Carbohydrate Research 344 (2009) 1175–1179

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Substrate recognition and hydrolysis by a fungal xyloglucan-specific family 12 hydrolase

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ARTICLE INFO

Article history:
Received 5 March 2009
Received in revised form 16 April 2009
Accepted 19 April 2009
Available online 22 April 2009

Keywords:
Xyloglucan
Oligosaccharides
Xyloglucanase
Glycoside hydrolase
Kinetics

ABSTRACT

Biochemical studies to elucidate the structural basis for xyloglucan specificity among GH12 xyloglucanases are lacking. Accordingly, the substrate specificity of a GH12 xyloglucanase from *Aspergillus niger* (*An*XEG12A) was investigated using pea xyloglucan and 12 xylogluco-oligosaccharides, and data were compared to a structural model of the enzyme. The specific activity of *An*XEG12A with pea xyloglucan was 113 μmol min^{-1} mg^{-1}, and apparent k_{cat} and K_m values were 49 s^{-1} and 0.54 mg mL^{-1}, respectively. These values are similar to previously published results using xyloglucan from tamarind seed, and suggest that substrate fucosylation does not affect the specific activity of this enzyme. *An*XEG12A preferred xylogluco-oligosaccharides containing more than six glucose units, and with xylose substitution at the −3 and +1 subsites. The specific activities of *An*XEG12A on 100 μM XXXGXXXG and 100 μM XLLGXLLG were 60 ± 4 and 72 ± 9 μmol min^{-1} mg^{-1}, respectively. *An*XEG12A did not hydrolyze XXXXXXXG, consistent with other data that demonstrate the requirement for an unbranched glucose residue for hydrolysis by this enzyme.

1. Introduction

Xyloglucan is the predominant hemicellulose in primary cell walls of dicots and several monocots, and is a storage polymer in the seed endosperm of many plants. In primary cell walls, xyloglucan is bound to cellulose through hydrogen bonds, thereby enabling cell elongation and expansion by cross-linking cellulose microfibrils and preventing their aggregation.[1] Accordingly, enzymes that are involved in the synthesis and degradation of xyloglucan are potential targets for fiber engineering.

Structural and seed storage xyloglucans consist of a β-(1→4)-linked β-D-Glc*p* (β-D-glucopyranose) backbone having up to 75% of the β-D-Glc*p* residues covalently linked to α-D-Xyl*p* (α-D-xylopyranose) at the O-6 position. Xylose can be further substituted by galactose or arabinose, and galactose may be fucosylated. The precise xyloglucan structure is dependent upon plant species, as well as tissue.[2,3] While the extent of xylose substitution varies considerably, the glucose backbone structure is typically substituted by 50% or 75% xylose. As a result, xyloglucans are classified as XXXG-type or XXGG-type.[4] Given the variability of xyloglucan side-groups, a single letter nomenclature was developed to describe xyloglucan structures (Fig. 1).[5] For instance, xyloglucan from tamarind seed is built from the heptasaccharide XXXG, octasaccharides XXLG and XLXG, and nonasaccharide XLLG, whereas pea xyloglucan mostly contains the heptasaccharide described above, and the fucosylated nonasaccharides XXFG and XFXG.[6]

Glycoside hydrolases (GHs) have been classified into over one hundred distinct families,[7] and xyloglucan-specific β-(1→4)-glucanases have been identified (http://www.cazy.org) in families GH5 12, 16, 44, and 74.[8–10] While many endoglucanases with activity toward several of β-(1→4)-glucose polymers have been described, xyloglucan-specific glycoside hydrolases constitute a relatively new class of enzymes,[11] and were recently ascribed

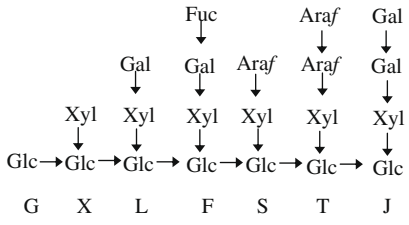

Figure 1. Nomenclature used to describe the individual segments of xyloglucan, where Glc: glucose; Xyl: xylose; Gal: galactose; Fuc: fucose; Ara*f*: arabinose.

Abbreviations: MALDI, matrix-assisted laser desorption ionization; XEG, xyloglucan endoglucanase; Glc, glucose.

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doi:10.1016/j.carres.2009.04.020

new EC entries (EC 3.2.1.151 and 3.2.1.155). To date, the majority of described xyloglucan-specific hydrolases belong to family GH74. At least seven GH74 xyloglucanases have been biochemically characterized, and structures for both *endo*-acting and *exo*-acting xyloglucanases are available.[12,13] By contrast, biochemical characterization data have been published for only two xyloglucanases from GH5, one xyloglucanase from GH16, and three xyloglucanases from GH12, with structural data available for one GH5, one GH12, and one GH16 xyloglucanase.[14–18] A more complete account of the reaction mechanisms and the structural diversity of xyloglucanases are described in a recent review.[10]

The recombinant production and characterization of a GH12 xyloglucanase from *Aspergillus niger*, *An*XEG12A, was recently published.[16] While the specific activity of *An*XEG12A with tamarind xyloglucan is similar to other xyloglucanases, it demonstrates comparatively high thermostability. *An*XEG12A cleaves tamarind xyloglucan at unbranched glucose residues, and preferentially hydrolyzes the polymeric substrate at regions with low branching structure. Defined xylogluco-oligosaccharides have been used to study the structural basis for xyloglucan specificity among GH74 xyloglucanases. However, similar investigations are lacking among GH12 xyloglucanases. Biochemical analysis of GH12 xyloglucanases would expand the application of xyloglucanases for compositional characterization of plant fiber and hydrolysis of biomass feedstocks. Given the sequence similarity to a GH12 xyloglucan endotransglycosylase, further biochemical analysis of fungal GH12 xyloglucanases will also help to elucidate the structural basis for substrate preference and hydrolytic versus transglycosylation activity within this GH family, as was previously done for GH family 16 enzymes.[19] Accordingly, this report characterizes *An*XEG12A using pea xyloglucan and defined xylogluco-oligosaccharides, and reveals the impact of the extent and position of branching sugars on rates of hydrolysis by this enzyme. These data are discussed in terms of substrate binding among GH12 xyloglucanases.

2. Results and discussion

2.1. Activity of *An*XEG12A on pea xyloglucan

The specific activity of *An*XEG12A with pea xyloglucan was 113 μmol min^{-1} mg^{-1}. The apparent k_{cat} and K_m values for *An*XEG12A with pea xyloglucan were 49 s^{-1} and 0.54 mg mL^{-1}, respectively, and were similar to values previously obtained using tamarind xyloglucan.[16] Pea and tamarind xyloglucan are both XXXG-type polysaccharides, and contain xylosyl residues that are covalently linked to galactose through β-(1→2)-linkages. Approximately 50% of galactose in pea xyloglucan are further decorated by α-(1→2)-fucose.[6] Previous MALDI-TOF analyses of end-products from tamarind xyloglucan hydrolysis suggest that *An*XEG12A preferentially attacks glycosidic linkages between heptasaccharide subunits.[16] The current activity data revealed that further substitution by fucose at the −2 subsite of xyloglucan subunits did not impact substrate binding and hydrolysis.

Reaction products from standard activity assays with pea xyloglucan and *An*XEG12A were analyzed by MALDI-TOF mass spectrometry following incubation of pea xyloglucan with 0.06 ng μL^{-1} (0.029 μM, final concentration) enzyme at 50 °C for 5, 10, 30, and 60 min; the increase in concentration of reducing sugars over this time course was approximately linear (data not shown). From 5 to 60 min, the main product peaks that were observed matched the predicted masses of sodium adducts of XXFG+XXXG (*m/z* 2438), although sodium adducts of XXFG (*m/z* 1394) and XXXG (*m/z* 1085) were also detected. Additional peaks near *m/z* 2438 corresponded to sodium adducts of Glc_8 xylogluco-oligosaccharides that were present in comparatively low abundance, including XXXG+XXLG or XXXG+XLXG (*m/z* 2293), XXFG+XXLG (or XLXG), and XLFG+XXXG (*m/z* 2602).[20] Overnight incubations showed that these Glc_8 xylogluco-oligosaccharides were eventually degraded into corresponding Glc_4 subunits: XXXG, XXLG (XLXG), XXFG, XLFG. Notably, fucosylation of xyloglucan did not affect the preference of *An*XEG12A to cleave this substrate at unbranched Glc residues. However, it is not possible from these data to distinguish the orientation of Glc_4 subunits present in the Glc_8 xylogluco-oligosaccharides that remained after 60 min of incubation with *An*XEG12A. Therefore, to directly investigate the impact of substrate length and substitution on enzyme activity, the activity of *An*XEG12A was tested against a broad range of defined xylogluco-oligosaccharide structures.

2.2. Activity of *An*XEG12A on xylogluco-oligosaccharides

Hydrolysis of selected Glc_5 xylogluco-oligosaccharides by *An*XEG12A was not observed by HPLC. However, mass spectra consistently revealed peaks corresponding to Glc_4 products in reactions containing XXXGG, XLLGG, and XXLGG (Table 1). Enzymatic production of glucose from XXXGG, XLLGG, and XXLGG was confirmed by performing a glucose oxidase-based assay with corresponding reaction mixtures. The rate of glucose production from XXXGG by *An*XEG12A was 0.35 nmol min^{-1} mg^{-1}, which is more than 10^5 times lower than rates of XXXG and XLLG production from XXXGXXXG and XLLGXLLG, respectively, indicating that cleavage of Glc_5 xylogluco-oligosaccharides ending in GG is a very minor activity. Moreover, since *An*XEG12A did not hydrolyze XLLG, the production of XLL in reactions containing XLLGXLLG most likely resulted from the hydrolysis of XLLGXLL impurities that were detected in the XLLGXLLG sample. Taken together, these data suggest that xylosyl substitution at the +1 position promotes substrate binding and hydrolysis. Alternatively, more than one positive subsite might be required.[21] Hydrolysis of XXGXXXG, but no detectable hydrolysis of GXGXXXG, implies that α-D-Xyl*p* substitution at the −3 position is also necessary for efficient hydrolytic activity by *An*XEG12A. The occupation of a −4 subsite was not necessary for hydrolysis. However, the significantly lower specific activity of *An*XEG12A on XXGXXXG compared to XXXGXXXG suggests that occupation of the −4 position increases the rate of hydrolysis and/or improves substrate binding by this enzyme (Table 2). A similar observation was made regarding the −4 subsite

Table 1
Substrate specificity of *An*XEG12A toward xylogluco-oligosaccharides after overnight incubation

Substrate	Products detected by HPLC	Major peaks detected by mass spectrometry ($[M+Na]^+$)[b]
XXXGG	ND[a]	**XXXGG (1247.406)**, XXXG (1085.284),
XLLGG	ND	**XLLGG (1571.466)**, XLLG (1409.5128), XLL (1247.389)
GXXXG	ND	**GXXXG (1247.338)**
XXLGG	ND	XXLGG (1571.464), **XXLG (1409.4442**)
XXXGXX	XXXG, XX	**XXXG (1085.387); XX (628.993)**
GXGXXXG	ND	Not acquired due to limited substrate
XXGXXXG	XXG, XXXG	**XXG** (791.181); **XXXG (1085.292);** XXGXXXG (1835.689)
XXXGXXXG	XXXG	**XXXG (1085.222)**
GXXGXXXG	GXXG, XXXG	**GXXG (953.170); XXXG (1085.247)**
XLLGXLLG	XLLG	**XLLG (1409.438)**, XLL (1247.292)
XXXXXXG	ND	Not acquired due to limited substrate
XXXGXXXGXXXG	XXXG	Not acquired due to limited substrate

[a] Not detected.
[b] The most abundant peaks are shown in bold. In some cases control samples showed small amounts of potential product peaks present, probably from minor impurities in the substrate. Peaks are only listed if the ratio with the substrate peak changed significantly after incubation with enzyme.

Table 2
The effect of xylogluco-oligosaccharide substitution on rates of hydrolysis by *An*XEG12A

Xylogluco-oligosaccharide (100 μM)	Specific activity (μmol min^{-1} mg^{-1})
XXGXXXG	2.1 ± 4
XXXGXX	63 ± 1
XXXGXXXG	60 ± 4
XLLGXLLG	72 ± 9

Reactions were terminated after 5 min by adding sodium hydroxide to 60 mM.

of XEG from *Geotrichum* sp. M128.[22] Notably, the absence of detectable hydrolysis products from XXXXXXG is consistent with data collected using polymeric substrates, which suggest that *An*XEG12A uniquely hydrolyzes xyloglucan at unbranched Glc residues. Thus, activity analyses using available xylogluco-oligosaccharides did not reveal an ability to hydrolyze xyloglucan substrates at branched Glc residues, unlike Cel74A from *Hypocrea jecorina*, the GH74 oligoxyloglucan reducing end-specific cellobiohydrolase from *Geotrichum* sp. M128 (OXG-RCBH), and XEG74 from *Paenibacillus* sp. KM21.[23–25]

To our knowledge, the current study is the first report to directly compare the activity of a family 12 xyloglucan-specific hydrolase with oligosaccharides containing X and L residues in both positive and negative substrate-binding sites (Table 2). Nevertheless, chromogenic xylogluco-oligosaccharide aryl glycosides were previously used to characterize the activity of *Bl*XG12, a family 12 endoglucanase from *Bacillus licheniformis* with partial preference for tamarind xyloglucan.[15,18] Notably, while the ratio of k_{cat}/K_m indicates that *Bl*XG12 efficiency is inversely related to substrate branching,[15] similar specific activities of *An*XEG12A with XXXGXXXG and XLLGXLLG suggest that branching structures do not significantly affect the hydrolytic efficiency of this enzyme. These differences are perhaps not surprising since *Bl*XG12 shows considerable activity on carboxyl methyl cellulose (CMC) while *An*XEG12A does not, suggesting that branching structures promote substrate binding and/or hydrolysis by *An*XEG12A.

2.3. Modeling substrate binding

The superposition of *Bl*XG12 and a family 12 endoglucanase from *Humicola grisea* (*Hg*GH12, Protein Data Bank (PDB) entry 1UU6)[26] reveals amino acid differences at −3 and +2 substrate-binding subsites that are predicted to promote xyloglucanase activity.[15] Specifically, Gloster et al. (2007) suggest that the substitution of Y9, Y132, and R97 in *Hg*GH12 to a serine, threonine, and glycine, in *Bl*XG12, respectively, promotes productive interactions with xylose residues of xyloglucan substrates. While Y9 in *Hg*GH12 is predicted to clash with xylose in the −3 subsite, Y132 and R97 are predicted to interfere with xylose binding in the +2 subsite of the enzyme.

To further identify amino acid signatures that predict xyloglucanase activity, a model structure of *An*XEG12A was generated and superimposed with *Bl*XG12 in complex with XXXG/XX (PDB entry: 2JEN) (Fig. 2). Similar to *Bl*XG12, amino acids that correspond to R97 and Y132 in *Hg*GH12 are valine and glycine in *An*XEG12A (Fig. 2). Val94 and Gly127 (or threonine in *Bl*XH12) at these positions were shown to create a comparably open substrate-binding architecture.[15] Previously reported sequence alignments also reveal equivalent amino acid substitutions for other fungal xyloglucanases.[16] Although fungal and bacterial xyloglucanases appear to share structurally conserved amino acids at the +2 subsite, Y9 at the −3 subsite of *Hg*GH12 is equivalent to W7 in *An*XEG12A and aromatic amino acids in other fungal xyloglucanases.[16] W7 in *An*XEG12A could promote substrate binding through van der Waals interactions with xylose (Fig. 2).

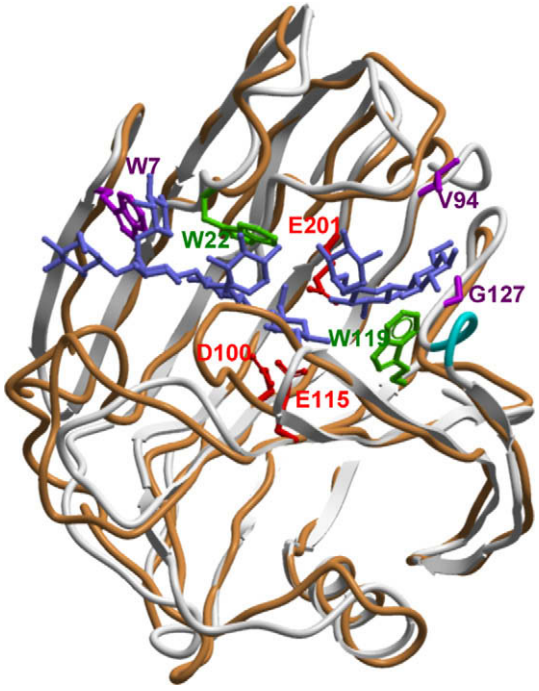

Figure 2. Predictive model of *An*XEG12A (gray) built by homology to an *Humicola grisea* GH12 endoglucanase (PDB entry 1UU6) superposed with the experimental structure of *Bl*XG12 (orange) in complex with XXXG/XX (PDB entry 2JEN). Catalytic residues (red) D100, E115, E201, predicted substrate binding residues (green) W22, W119, residues that are predicted to influence substrate specificity in GH12 xyloglucanases (purple) W7, V94, G127, and the SST insertion sequence (130–132, blue) are highlighted in *An*XEG12A.

The *An*XEG12A sequence is distinguished from both *Bl*XG12 and *Hg*GH12 by an SST insertion positioned at the reducing end of the substrate binding cleft (*An*XEG12A residues 130–132, Fig. 2). This insertion sequence is conserved among fungal GH12 xyloglucanases and is adjacent to the so-called 'cord' region that forms the bottom of the +2 and +3 subsites in GH12 endoglucanases.[16,27] The insertion of the SST sequence is predicted to increase the polarity of the reducing end of the substrate binding cleft. This in turn could facilitate xyloglucan binding and hydrolysis by promoting productive hydrogen-bond interactions with xylose, and increasing the conformational flexibility of the substrate cleft.

The surface topology of the model *An*XEG12A protein structure and experimental *Hg*GH12 protein structure were generated to gain additional insight into structural determinants of substrate specificity by these enzymes (Fig. 3). Notably, the more open binding cleft predicted for *An*XEG12A compared to *Hg*GH12 can be expected to generate weaker interactions with linear polymeric substrates, which is consistent with the absence of cellulolytic activity by *An*XEG12A. Further, the narrower substrate binding cleft of *Hg*GH12 compared to *An*XEG12A can be expected to reduce the activity of *Hg*GH12 on branched polysaccharides.

2.4. Conclusion

The present study reports detailed biochemical analysis of a GH12 xyloglucanase using polymeric and oligomeric substrates. *An*XEG12A accommodates fucosylated xyloglucan polymers, and maximum activity of *An*XEG12A was observed using substrates that are predicted to occupy the −4 to +2 subsites of the enzyme active centre. Superposition of the three-dimensional model structure for *An*XEG12A and the bacterial xyloglucanase *Bl*XH12 refined amino acid signatures that are conserved among GH12 xyloglucanases; productive interactions between the SST insertion in *An*XEG12A and xylogluco-oligosaccharides were also predicted. Site-directed

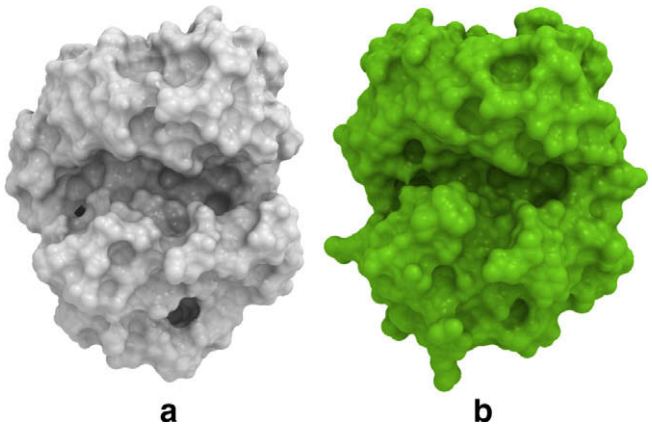

Figure 3. A molecular surface representation of our model of *An*XEG12A reveals a wider substrate binding cleft compared to *Hg*GH12, suggesting increased affinity for branched substrates. (a) The predicted van der Waal's surface for *An*XEG12A, and (b) van der Waal's surface for *Hg*GH12, the experimentally solved cellulase structure from *Humicola grisea* (PDB entry: 1UU6).

mutagenesis studies are now underway to further elucidate the structural basis of substrate specificity in GH12 enzymes.

3. Experimental

3.1. General

Buffers were prepared using water purified on a Milli-Q® system (Millipore®) with a resistivity ⩾ 18.2 MΩ cm. Protein concentration was determined using the DC Protein Assay from BioRad with bovine serum albumin as the standard, according to the manufacturer's instructions. Glucose oxidase assays were performed using the Glucose (GO) Assay kit from Sigma (product number GAGO20). Spectrophotometric measurements were performed using a Cary 50 UV–vis spectrophotometer (Varian Instruments).

3.2. Enzyme substrates

Pea xyloglucan was a kind gift from Professor Takahisa Hayashi (Kyoto University, Japan). Xylogluco-oligosaccharides used in this study were kind gifts from Dr. Katsuro Yaoi (National Institute of Advances Industrial Science and Technology, Tsukuba, Japan). Exceptions were the heptasaccharide, XXXG, and nonasaccharide, XLLG, which were purchased from TCI America (Portland, Oregon).

3.3. Enzyme activity with pea xyloglucan

Standard activity assays were performed at 50 °C in 50 mM acetate buffer (pH 5.0). The concentration of pea xyloglucan was 0.05% (w/v). Reactions were initiated by adding 0.06 ng μL^{-1} (0.029 μM) of *An*XEG12A, and replicate samples were incubated for 5, 10, 30, and 60 min, and overnight. Reducing sugar products were quantified using the disodium 2,2′-bicinchoninate (BCA) assay for reducing sugars; a standard curve was prepared using glucose.[28] The identity of reaction products was determined by mass spectrometry as described below. *An*XEG12A used in this study was recombinantly expressed in *A. niger*, and purified to homogeneity.[16] The specificity of purified *An*XEG12A for xyloglucan substrates and absence of activity on cellulose and other polysaccharides were previously demonstrated.[16]

3.4. Kinetic measurements with pea xyloglucan

Kinetic parameters were measured using the standard activity assay and twenty concentrations of pea xyloglucan, from 0.001% to 0.2% (w/v). Reactions were initiated by adding *An*XEG12A (0.06 ng μL^{-1}) and were incubated for 5 min at 50 °C. Reactions were terminated and analyzed using the BCA assay for reducing sugars; reducing ends were quantified relative to known concentrations of glucose. Triplicate reactions were prepared for each substrate concentration. Kinetic constants were calculated from nonlinear regression of Michaelis–Menten plots using the PRISM5 GraphPad software.

3.5. Enzyme activity with xylogluco-oligosaccharides

The activities of enzymes with xylogluco-oligosaccharides containing a backbone of 4–12 β-(1→4)-linked glucose units, and varying side groups, were tested using the standard activity assay with 100 μM of each oligosaccharide. Reactions were initiated by adding enzyme (1.3–0.06 ng μL^{-1}) and replicate samples were incubated for 5 min and overnight at 50 °C; controls were prepared lacking enzyme. Reactions were terminated by adding sodium hydroxide to 60 mM. The products of enzymatic hydrolysis were analyzed by high performance anion-exchange chromatography with a pulsed amperometric detector (HPAEC-PAD, Dionex DX600) and a CarboPac PA-200 column (3 × 250 mm, Dionex). The eluents were 100 mM sodium hydroxide (eluent A) and 100 mM sodium hydroxide with 1 M sodium acetate (eluent B). Elution was performed at a constant flow rate of 0.75 mL min^{-1} at room temperature using a linear gradient from 0% to 10% of eluent B over 20 min, followed by a linear gradient from 10% to 50% of eluent B over 10 min, and a linear gradient from 50% to 100% eluent B over 15 min. The injection volume was 500 μL in each case. The reaction products were identified and quantified relative to known standards (Table 1).

3.6. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF)

3.6.1. Oligosaccharide analysis

Samples of xyloglucan degradation products were analyzed by MALDI-TOF mass spectrometry. Reaction mixtures were as described above for the standard activity assays except the buffer was 10 mM sodium acetate, pH 5. Aliquots were withdrawn from reaction mixtures at different times and frozen immediately at −70 °C until analysis. For analysis, samples were thawed rapidly and diluted 10- to 50-fold in ice-cold 50% acetonitrile prior to mixing with an equal volume of matrix. The matrix used was 2,5-dihydroxybenzoic acid (10 mg mL^{-1} in water).[29] Samples (1 μl) were then spotted on the MALDI plate and analyzed using a Micromass M@LDI-LR mass spectrometer operated in positive ion mode. The spectrometer was calibrated using a mixture of 6 peptides, and spectra were corrected using ACTH (18–39 clip) as a lockmass, as described above: the matrix used for peptide samples was α-cyanohydroxycinnamic acid. Spectra were compared to previously published spectra of tamarind xyloglucan degradation products.[29,30]

3.7. Modeling substrate binding by *An*XEG12A

A model of the *An*XEG12A structure was built by homology to an *H. grisea* GH12 endoglucanase following the ICM method (Molsoft LLC).[31] Briefly, the *An*XEG12A sequence was mapped onto *Hg*GH12, the experimentally solved cellulase structure from *H. grisea* (PDB entry: 1UU6).[26] The energy of the system was minimized in the internal coordinate space by a biased probability Monte Carlo simulation, and the model *An*XEG12A structure was superimposed with *Bl*XG12, a xyloglucan-specific GH12 hydrolase from *B. licheniformis* (PDB entry: 2JEN).[15] *Bl*XG12 in complex with XXXG/XX was used in this analysis to further elucidate amino acid

residues that participate in substrate recognition by GH12 xyloglucanases.

Acknowledgments

This work was supported by Grants from the Natural Sciences and Engineering Research Council to E. R. M. and J. P. We would like to acknowledge the technical assistance of Mathew Patenaude for the mass spectrometry measurements. We thank Professor Takahisa Hayashi (Kyoto University, Japan) for generously providing the pea xyloglucan that was used in this study, Dr. Katsuro Yaoi (National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan) for kindly providing the xylogluco-oligosaccharides and for insight into xylogluco-oligosaccharide analysis.

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