mainly through hydroxycinnamic acids, such as *p*-coumaric acid and ferulic acid in grasses and other graminaceous plants (Sun *et al.*, 2001). The two hydroxycinnamic acids are mostly found as engaged in ester linkages to arabinose units in xylan and ether linkages to hydroxyl groups in lignin (Jeffries, 1990). The cross linking of plant cell wall polysaccharides and lignin through ferulic acid and *p*-coumaric acid is known to negatively impact hydrolysis to monomeric sugars and ultimately biodegradation. Delignification of biomass is, therefore, a critical step in bioconversion of biomass to biofuels.

Current strategies to delignify feedstock materials, including mild acid treatment, often lead to the production of a number of inhibitors of fermentation by ethanologenic yeast. These inhibitors include furfural and 5-hydroxymethylfurfural, which are produced by the dehydration of pentose and hexose sugars, respectively (Dunlop, 1948; Antal *et al.*, 1990; Larsson *et al.*, 1999). Significant improvements in plant cell wall hydrolysis and fermentation can be achieved if treatment methods that reduce toxic compounds and increase conversion of plant cell wall polysaccharides to monosaccharides are developed. Having evolved over millions of years for the purpose of extracting nutrients from plant cell walls, microbial enzymes represent an important alternative to the current treatment methods. More importantly, their products of hydrolysis are unlikely to exhibit the reported inhibitory effects.

The emphasis accorded to deconstruction of cellulose is due to the use of its component sugar, glucose, as the primary substrate for microorganisms, such as the yeast *Saccharomyces cerevisiae* and the bacterium *Zymomonas mobilis*, in the industrial production of ethanol. However, both microorganisms are incapable of fermenting the pentose sugars xylose and arabinose, commonly found in hemicellulose. There is, therefore, great interest in naturally-occurring or engineered organisms that can ferment the monosaccharide components of hemicellulose the second most abundant polysaccharide, to biofuels (Jeffries & Jin, 2000; Agbogbo & Coward-Kelly, 2008).

Xylan represents the most abundant hemicellulose (Saha, 2003). Therefore, its deconstruction to the constituent sugars, mainly xylose and arabinose, (Fig. 2) for subsequent fermentation by ethanol producing microbes is critical to the efficient use of plant biomass for biofuel production. About two decades ago, Wong *et al.* (1988) published a review that remains a classic introduction to the biochemistry of xylan deconstruction. Since then, there have been other reviews mainly focused on plant cell wall degradation (Shallom & Shoham, 2003; Cann *et al.*, 2007; Gilbert *et al.*, 2008), including an article that

addresses the structure of plant cell wall and its deconstruction through both chemical and enzymatic treatments (Saha, 2003). Unlike cellulose depolymerization, the optimization of which has received much recent attention (Percival Zhang *et al.*, 2006; Bayer *et al.*, 2007; Himmel *et al.*, 2007), increased interest in the use of xylan as feedstock in industrial processes has not occurred with a commensurate increase in review articles addressing, in detail, xylan deconstruction by microbial enzymes. Furthermore, it is anticipated that the current effort in the emerging biofuel industry will emphasize a better understanding of xylan deconstruction, in order to increase the overall efficiency of biomass conversion to biofuels. Thus, in this review, we will concentrate our efforts on the enzymatic depolymerization of xylan to its component monosaccharides.

Xylan is a heteropolymeric substrate consisting of a repeating β -1,4-linked xylose backbone decorated with acetyl, arabinofuranosyl, and 4-*O*-methyl glucuronyl groups and as stated above, xylan may be cross linked to lignin by aromatic esters. In order to efficiently depolymerize xylan to the component monosaccharides, a mixture of different enzymatic functionalities are required, including endo-1,4- β -xylanases (EC 3.2.1.8), β -D-xylosidases (EC 3.2.1.37), α -L-arabinofuranosidases (AFs) (EC 3.2.1.55), α -glucuronidases (EC 3.2.1.139), acetyl xylan esterases (EC 3.1.1.72), and ferulic/coumaric acid esterases (EC 3.1.1.73).

Much is known about the individual enzymatic functionalities required to depolymerize xylan and hundreds, perhaps thousands, of xylanolytic enzymes from a variety of microbial sources have been identified (CAZy; <http://www.cazy.org/>). A significant limitation to optimizing xylan depolymerization is the lack of detailed knowledge of both the structural diversity in xylan and the corresponding enzymatic strategies employed by microbes to hydrolyze the linkages within this complex heteropolymer. However, current efforts in microbial and structural genomics are gradually leading to a better understanding of the structural basis for substrate specificity of a number of xylanolytic enzymes belonging to different glycoside hydrolase (GH) families. The subject of the current review is to summarize these recent insights and to discuss their potential application in improving biocatalytic saccharification of xylan for biofuel production.

Structure and prevalence of xylans

Xylans are polysaccharides consisting of a backbone of β -1,4-linked xylopyranosyl groups that are further decorated with different side chain residues; the proportions of which vary based on the source of the plant tissue. Xylans can be broadly classified as homoxylans, arabinoxylans, glucuronoxylans, and arabinoglucuronoxylans. Homoxylans, which consist of a chain of β -1,4- and β -1,3-linked xylose units are relatively rare in higher plants, but represent a significant structural component of cell walls in red seaweeds (Painter, 1983). All of the xylans of higher plants are based on a backbone of β -1,4-linked xylopyranose sugars and are typically substituted with acetyl groups and other sugar residues (Fig. 2). Arabinoxylans are principal components of the plant cell walls, especially in cereal grains including wheat, and consist of a xylose backbone with arabinose residues linked to the *O*-2 or *O*-3 of xylose. Xylose residues may be singly or doubly substituted with arabinose, and the arabinose residues may have additional linkages to the phenolic compound ferulic acid which may form covalent crosslinks to lignin or ferulic acid groups in other arabinoxylan chains. Glucuronoxylans are mainly found in hardwoods, herbs, and woody plants (Timell, 1964; Ebringerova & Heinze, 2000) and typically consist of a xylose backbone with 4-*O*-methyl- α -D-glucuronic acid (MeGA) residues linked off of the *O*-2 of xylose. Arabinoglucuronoxylans are typically found in the lignocellulose isolated from grasses and have arabinofuranosyl, MeGA, and acetyl side chains linked to the xylose backbone and reflect the composition of biomass currently targeted for biofuel production. While the relative proportions of plant cell wall polysaccharides within biofuel feedstocks are

generally known, the dearth of knowledge on how individual sugar components are chemically linked within the cell wall represents an important limitation to designing enzyme cocktails for efficient hydrolysis of these polysaccharides.

Enzymatic activities required for xylan deconstruction

The structural diversity of plant cell wall xylans necessitates an equal diversity in the repertoire of enzymes for deconstruction of these polysaccharides. Thus, xylan-degrading bacteria and fungi have evolved diverse enzymatic machineries for the hydrolysis of xylan, and evidence is readily gleaned from the genome sequences of such organisms (<http://www.ncbi.nih.gov/genomes/lproks.cgi>). Detailed structural and biochemical studies of the substrate specificities for these enzymes are beginning to provide important insights into the function and evolution of these proteins.

Plant cell wall polysaccharides are highly stable polymers composed of repeating sugar units whose glycosidic linkages exhibit half lives at room temperature on the order of 20 million years (Wolfenden *et al.*, 1998). Organisms across the three domains of life (eukaryotes, archaea, and bacteria) have evolved GHs, which are extraordinarily efficient catalysts that enhance the rate of hydrolysis of glycosidic linkages over the noncatalyzed reaction by up to 17 orders of magnitude (Wolfenden *et al.*, 1998). Xylan degrading GHs hydrolyze glycosidic linkages by inversion or retention of stereo-chemical configuration at the anomeric carbon. These two different mechanisms both employ a pair of carboxylic acid residues in the active site. Retaining glycosidases employ a double displacement mechanism involving two consecutive bimolecular substitution (S_N2) reactions. The first substitution is carried out by a catalytic carboxylic acid nucleophile which results in the formation of a covalent enzyme–substrate intermediate (Vocadlo *et al.*, 2001), and the second is performed by an activated water molecule (Fig. 3a). Inverting glycosidases, on the other hand, employ a single displacement reaction wherein a carboxylic acid group acts as a base to activate a water molecule for attack at the anomeric carbon (Fig. 3b). Henceforth, we describe the enzymes required for xylan hydrolysis into its component sugars for subsequent fermentation to biofuels.

The endo-1,4- β -xylanases

One of the critically important enzymatic activities required for the depolymerization of xylans is endo-1,4- β -xylanase (xylanase) activity. These enzymes cleave the β -1,4 glycosidic linkage between xylose residues in the backbone of xylans. Xylanases have been classified into GH families 5, 7, 8, 10, 11, and 43 on the basis of their amino acid sequences, structural folds, and mechanisms for catalysis (Cantarel *et al.*, 2009). Previous reviews have discussed the biochemical and mechanistic properties of these enzymes in great detail (Wong *et al.*, 1988; Collins *et al.*, 2005; Berrin & Juge, 2008). The binding sites for xylose residues in xylanases are termed subsites with bond cleavage occurring between sugar residues at the -1 subsite (nonreducing end) and the $+1$ subsite (reducing end) of the polysaccharide substrate (Fig. 4) (Davies *et al.*, 1997). GH 10 and 11 xylanases represent the best studied xylanase families and they differ in the number of subsites they possess, with GH 10 having four or five subsites (Biely *et al.*, 1981, 1997b; Derewenda *et al.*, 1994) and GH 11 having at least seven subsites (Vrsanska *et al.*, 1982; Bray & Clarke, 1992). While commercially available nondecorated xylo-oligosaccharide substrates are convenient for assessing xylanase activity, limited information can be drawn from studies with these substrates since they do not represent the natural xylan substrates that these enzymes would encounter when deconstructing plant cell walls. A recent study assessed the activity of several GH 10 and 11 proteins with purified xylo-oligosaccharides substituted with MeGA and revealed that GH 10 enzymes cleave xylan chains when MeGA is linked to xylose at the $+1$ subsite, whereas GH 11 enzymes cleave xylan when MeGA is appended at the $+2$ subsite

(Fig. 5) (Kolenova *et al.*, 2006). Direct support for these results was reported in a recent study on the mass spectra of the products of hydrolysis for GH 10 and 11 with arabinoxylan substrates. The results indicated that GH 10 products have arabinose residues substituted on xylose at the +1 subsite, whereas GH 11 products have arabinose residues substituted at the +2 subsite (Maslen *et al.*, 2007). These results suggest that GH 10 enzymes are able to hydrolyze xylose linkages closer to side chain residues and thus help to explain why GH 10 enzymes release shorter products than GH 11 enzymes when incubated with arabinoglucuronoxylan substrates (Biely *et al.*, 1997b).

Recent structural and other biochemical studies support the data above and indicate that while GH 10 and 11 enzymes are both able to bind decorated xylo-oligosaccharides (Fujimoto *et al.*, 2004; Pell *et al.*, 2004b; Vardakou *et al.*, 2005, 2008), GH 10 enzymes can accommodate linkages on xylose at the +1 subsite (Fig. 6a) (Kaneko *et al.*, 2004; Pell *et al.*, 2004a), whereas GH 11 enzymes can accommodate linkages on xylose at the +2 subsite (Fig. 6b) (Vardakou *et al.*, 2008). Furthermore, the structure for a GH 10 enzyme complexed with feruloyl arabinoxylodisaccharide (FAX₂) revealed that the arabinose substituent appended at the +1 subsite was a significant determinant of substrate specificity for a GH 10 xylanase from the thermophilic fungus, *Thermoascus aurantiacus* (Vardakou *et al.*, 2005). Taken together these results support the idea that xylan side chain decorations are recognized by xylanases, and the degree of substitution in xylan will influence the products of hydrolysis for xylanases. This difference in substrate specificity for xylanases has important implications in the deconstruction of xylan. For example, α -glucuronidases can only release MeGA from a terminal nonreducing end xylose unit, thus the products of a GH 10 enzyme acting on glucuronoxylan will be a direct substrate for α -glucuronidase (Fig. 5a), whereas α -glucuronidases will be unable to hydrolyze GH 11 products since the MeGA will be substituted off of the penultimate xylose residue (Fig. 5b).

The foregoing discussions, therefore, underscore the importance of understanding the natural substrate specificities for xylanolytic enzymes and indicate that comprehensive knowledge of the structure of the xylan substrate will be indispensable for designing optimized enzyme cocktails.

The AFs

The function of AF in xylan deconstruction is to remove arabinose side chains from the xylose backbone of arabinoglucuronoxylan. These enzymes are grouped into four different families of (GH 43, 51, 54, and 62), and can hydrolyze glycosidic linkages with net inversion (GH 43) or retention (GH 51, 54) of stereochemical configuration at the anomeric carbon. In the context of arabinoglucuronoxylans, there is diversity in the types of linkages for arabinosyl units, and this has significant consequences in terms of the type of enzyme that removes arabinose side chains. Specifically, arabinose may be linked to the O-2 or O-3 of xylose in the xylan backbone, and these xylose sugars may be either singly or doubly substituted with arabinose. Furthermore, some arabinose molecules may have ferulic acid molecules esterified to the 5'-OH which may subsequently be covalently tethered to ferulic acid residues attached to other arabinoxylan chains (Fig. 2).

Members of the GH family 43 AF's display a variety of different substrate specificities that is beginning to be understood in greater molecular detail as more three dimensional (3D) structures are solved for these enzymes. Enzymes with AF activity may be grouped into three major classes: (a) AF type A enzymes that are only active on short arabinooligosaccharides and *p*NP- α -L-arabinofuranoside (Pitson *et al.*, 1996), (b) AF type B enzymes that are active on a wider variety of substrates including short oligosaccharides and longer polysaccharides such as arabinoxylan and arabinan (Pitson *et al.*, 1996), and (c) arabinoxylan arabinofuranohydrolases (AXHs) which are mainly active on arabinoxylan

(Kormelink *et al.*, 1991a, b, Bourgois *et al.*, 2007). The AXHs can further be classified based on whether they release α -L-arabinose from only mono-substituted xylose residues (AXH-m) (Kellett *et al.*, 1990; Kormelink *et al.*, 1991b, Bourgois *et al.*, 2007) or only disubstituted xylose residues (AXH-d) (Van Laere *et al.*, 1997, 1999; van den Broek *et al.*, 2005; Sorensen *et al.*, 2006).

Recently, crystal structures have been reported for a GH 43 AF type A β -xylosidase/ α -L-arabinofuranosidase from the rumen bacterium, *Selenomonas ruminantium* (Brunzelle *et al.*, 2008) and a GH 43 AXH-m from the soil bacterium, *Bacillus subtilis* (Vandermarliere *et al.*, 2009). These enzymes have distinct substrate preferences with the *S. ruminantium* enzyme (SXA) having high activity on *p*NP- β -D-xylopyranoside, *p*NP- α -L-arabinofuranoside (Whitehead & Cotta, 2001) and xylo-oligosaccharides (Jordan *et al.*, 2007), whereas the *B. subtilis* enzyme (BsAXH-m2,3) exhibited highest activity on *p*NP- α -L-arabinofuranoside and water extractable arabinoxylans (Bourgois *et al.*, 2007). The domain architectures for these two enzymes, revealed by the 3D structures, provide significant insight into the differences in the substrate specificities for these two enzymes. Although they both possess an N-terminal five-bladed β -propeller fold common to GH 43 enzymes, BsAXH-m 2,3 has a C-terminal CBM family 6 (CBM6)-like domain that is clear of the active site region (Fig. 7a), whereas SXA has a large C-terminal β -sandwich domain which projects a loop that closes off the active site pocket for the enzyme (Fig. 7b). For BsAXH-m2,3, a groove in the protein is located just above the active site that binds to xylo-oligosaccharides and allows for arabinose side chains substituted from either the *O*-2 or *O*-3 of xylose to enter the active site for hydrolysis. The SXA on the other hand does not possess this groove, and the β -sandwich loop restricts the size of the active site such that only one to two residues may enter the active site, resulting in an exo-type enzyme activity.

The xylan-1,4- β -xylosidases

Xylan-1,4- β -xylosidase (β -xylosidase) enzymes release xylose monomers from the nonreducing end of xylo-oligosaccharides. As described earlier, xylanases break down xylan polymers into shorter fragments, thereby increasing the total number of nonreducing ends available for hydrolysis by β -xylosidases. β -xylosidases are grouped into five different families (GH 3, 39, 43, 52, and 54) (Henrissat, 1991) and their reaction mechanisms result either in inversion (GH 43) or retention (GH 3, 39, 52, and 54) of stereochemical configuration at the anomeric carbon. The most abundant and best characterized β -xylosidases are from GH 3 and 43 (Sunna & Antranikian, 1997).

The crystal structures for two biochemically characterized GH 43 β -xylosidase enzymes have revealed the presence of two domains, an N-terminal five-bladed β -propeller domain, and a C-terminal α/β sandwich domain (Brux *et al.*, 2006; Brunzelle *et al.*, 2008). The catalytic residues are located in a shallow active site cleft in the center of the β -propeller domain which is closed off by a phenylalanine residue contributed from the C-terminal α/β sandwich domain. These enzymes possess two subsites for sugar binding and it is anticipated that when hydrolyzing oligo-saccharides longer than two sugar residues, the remaining residues will extend out into solution (Fig. 8). This prediction is supported by biochemical analyses of GH 43 β -xylosidases that reveal a decrease in the catalytic efficiency (k_{cat}/K_M) when active on xylo-oligosaccharides with a degree of polymerization greater than two (Van Doorslaer *et al.*, 1985; Jordan *et al.*, 2007; Wagschal *et al.*, 2009), suggesting that these enzymes possess just two xylose binding sites.

The GH 3 represents a large group of glycosidic enzymes and includes members that possess several distinct enzymatic activities including β -D-glucosidase, β -D-xylosidase, AF and *N*-acetyl- β -D-glucosaminidase (NagZ) activities (Harvey *et al.*, 2000; Faure, 2002). Despite the large number of β -xylosidase enzymes that have been characterized from this

gene family, there are currently no crystal structures available for enzymes with β -xylosidase activity from this family. There are two crystal structures for GH 3 enzymes, one for a putative NagZ from the bacterium *Vibrio cholera* (unpublished, protein databank accession no. 1y65), and a variety of structures with different substrate analogs and inhibitors for a β -D-glucosidase from *Hordeum vulgare* (Barley) (Varghese *et al.*, 1999; Hrmova *et al.*, 2001, 2002, 2005). In the case of the NagZ structure from *V. cholera*, a single N-terminal $(\alpha/\beta)_8$ triosephosphate isomerase (TIM) barrel domain exists (Fig. 9a), whereas analysis of the crystal structure for the *H. vulgare* β -D-glucosidase revealed the presence of an N-terminal $(\alpha/\beta)_8$ TIM barrel and an additional C-terminal $(\alpha/\beta)_6$ sandwich domain (Fig. 9b). Most of the catalytic residues for these enzymes map to the N-terminal $(\alpha/\beta)_8$ TIM barrel domain, however, for the Barley enzyme, a putative catalytic acid/base residue was located in the C-terminal domain (Varghese *et al.*, 1999).

The β -hexosaminidases from GH family 20 employ substrate-assisted catalysis whereby the *N*-acetamido group on the C2 of the *N*-acetyl- β -D-glucosaminy substrate acts as the catalytic nucleophile (Tews *et al.*, 1996; Drouillard *et al.*, 1997). If this were the case for the GH 3 NagZ, then these enzymes might not require the additional acidic residue provided by the C-terminal $(\alpha/\beta)_6$ sandwich domain (Harvey *et al.*, 2000). These observations support the possibility that the N-terminal $(\alpha/\beta)_8$ TIM barrel domain (Fig. 9a) evolved initially to encode NagZ activity, a function that is important for cell wall recycling in bacteria (Cheng *et al.*, 2000). Gene duplication and the acquisition of an additional $(\alpha/\beta)_6$ sandwich domain may then have led to the evolution of β -glucosidase and β -xylosidase activities on this protein scaffold (Table 1). Further support for this idea comes from the fact that a number of GH 3 genes have been identified that have both the $(\alpha/\beta)_6$ and $(\alpha/\beta)_8$ TIM barrel domains, however the regions of these domains in the polypeptide sequence are switched, yet these proteins retain their enzymatic activities (Honda *et al.*, 1988; Ohmiya *et al.*, 1990). An additional protective antigen 14 kDa (PA14)-like domain has been identified for GH 3 proteins, which forms an insertion within the N-terminal $(\alpha/\beta)_6$ sandwich domain (Table 1). The functional significance of the PA14-like domain insertion in GH 3 enzymes is currently unknown, however several lines of evidence support the possibility that the PA14 domain represents a novel CBM (Rigden *et al.*, 2004; Zupancic *et al.*, 2008).

The active site for the Barley GH 3 β -glucosidase is a small, coin-shaped cavity that, similar to GH 43 β -xylosidases, possesses only two substrate binding sites. Subsite mapping studies for this enzyme using gluco-oligosaccharides of varying lengths confirmed the presence of just two glucoside binding sites in the enzyme (Hrmova *et al.*, 2002). The structure of the Barley β -glucosidase is useful for making generalizations on the structure–activity relationships for GH 3 β -xylosidases. However, the amino acid residues that confer substrate specificity (β -glucosidase vs. β -xylosidase) for this family of enzymes remain to be elucidated. Thus, determination of the 3D structure for β -xylosidases in this family will yield insight into the molecular determinants of substrate specificity which will prove invaluable for characterizing the function and substrate specificity for members of this large family of enzymes.

The α -glucuronidases

Xylose residues within arabinoglucuronoxylan may be substituted at *O*-2 with MeGA (Fig. 2). The α -glucuronidases cleave MeGA from xylose residues when the MeGA is attached to the terminal, nonreducing end of xylo-oligosaccharides (Puls *et al.*, 1987; Siikaaho *et al.*, 1994). An additional limitation to the release of MeGA by α -glucuronidases is the extent of acetylation of the xylose chain in proximity to the MeGA substituent (Puls & Schuseil, 1993). The crystal structures for two bacterial α -glucuronidases (*Geobacillus stearothermophilus* AguA and *Cellvibrio japonicus* GlcA67A) provided insight into the specific requirements of α -glucuronidases (Nurizzo *et al.*, 2002; Golan *et al.*, 2004). These

structures revealed a $(\beta/\alpha)_8$ barrel enzyme possessing a deep active site pocket which explains the requirement for terminally appended MeGA sidechains on the xylan backbone. The biological significance of this deep active site pocket and absolute requirement for MeGA linked to the *O*-2 of xylose residues at the nonreducing end is that α -glucuronidases require xylanase activity to generate their cognate substrates. This suggests that α -glucuronidases function downstream of xylanases to deconstruct plant cell wall polysaccharides (Nurizzo *et al.*, 2002).

The xylanolytic esterases

Arabinoglucuronoxylans are typically acetylated at *O*-2 and *O*-3 positions of the xylose chain and frequently have ferulic acid or coumaric acid groups esterified to the 5'-OH of arabinofuranosyl groups. The acetyl groups affect the capacity of other xylanolytic enzymes such as xylanases (Biely *et al.*, 1986; Grohmann *et al.*, 1989) and α -glucuronidases (Puls & Schuseil, 1993) to bind and hydrolyze backbone or side chain linkages. Ferulic and coumaric acid groups may be covalently linked either to lignin or to other ferulic or coumaric acid groups in xylans, thus these chemical linkages impart significant limitations to deconstructing xylan substrates. Correspondingly, many xylanolytic microbes harbor genes encoding ferulic/coumaric acid esterases and acetyl xylan esterases. Ferulic/coumaric acid esterases belong to the carbohydrate esterase (CE) family 1, whereas acetyl xylan esterase activity has been described for members of CE 1–7, 12 and the recently discovered family 16 (Li *et al.*, 2008). With the exception of acetyl xylan esterases in CE 4, all of the ferulic/coumaric acid esterases and acetyl xylan esterases employ a Ser–His–Asp(Glu) catalytic triad analogous to the mechanism utilized by serine proteases (Kraut, 1977). This mechanism involves two phases, the initial acylation of the nucleophilic serine residue followed by deacylation with water acting as a nucleophile (Fig. 10). The alternative mechanism identified for members of CE 4 involves divalent cations coordinated by histidine residues and a nucleophilic aspartic acid residue (Taylor *et al.*, 2006).

Modularity of xylanolytic enzymes

GHs are modular in general, and likewise many xylanolytic enzymes exhibit a modular domain organization and link various catalytic domains or carbohydrate binding domains within the same polypeptide.

CBMs increase the effective concentration of catalytic modules on the surface of insoluble substrates and may change the structure of the sugar by disrupting hydrogen bonding interactions (Shoseyov *et al.*, 2006). While the structure of cellulose is quite homogeneous, as mentioned above, the structure of xylan can vary greatly with different residues substituted off of the xylose backbone. This difference in structure imparts significant limitations to CBMs targeted towards xylan relative to the CBMs that bind cellulose, which is a homogenous substrate. Similar to the binding of decorated xylo-oligosaccharides by xylanases, the decorations on xylan will likely influence the way that CBMs bind to xylan. Indeed, the crystal structure for the family 15 CBM from the saprophytic soil bacterium, *C. japonicus* revealed that when bound to xylopentaose, the oligosaccharide adopts a helical conformation such that the *O*-2 and *O*-3 for all but one of the xylose residues face outward into solution (Fig. 11) (Szabo *et al.*, 2001). It is anticipated that this conformation will allow the CBM to accommodate decorations on the xylan chain, thus permitting the *C. japonicus* CBM to bind substituted arabinoglucuronoxylans (Szabo *et al.*, 2001).

Coordinated function of xylanolytic Enzymes

The biocatalytic conversion of xylan to the constituent monosaccharides, xylose, arabinose, and glucuronic acid will require the coordinated function of these xylanolytic enzymes. In our model presented in Fig. 12, we can envision xylanases, acetyl xylan esterases, and

ferulic acid esterases functioning together to produce short, substituted xylo-oligosaccharides with the concomitant release of ferulic and acetic acid byproducts (Fig. 12a). The substituted xylo-oligosaccharides then become the substrates for arabinofuranosidases and glucuronidases that liberate arabinose and glucuronic acid, leaving linear, nonbranched xylo-oligosaccharides (Fig. 12b). Xylosidases then convert the xylo-oligosaccharides into their constituent xylose sugars (Fig. 12c). Finally, fermenting microorganisms, naturally capable or engineered to ferment pentose sugars, will take up the xylose and arabinose and shuttle them into the pentose phosphate pathway for subsequent fermentation to biofuels (Fig. 12d). Indeed, a strain of the bacterium *Escherichia coli* has been genetically modified to produce ethanol from mixed sugars (glucose, xylose, and arabinose) that represent the products of hydrolysis of cellulose and xylan (Nichols *et al.*, 2001). In addition, naturally-occurring yeasts such as *Pachysolen tannophilus*, *Pichia stipitis*, and *Candida shehate* are capable of fermenting xylose to ethanol, and their commercial utilization may only require genetic manipulation to increase their ethanol tolerance and rates of growth (Wang *et al.*, 1980; Schneider *et al.*, 1981; Saha, 2003). Finally, through genetic engineering, the metabolic pathways that allow these microorganisms to produce ethanol from pentose sugars, especially xylose and arabinose, may be introduced into the yeast *S. cerevisiae* and the bacterium *Z. mobilis* to facilitate utilization of these substrates. Although not a simple feat, it is anticipated that these accomplishments are likely to be reported in the near future due to the current efforts in biofuel research.

Future perspectives

Plant cell walls are complex structures that have a variety of components including cellulose, hemicellulose, pectin (galacturonic acid polymers), and lignin. The relative proportions of these polymers vary among different species of plants, thus generalizations made based on the structure of cell wall polysaccharides studied from one species may not hold true when considering the cell wall structure of another species. Correspondingly, to generate optimized enzyme cocktails for biofuels production from lignocellulose, it will be essential to have a detailed knowledge of the relative compositions and fine structure of the plant cell wall components for the specific biomass feed-stocks being targeted, such as Miscanthus and Switch-grass.

Artificial substrates, while useful as initial screens for enzymatic activity, may be misleading. For example, the two GH 43 enzymes discussed above that have significant activity with pNPA as a substrate target completely different chemical linkages with BsAXH-m2,3 releasing arabinofuranosyl units from xylan fragments and SXA hydrolyzing xylo-oligosaccharides to monomeric xylose units. These results illustrate the catalytic promiscuity exhibited by many GHs and underscore the limitations of using artificial substrates to assign function to unknown proteins. Testing for enzymatic activity of proteins with natural plant cell wall polysaccharides will be indispensable for evaluating the true molecular function of newly isolated proteins. Furthermore, a standardized set of chemically and structurally defined plant cell wall polysaccharide substrates isolated from biomass feedstocks would prove valuable. Such substrates will permit the reconstitution of customized enzyme cocktails and will provide a platform for comparing the efficiencies of enzymes isolated from various microbial sources. Standardizing substrates and methods has already proved to be useful for interlaboratory comparison of xylanase activity (Bailey *et al.*, 1992). Establishment of materials and methods with substrates from biomass feedstocks will have additional benefits. Structural studies of xylanolytic enzymes have already proven valuable for identifying the substrate specificity of xylan degrading enzymes, and future studies in this important arena will likely aid us in identifying optimized enzyme cocktails for the deconstruction of hemi-cellulosic polysaccharides.

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Nomenclature

AF	α -L-arabinofuranosidase
AXH	arabinoxylan arabinofuranohydrolase
β -xylosidase	xylan-1,4- β -xylosidase
CBM	carbohydrate binding module
CE	carbohydrate esterase
FAX ₂	feruloyl arabinoxylodisaccharide
GH	glycoside hydrolase
MeGA	4-O-methyl- α -D-glucuronic acid
PA14	protective antigen 14 kDa
TIM	triosephosphate isomerase
Xylanase	endo-1,4- β -xylanase

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Switchgrass (Alamo)

Fig. 1.

Compositional analysis of the Alamo cultivar of Switch-grass. As indicated, glucans are predominantly composed of cellulose. Hemicellulosic components include galactan, mannan, xylan, arabinan, and uronic acids, although xylan represents the most abundant hemicellulosic polymer (Vogel, 2008). These data were obtained from the US DOE Biomass Feedstock Composition and Property Database (<http://www.afdc.energy.gov/biomass/progs/search1.cgi>).

Fig. 2.

General structure showing the various linkages found in a variety of xylans isolated from plant cell walls. As described in the text, xylans isolated from different sources may not possess all of the linkages shown. Ferulic acid may form a di-ferulic acid bridge with ferulic acid residues attached to other arabinoxylan chains. Xylose residues may be di-substituted or mono-substituted with arabinose at the *O*-2 and *O*-3 positions.

Fig. 3. General glycosidase mechanisms for (a) retaining glycosidases and (b) inverting glycosidases. In (a), a deprotonated carboxylic acid nucleophile attacks the anomeric carbon, displacing the attached sugar residue (indicated as R) and forming a covalent enzyme-sugar adduct. Subsequently an activated water molecule displaces the enzymic carboxylic acid resulting in net retention of stereochemical configuration at the anomeric carbon. In (b), an activated water molecule displaces the attached sugar residue resulting in net inversion of stereochemical configuration. In both of these mechanisms, the glycoside leaving group is assisted through protonation by a catalytic acid residue.

Fig. 4. Diagrammatic representation of the sugar binding sites in glycosidases based on the scheme proposed by Davies *et al.* (1997).

Fig. 5.

Differences in the products of hydrolysis between GH 10 and 11 endo-xylanases when incubated with substituted xylans (Biely *et al.*, 1997b; Maslen *et al.*, 2007). (a) For GH family 10 enzymes, substitutions on the xylan chain are accommodated at the +1 site, thus these enzymes can release xylo-oligosaccharides in which the terminal nonreducing xylose residue is substituted. (b) For GH family 11 enzymes, substitutions on the xylan chain are not accommodated at the +1 site, thus these enzymes produce xylo-oligosaccharides with substitutions at the penultimate xylose residue. GH, glycoside hydrolase.

Fig. 6.

Structural surface representation of the (a) *Neocallimastix patriciarum* GH family 11 xylanase in complex with ferulic acid arabinoxylotrisaccharide (PDB accession no. 2VGD) (Vardakou *et al.*, 2008) and the (b) *Cellvibrio mixtus* GH family 10 xylanase in complex with aldotetrauronic acid (PDB accession no. 1UQZ) (Pell *et al.*, 2004b). For (a), the active site cleft of the GH 11 enzyme excludes the possibility of a substitution at the +1 subsite of the xylose chain, whereas substitutions may be accommodated at the +2 subsite. For (b), the open topology of the active site of the GH 10 enzyme permits the accommodation of a substitution at the +1 subsite. All structural representations in this and subsequent figures were generated with the UCSF Chimera software package (Pettersen *et al.*, 2004). GH, glycoside hydrolase.

Fig. 7.

Structural representations of the (a) *Bacillus subtilis* GH family 43 arabinoxylan arabinofuranohydrolase (AXH-m2,3) in complex with xylootetraose (PDB accession no. 3C7G) (Vandermarliere *et al.*, 2009) and the (b) *Selenomonas ruminantium* GH family 43 β -xylosidase (SXA) in complex with 1,3-bis[tris(hydroxymethyl)methylamino]propane (PDB accession no. 3C2U) (Brunzelle *et al.*, 2008). Both enzymes possess two domains, an N-terminal β -propeller domain and a C-terminal mainly β -sheet domain, although the C-terminal domain for SXA is much larger and projects a loop that contacts the active site for the enzyme. GH, glycoside hydrolase.

Fig. 8. Schematic representation of the GH family 43 β -xylosidase (SXA) from *Selenomonas ruminantium* indicating the two xylose binding sites in the active site and the projection of extended xylose chains out into solution. GH, glycoside hydrolase.

Fig. 9.

Structural representations of the (a) *Vibrio cholerae* putative GH family 3 *N*-acetyl- β -glucosaminidase in complex with *N*-acetyl- β -glucosaminidase (PDB accession no. 1Y65) and the (b) *Hordeum vulgare* GH family 3 β -xylosidase in complex with glucose (PDB accession no. 1EX1) (Varghese *et al.*, 1999). As described in the text, acquisition of the second $(\alpha/\beta)_6$ sandwich domain may have led to the evolution of β -glucosidase activity within this family of proteins. GH, glycoside hydrolase.

Fig. 10.

General mechanism for esterases employing the Ser-His-Asp(Glu) catalytic triad. Analogous to serine proteases, the first set of reactions leads to acylation of the enzyme followed by de-acylation of the enzyme involving attack of the ester linkage by an activated water molecule.

Fig. 11.

Structural surface rendering of the *Cellvibrio japonicus* family 15 carbohydrate binding module (CBM) in complex with xylopentaose (PDB accession no. 1GNY) (Szabo *et al.*, 2001). The xylopentaose sugar adopts a helical conformation wherein most of the *O*-2 and *O*-3 hydroxyl groups point out into solution suggesting that this CBM could accommodate a highly decorated xylan chain. The only exception is the fourth xylose residue which makes hydrogen bond contacts from both the *O*-2 and *O*-3 hydroxyl groups to the protein.

Fig. 12.

Schematic outline depicting the functional coordination of xylanolytic enzymes in the deconstruction of xylan for biofuels production. (a) Xylanases, acetyl xylan esterases, and ferulic acid esterases function together to produce short, substituted xylo-oligosaccharides with the concomitant release of ferulic and acetic acid byproducts. (b) Arabinofuranosidases and glucuronidases then liberate arabinose and glucuronic acid from these substituted xylo-oligosaccharides. (c) Xylosidases convert the xylo-oligosaccharides into their constituent xylose sugars. (d) Fermenting microorganisms take up the xylose and arabinose and shuttle them into the pentose phosphate pathway for subsequent fermentation to biofuels.

Table 1

Domain architecture for GH family 3 genes.

Domain organization*	Biochemical activities	Pfam hits†
<i>N</i> -acetyl- β -D-glucosaminidase	693
β -D-glucosidase β -D-xylosidase α -L-arabinofuranosidase	898
β -D-glucosidase	42
β -D-glucosidase β -D-xylosidase α -L-arabinofuranosidase	129

* Functional domains were assigned utilizing the Pfam online server (<http://www.sanger.ac.uk/Software/Pfam/>) (Finn *et al.*, 2006). Domain hits were included if the expect value (*E* value) was smaller than 1×10^{-8} .

† The number of domains for each domain organization was estimated from values provided on the Pfam online server. GH, glycoside hydrolase.