Kinetic Analyses of Retaining *endo*-(Xylo)glucanases from Plant and Microbial Sources Using New Chromogenic Xylogluco-Oligosaccharide Aryl Glycosides[†]

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ABSTRACT: A library of phenyl β-glycosides of xylogluco-oligosaccharides was synthesized via a chemoenzymatic approach to produce new, specific substrates for xyloglucanases. Tamarind xyloglucan was completely hydrolyzed to four, variably galactosylated component oligosaccharides based on Glc_4 backbones, using a *Trichoderma endo*-glucanase mixture. Oligosaccharide complexity could be further reduced by β-galactosidase treament. Subsequent per-*O*-acetylation, α-bromination, phase-transfer glycosylation, and Zemplén deprotection yielded phenyl glycosides of XXXG and XLLG oligosaccharides with a broad range of aglycon pK_a values. Kinetic and product analysis of the action of the archetypal plant *endo*-xyloglucanase, *Tropaeolum majus* NXG1, on these compounds indicated that formation of the glycosyl−enzyme intermediate was rate-limiting in the case of phenol leaving groups with pK_a values of >7, leading exclusively to substrate hydrolysis. Conversely, substrates with aglycon pK_a values of 5.4 gave rise to a significant amount of transglycosylation products, indicating a change in the relative rates of formation and breakdown of the glycosyl−enzyme intermediate for these faster substrates. Notably, comparison of the initial rates of XXXG-Ar and XLLG-Ar conversion indicated that catalysis by *Tm*NXG1 was essentially insensitive to the presence of galactose in the negative subsites for all leaving groups. More broadly, analysis of a selection of enzymes from CAZy families GH 5, 12, and 16 indicated that the phenyl glycosides are substrates for anomeric configuration-retaining *endo*-xyloglucanases but are not substrates for strict xyloglucan *endo*-transglycosylases (XETs). The relative activities of the GH 5, 12, and 16 *endo*-xyloglucanases toward GGGG-CNP, XXXG-CNP, and XLLG-CNP reflected those observed using analogous high molar mass polysaccharides. These new chromogenic substrates may thus find wide application in the discovery, screening, and detailed kinetic analysis of new xyloglucan-active enzymes.

The xyloglucans comprise a family of extensively $\alpha(1\rightarrow6)$ xylosylated $\beta(1\rightarrow4)$ glucans that are widely distributed in the seeds and vegetative tissues of land plants. Indeed, fucogalactoxyloglucans are some of the predominant matrix polysaccharides in the primary cell wall of all dicots and non-commelinoid monocots, in which they can account for up to 20% of the dry mass (*1*). Nonfucosylated galactoxyloglucans are employed as the main carbohydrate reserve in the seeds of several plant species (*1, 2*). Although xyloglucans exhibit local, sometimes complex (*3*), microheterogeneity of the subtending side chain residues, a hallmark of these polysaccharides is very regular backbone xylosylation (*4*). The XXXG motif is the most common repeating unit found in a variety of species and tissues [where G = Glc*p* and X = α-D-Xyl*p*(1→6)-Glc*p* (*5*)], while, for example, XXGG, XGGG, XXGGG, XXXXG, and XXXXXG motifs have also been observed, sometimes exclusively (*1, 4, 6*).

Xyloglucans are receiving continued attention due to their key role in plant morphogenesis (*7*) and biotechnological applications (*8, 9*), and interest in specific xyloglucan hydrolases has also blossomed. Three new EC entries have been created since 2003 (NC-IUBMB) to catalog the action of oligoxyloglucan reducing-end-specific cellobiohydrolases (EC 3.2.1.150) (*10*), xyloglucan-specific *endo*-$\beta(1\rightarrow4)$-glucanases (EC 3.2.1.151) (*11*), and xyloglucan-specific *exo*-$\beta(1\rightarrow4)$-glucanases (EC 3.2.1.155) (*12*). The ability of fungal *endo*-$\beta(1\rightarrow4)$-glucanases ("cellulases") to hydrolyze xyloglucan has long been known (*2, 13, 14*), and a specific plant *endo*-xyloglucanase involved in seed germination has been characterized (refs *15* and *16* and references therein). More recently, a diverse array of xyloglucan-active microbial enzymes has been isolated, recombinantly expressed, and subjected to detailed structure−function studies (e.g., refs *17*−*21*). Specific xyloglucanases have thus far been identified in glycoside hydrolase (GH)[1] families GH5, GH12, GH16, GH44, and GH74 (reviewed in ref *22*; see also http://www.cazy.org/). Most of the fully characterized enzymes from these families cleave the anomeric bond of unbranched Glc residues in the xyloglucan backbone exclusively, al-

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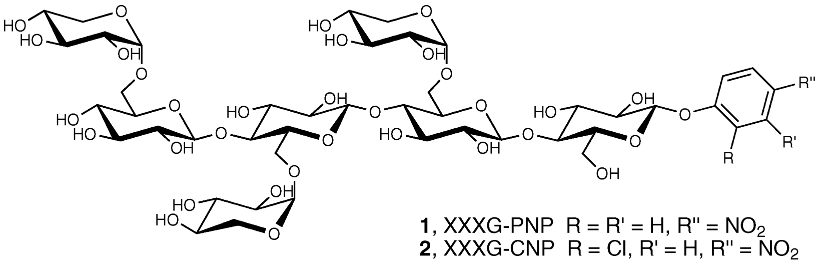
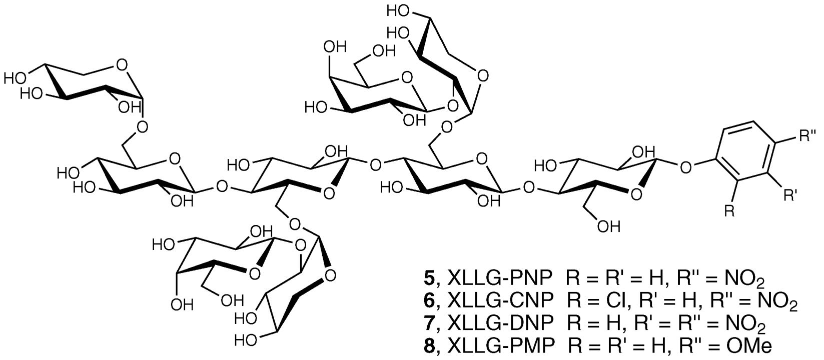


1, XXXG-PNP R = R' = H, R'' = NO_2
2, XXXG-CNP R = Cl, R' = H, R'' = NO_2
3, XXXG-DNP R = H, R' = R'' = NO_2
4, XXXG-PMP R = R' = H, R'' = OMe



5, XLLG-PNP R = R' = H, R'' = NO_2
6, XLLG-CNP R = Cl, R' = H, R'' = NO_2
7, XLLG-DNP R = H, R' = R'' = NO_2
8, XLLG-PMP R = R' = H, R'' = OMe

FIGURE 1: Chromogenic aryl xylogluco-oligosaccharide (XGO) β-glycosides. The XGO nomenclature corresponds to that described in ref *5*.

though certain GH74 enzymes accommodate or even require xylose branching at the −1 subsite (*10, 18, 23–25*) (active site nomenclature of ref *26*).

The current and growing interest in these enzymes is stimulated, in part, by their roles in plant−microbe interactions (*27–29*) and potential use in the saccharification of plant matter for ethanol production (*30*). In spite of their fundamental and applied importance, there currently is a paucity of convenient and precise assays for xyloglucanases; at present, only discontinuous viscometric, reducing-sugar, or HPLC-based assays are used, typically employing polydisperse xyloglucans as substrates. Chromogenic aryl mono- and oligosaccharide glycosides have a rich history of use in the dissection of mechanistic details of catalysis by a variety of *endo*- and *exo*-acting glycosidases. Substrates of this type are chemically well-defined, and aglycon release can be conveniently followed spectrophotometrically, often in continuous assays (e.g., refs *31*−*35*). In particular, access to a library of glycosides of phenols with pK_a values spanning a wide range allows Brønsted analysis, as well as kinetic identification of active site residues (refs *36*−*41* and references therein).

Because of our interest in the characterization of xyloglucanases and xyloglucan *endo*-transglycosylases from both plant and microbial sources (*16, 19, 20, 42*), we have synthesized a set of phenyl β-glycosides of the differentially galactosylated, Glc$_4$-based xylogluco-oligosaccharides XXXG and XLLG [L = β-D-Gal*p*(1→2)-α-D-Xyl*p*(1→6)-D-Glc*p*] as new chromogenic substrates for these enzymes (Figure 1). We present here their chemoenzymatic syntheses and application for the detailed kinetic analysis of an archetypal plant *endo*-xyloglucanase involved in seed germination. Further, we demonstrate that comparative analysis using phenyl (xylo)gluco-oligosaccharide congeners can be used to categorize the substrate specificity of *endo*-β(1→4)-glucanases from various sources.

EXPERIMENTAL PROCEDURES

General Methods. All chemicals were obtained from Sigma/Aldrich/Fluka and were of reagent grade or better. Ultrapure water ($\rho \geq 18$ MΩ cm) was produced on a Milli-Q system (Millipore). The crude *Trichoderma reesei* cellulase mixture was from Fluka (catalog no. 22173) or Sigma (catalog no. C8546), and *Aspergillus niger* β-galactosidase was from Megazyme (catalog no. E-BGLAN). Nasturtium (*T. majus*) seed *endo*-xyloglucanase *Tm*NXG1 (*16*), *Populus Ptt*XET16-34 (previously called *Ptt*XET16A) (*43*), and cauliflower *Bob*XET16A (*44*) were heterologously expressed in *Pichia pastoris* and purified from culture medium as previously described. XXXGXXXG was produced by limited *endo*-glucanase digestion of tamarind (*Tamarindus indica*) xyloglucan (Megazyme), degalactosylation with *A. niger* β-galactosidase, and purification by size-exclusion chromatography (Biogel P6, Bio-Rad) (*19*).

Preparation of Per-O-acetylated Xylogluco-Oligosaccharides. Powdered xyloglucan from tamarind (*Ta. indica*) seeds (20 g) was added portionwise over 15−20 min to a

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[1] Abbreviations: GH, glycoside hydrolase; XET, xyloglucan *endo*-transglycosylase; XGO, xyloglucan oligosaccharide; G, Glc*p*; X, α-D-Xyl*p*(1→6)-Glc*p*; L, β-D-Gal*p*(1→2)-α-D-Xyl*p*(1→6)-D-Glc*p*; PDB, Protein Data Bank; PMP, *p*-methoxyphenol; PNP, *p*-nitrophenol; CNP, 2-chloro-4-nitrophenol; DNP, 3,4-dinitrophenolate; HPAEC-PAD, high-performance anion exchange chromatography-pulsed amperometric detection; HP-SEC, high-performance size exclusion chromatography. Enzymes: BlXG12, *Bacillus licheniformis endo*-xyloglucanase (EC 3.2.1.151, GenPept entry AAR65335, PDB entries 2JEM and 2JEN); *Bob*XET16A, *Brassica oleracea* var. *botrytis* xyloglucan *endo*-transglycosylase (EC 2.4.1.207, GenPept entry AAO00727); *Pp*XG5, *Paenibacillus pabuli endo*-xyloglucanase (EC 3.2.1.151, PDB entries 2JEP and 2JEQ); *Ptt*XET16-34, *Populus tremula* × *tremuloides* xyloglucan *endo*-transglycosylase (EC 2.4.1.207, GenPept entry AAN87142, PDB entries 1UN1 and 1UMZ); *Tm*NXG1, *Tropaeolum majus endo*-xyloglucanase 1 (EC 3.2.1.151, GenPept entry CAA48324).

vigorously stirred solution of *Tr. reesei* cellulase (200 units, Fluka) in 0.01 mM ammonium acetate buffer (pH 5.0) (500 mL). The reaction mixture was stirred at 45 °C for 20 h, filtered, concentrated in vacuo to ca. one-third of the initial volume, and freeze-dried to yield a mixture of XXXG, XXLG, XLXG, and XLLG (*45*) [yield, 95% (w/w)]. XXXG was routinely prepared by addition of *A. niger* β-galactosidase (1000 units) to the digest after incubation of the xyloglucan with the cellulase for 5−6 h. Crude oligosaccharides (20 g) were powdered and acetylated in a pyridine/acetic anhydride mixture (2:1, v/v, 300 mL) in the presence of a catalytic amount of DMAP (0.5 g) by being stirred overnight at 60 °C. The reaction mixture was concentrated in vacuo, dissolved in CH_2Cl_2 (300 mL), and washed with water, 5% H_2SO_4(aq), water, and saturated $NaHCO_3$(aq). The organic phase was dried over Na_2SO_4(s) and evaporated. The residue was purified by flash chromatography using a stepwise toluene/acetone gradient (4:1 to 1:1) as the eluent to give the desired acetylated xylogluco-oligosaccharides as amorphous solids in 27 and 62% yields, respectively, for acetylated XLLG and XXXG. NMR and MS data were consistent with those previously reported (*45*).

*General Procedure for the Synthesis of Aryl β-Glycosides of Xylogluco-Oligosaccharides (**1−8**) via the Corresponding α-Glycosyl Bromides.* To a solution of acetylated XGO (XXXG or XLLG, 1.5 mmol) dissolved in CH_2Cl_2 (25 mL) at 0 °C was added a 33% solution of HBr in glacial HOAc (10 mL, 56 mmol). The reaction mixture was kept at this temperature for 2−3 h and then poured into ice (50 g). The water phase was separated and extracted with CH_2Cl_2 (30 mL). The organic extracts were combined and washed with ice-cold water (50 mL) and ice-cold saturated $NaHCO_3$(aq) (2 × 40 mL), dried over Na_2SO_4(s), and concentrated in vacuo to yield the corresponding α-glycosyl bromide as an amorphous solid. A solution of the crude bromide (0.4 mmol), benzyl tri-*n*-butylammonium chloride (125 mg, 0.4 mmol), and appropriate phenol (1.2 mmol) in CH_2Cl_2 (8 mL) was vigorously stirred with a 0.75 M solution of sodium hydroxide (2.1 mL, 1.6 mmol) at room temperature for 20−24 h (*46, 47*). The reaction mixture was diluted with CH_2Cl_2 (20−30 mL), washed with water (2 × 20 mL), dried over Na_2SO_4(s), and concentrated in vacuo. The residue was reacetylated in a pyridine/acetic anhydride mixture (2:1, 6 mL) in the presence of DMAP (20 mg) for 30 min at room temperature. The resulting mixture was concentrated under reduced pressure, and the residue was purified by flash chromatography using a toluene/acetone (4:1 to 1:1) gradient as the eluent. Per-*O*-acetylated derivatives of compounds **1**−**8** were thus obtained in 25−60% yields and exhibited satisfactory ^{13}C NMR and MS spectra (yields and spectral data available as Supporting Information). The pure acetylated phenyl glycosides were deprotected in a CH_2Cl_2/MeOH mixture using a catalytic amount of NaOMe. The reaction mixture was kept for several hours at ambient temperature, neutralized with acetic acid, and evaporated. The residue was dissolved in water (2−3 mL) and purified by reversed-phase chromatography using a 20 mL RP-18 cartridge (Supelco) and stepwise elution by a gradient of acetonitrile in water. Compounds **1**−**8** were thus obtained in 40−80% yield after purification and gave satisfactory ^{13}C NMR and MS spectra (Supporting Information).

2-Chloro-4-nitrophenyl cellotetraose (GGGG-CNP) (*32, 33*) was synthesized in the same manner from per-*O*-acetyl cellotetraose obtained by acetolysis of cellulose using standard procedures (*48*); the mixture of per-*O*-acetylated cellooligosaccharides so obtained was fractionated on silica gel using gradient elution with acetone in toluene. GGGG-CNP was characterized by NMR and MS spectrometry (Supporting Information).

Initial Rate Enzyme Kinetics. The enzymatic hydrolyses of the CNP and DNP glycosides of GGGG, XXXG, and XLLG were followed by continuous assays assessing the release of 2-chloro-4-nitrophenolate (λ_{max} = 405 nm, ε = 5117 M^{-1} cm^{-1}, 5 mM NaOAc buffer, pH 5.0) or 3,4-dinitrophenolate (λ_{max} = 400 nm, ε = 4545 M^{-1} cm^{-1}, 5 mM NaOAc buffer, pH 5.0) using a Cary 300 Bio UV−visible spectrophotometer (Varian). A total assay volume of 100 μL was used in 1 cm path length quartz cells equilibrated and maintained at 30 ± 0.1 °C in a Peltier-controlled cell block. Initial rates were determined from the slope of the linear region of the reaction time course corresponding to no more than 10% substrate conversion (often <1%). In these assays, the total concentration of *Tm*NXG1 was 11 μg/mL (0.035 μM).

The rates of enzyme-catalyzed hydrolysis of PMP and PNP glycosides were determined by incubation in 5.0 mM NaOAc (pH 5.0) with the temperature maintained in a thermostated block at 30 ± 0.1 °C (total assay volume of 100 μL). In assays using XXXG-PNP and XLLG-PNP, the total protein concentration was 11 μg/mL (0.035 μM) and the incubation time was 30 min. In assays using the corresponding PMP glycosides, the total protein concentration was 55 μg/mL (0.17 μM) and the incubation time was 60 min. The reactions were stopped by addition of 0.2 M sodium carbonate (100 μL), and the amounts of released phenolates were determined (*p*-methoxyphenolate, 305 nm, ε = 2656 M^{-1} cm^{-1}; *p*-nitrophenolate, 410 nm, ε = 18500 M^{-1} cm^{-1}), using a Cary 50 Bio UV−visible spectrophotometer (Varian). Background absorbance values for each corresponding glycoside were substracted; in all stopped assays, the level of substrate conversion was <10% (often <1%). Initial rates of enzymic hydrolysis of XXXGXXXG were determined by HPAEC-PAD as described in ref *16*. Kinetic constants were obtained from nonlinear curve fits of the Michaelis−Menten equation to plots of $v_o/[E]_t$ versus [S] using Microcal Origin version 6.0; $[E]_t$ was assumed to be equivalent to the total protein concentration in all cases, i.e., 100% active enzyme.

Product Analysis. Enzymic reactions (total volume of 150 μL) for product analysis were performed in 25 mM NaOAc (pH 5.0) containing 5 mM XXXG-CNP (**2**), XLLG-CNP (**6**), XXXG-DNP (**3**), or XLLG-DNP (**7**), and 0.067 mg/mL (2.1 μM) *Tm*NXG1. Reaction mixtures were maintained at 30 ± 0.1 °C. Samples (10 μL) were taken at defined times, quenched with HOAc (1 μL), and freeze-dried. The solids were dissolved in DMSO (200 μL), and 100 μL of the resulting solution was analyzed by high-performance size-exclusion chromatography (HP-SEC) using Tosoh G5000HHR and G3000HHR columns (both 7.8 mm × 300 mm) in series with DMSO as the eluent, as previously described (*49*). Using this system, Glc_4- and Glc_8-based xylogluco-oligosaccharides were well-resolved, while individual Glc_4- and Glc_8-based sugars were not resolved (e.g., XXXG and XXXG-CNP). Amounts of Glc_4- and Glc_8-based xylogluco-oligosaccharides were thus estimated from standard curves for XXXG, XXXG-CNP, and XXXGXXXG. The remaining sample in DMSO (100 μL) was diluted with 0.2 M Na_2CO_3 (900 μL), and the adsorption of the released 2-chloro-4-nitrophenolate at 405 nm ($\varepsilon = 17570$ M^{-1} cm^{-1}) or 3,4-dinitrophenolate at 400 nm ($\varepsilon = 14290$ M^{-1} cm^{-1}) was measured using a Cary 50 Bio UV−visible spectrophotometer (Varian).

RESULTS AND DISCUSSION

Substrate Synthesis. A mixture of XXXG, XXLG, XLXG, and XLLG oligosaccharides (approximate ratio of 1:2:1:4) was readily obtained as the starting material for the synthesis of aryl xylogluco-oligosaccharide β-glycosides (XGO-Ar) using a well-established procedure for the digestion of tamarind seed xyloglucan (refs *19* and *45* and references therein). The complexity of this mixture could be further reduced by β-galactosidase treatment to yield XXXG and galactose. In either case, per-*O*-acetylation, followed by flash chromatography over silica gel, provided protected XXXG and XLLG in gram scales for subsequent glycosylation.

Although the synthesis of aryl glycosides of a wide range of mono- and oligosaccharides using various approaches has been described, it was not clear how successfully these methods could be extended to the large, branched XGOs. Somewhat surprisingly, acetylated XGOs were stable under acidic conditions ($HBr/HOAc/CH_2Cl_2$) and directly yielded the corresponding α-glycosyl bromides with no apparent loss of pendant α-xylose and β-galactose side chains. We have previously employed a variation of the Königs-Knorr glycosylation method to install the chromogen (*50*). In this work, we have found that a variety of per-*O*-acetyl XGO-Ar congeners can be conveniently prepared using phase-transfer-catalyzed (PTC) glycosylation conditions (*46, 47*), although partial deacetylation of the oligosaccharides was routinely observed. Attempts to use sodium and potassium phenolates as glycosyl acceptors in DMF were unsuccessful, due to decomposition of the starting glycosyl bromides. Deprotection using Zemplén conditions (catalytic NaOMe in MeOH) was straightforward for all XGO-Ar forms, with the exception of 2,4-dinitrophenyl XGO β-glycosides (data not shown) which decomposed under all deprotection conditions that were tested (Zemplén, hydrazine acetate, various primary amines). Compounds **1**−**8** were thus obtained in moderate yields following purification by reversed-phase chromatography.

*Application of XGO-Ar Substrates to the Kinetic Analysis of Nasturtium Seed Xyloglucanase. Tm*NXG1 from nasturtium is the best-characterized example of a plant xyloglucan-specific *endo*-β(1→4)-glucanase (EC 3.2.1.151) and is the only example whose enzymatic properties and gene sequence have been correlated (ref *16* and references therein). This enzyme is thus the archetype of GH16 *endo*-xyloglucanases, which nevertheless has some capacity to perform xyloglucan *endo*-tranglycosylation (EC 2.4.1.207) at elevated concentrations of minimal XGO donor substrates (ref *16* and references therein). Spectrophotometric analysis indicated that compounds **1**−**8** were all glycosyl donor substrates for recombinant *Tm*NXG1, with cleavage occurring at C-1 of the unbranced Glc residue to liberate the chromophoric aglycon. All substrates exhibited classical hyperbolic plots of $v_o/[E]_t$ versus [S] over the substrate concentration range of 0.05−5 mM, with no apparent deviation due to substrate inhibition; a typical plot of $v_o/[E]_t$ versus [S] is shown in Figure 2. Nonlinear least-squares curve fitting of the standard Michaelis−Menten equation was used to extract apparent kinetic constants (Table 1).

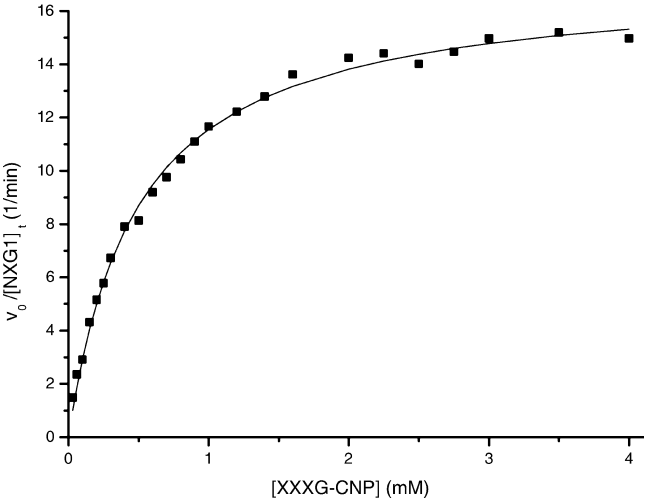

FIGURE 2: Dependence of the rate of nasturtium *endo*-xyloglucanase-catalyzed hydrolysis of XXXG-CNP on substrate concentration. Classic hyperbolic plots of $v_o/[E]_t$ vs [S] were observed for all chromogenic substrates **1**−**8** and GGGG-CNP with *Tm*NXG1.

Table 1: Kinetics of Hydrolysis of Aryl (Xylo)gluco-Oligosaccharide β-Glycosides and XXXGXXXG by Nasturtium *endo*-Xyloglucanase

substrate	leaving group pK_a[a]	$k_{cat(app)}$ (min^{-1})	$K_{m(app)}$ (mM)	$k_{cat}/K_{m(app)}$ (mM^{-1} min^{-1})
GGGG-CNP	5.45	2.36 ± 0.13	8.39 ± 0.74	0.28
XXXG-CNP **1**	5.45	17.2 ± 0.20	0.487 ± 0.019	35.3
XXXG-DNP **2**	5.36	6.00 ± 0.17	0.73 ± 0.068	8.2
XXXG-PNP **3**	7.18	0.80 ± 0.01	1.07 ± 0.052	0.75
XXXG-PMP **4**	10.27	0.35 ± 0.01	0.89 ± 0.067	0.39
XXXG-XXXG	(ca. 17)	9.32 ± 0.16	0.076 ± 0.007	123
XLLG-CNP **5**	5.45	8.93 ± 0.15	0.575 ± 0.033	15.5
XLLG-DNP **6**	5.36	12.6 ± 0.21	1.06 ± 0.049	11.9
XLLG-PNP **7**	7.18	1.32 ± 0.03	1.32 ± 0.069	1.0
XLLG-PMP **8**	10.27	1.08 ± 0.09	4.63 ± 0.645	0.23

[a] Phenol pK_a values are from ref *37* or *54*.

Transglycosylation is a potential feature of all GH16 enzymes (*22*), which operate through the classic "retaining" glycosidase mechanism (*51*) involving a covalent glycosyl−enzyme intermediate (*52*). Product analysis as a function of reaction time/substrate conversion by high-performance size-exclusion chromatography (HP-SEC) indicated that *Tm*NXG1 produced a significant amount of transglycosylation products from XXXG-CNP (**2**), XLLG-CNP (**6**), XXXG-DNP (**3**), or XLLG-DNP (**7**) (Figure 3). At saturating substrate concentrations (5 mM; cf. Figure 2 and Table 1), aglycon release was time-dependent and provided a semi-quantitative measure of the degree of conversion of the XGO-CNP and -DNP substrates. Although substrate hydrolysis predominated in all cases, Glc_8-based products[2] accumulated and were subsequently degraded over the course of the reactions, with XXXG-DNP and XLLG-DNP (Figure 3b) demonstrating levels of transglycosylation similar to those of the corresponding CNP glycosides (Figure 3a). Trace

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[2] The HP-SEC system and detection method, evaporative light scattering, do not distinguish XXXG from XXXG-CNP, XXXGXXXG from XXXGXXXG-CNP, etc.

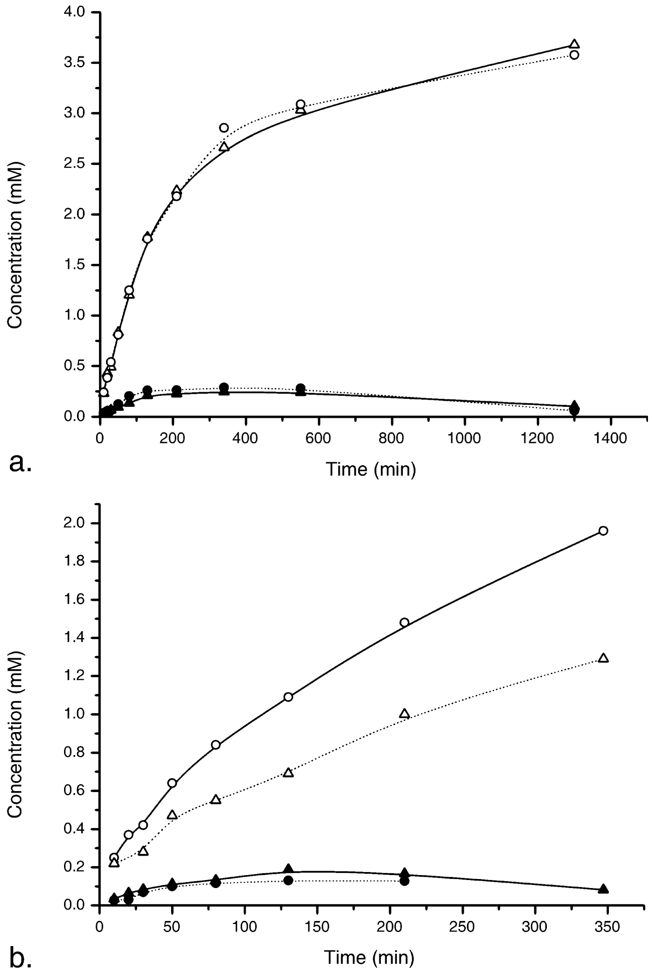

FIGURE 3: Product analysis of the conversion of 5 mM XXXG-CNP (**2**), XLLG-CNP (**6**), XXXG-DNP (**3**), and XLLG-DNP (**7**) by *Tm*NXG1 as a function of time: (a) (○) CNP released from XXXG-CNP, (●) XXXGXXXG produced with or without CNP, (△) CNP released from XLLG-CNP, and (▲) XLLGXLLG produced with or without CNP and (b) (○) DNP released from XXXG-DNP, (●) XXXGXXXG produced with or without DNP, (△) DNP released from XLLG-DNP, and (▲) XLLGXLLG produced with or without DNP.

amounts of Glc_{12}-based products were also observed in all of these cases due to successive transglycosylation events (data not shown). In contrast, the corresponding PNP and PMP glycosides yielded only hydrolysis products at all degrees of conversion, i.e., the free oligosaccharides and the aglycons.

This indicates that for substrates with comparatively good (low pK_a; see Table 1) aryl leaving groups, the relative rate of formation of the glycosyl−enzyme intermediate is fast enough, relative to the rate of breakdown, to allow accumulation at sufficient concentrations to facilitate transglycosylation. In these systems, the parameter k_{cat} will thus have a complex definition in terms of microscopic rate constants, including those involving formation of the glycosyl−enzyme intermediate and its breakdown via both transglycosylation and hydrolysis. In contrast, for substrates with poor (high pK_a; see Table 1) aryl leaving groups, k_{cat} is likely to reflect the first, rate-determining chemical step leading to formation of the intermediate. This change in rate-determining step and the potentially complex definition of k_{cat} prohibit analysis of linear free energy relationships based on k_{cat} alone. The value of k_{cat}/K_m, on the other hand, reflects the first chemical step of catalysis in all cases, i.e., formation of the glycosyl−enzyme intermediate. Although previous studies have used more extensive coverage (*36, 37, 41*), the range of leaving group pK_a values of the substrates allows an informative, minimal Brønsted analysis (*38, 40*) to be performed. Plots (not shown) of $\log(k_{cat}/K_m)$ versus phenol pK_a suggest Brønsted β_{lg} values of -0.3 ± 0.1 for both the XXXG and XLLG substrate series across the pK_a range of 5.4−10.3 (Table 1), thus indicating an asynchronous transition state typical of retaining glycosidases, in which leaving group departure preceeds nucleophilic attack by the enzyme nucleophile.

A number of other interesting features of *Tm*NXG1 catalysis emerged from the data. Comparison of the k_{cat}/K_m values of GGGG-CNP [$\beta(1\rightarrow4)Glc_4$-CNP (*32*)], XXXG-CNP, and XLLG-CNP, which all bear the "good" leaving group 2-chloro-4-nitrophenol, confirmed previous results using polysaccharide substrates that indicated *Tm*NXG1 is a specific xyloglucanase (*15, 16*). The presence of three Xyl units on the Glc_4 backbone improved selectivity by more than 2 orders of magnitude, whereas the presence of two additional Gal residues miminally attenuated catalysis. This effect of galactosylation on k_{cat}/K_m was indeed small (a factor of 2) and was not observed for other XXXG-Ar/XLLG-Ar pairs, for which k_{cat}/K_m values were nearly identical. XXXG-DNP and XLLG-DNP, whose 3,4-dinitrophenol leaving group has a pK_a nearly equivalent to that of 2-chloro-4-nitrophenol, have similar individual k_{cat} and K_m values, suggesting that the specificity for XXXG-CNP may be (slightly) anomolously high. The data nevertheless clearly indicate that *Tm*NXG1 does not discriminate against galactose, in keeping with its biological role in the mobilization of heterogeneously substituted seed galactoxyloglucan (*15*).

It is important to note that the aryl glycosides used in this study probe only catalytically productive binding interactions in the negative enzyme subsites during hydrolysis, while on the basis of structural homology with XETs, *Tm*NXG1 may also be expected to possess three positive substrate-binding subsites (*16, 42*). Initial rate kinetic analysis of the hydrolysis of XXXGXXXG by *Tm*NXG1 using HPAEC-PAD indicated that this substrate underwent the same mode of cleavage as XXXG-Ar substrates to yield two XXXG molecules. k_{cat} and K_m values (Table 1) indicate that sugar binding in the positive enzyme subsites contributes to catalysis. This same phenomenon has previously been observed in polysaccharidases, for example, *endo*-glucanases (*38*). Comparison with kinetic data for a natural mixture of variably galactosylated XXXGXXXG-based oligosaccharides [$k_{cat} = 4.28 \pm 0.14$ min^{-1}, and $K_m = 0.08 \pm 0.007$ mM (*16*)] again indicates that *Tm*NXG1 is indiscriminant with regard to the presence of subtending Gal residues, which was observed for the XXXG-Ar/XLLG-Ar pairs.

Use of Aryl Glycosides of (Xylo)gluco-Oligosaccharides in Comparative Assays of Xyloglucanases. To examine the general applicability of XGO-Ar as new probes in comparing xyloglucanase activity across CAZy families and supplementing or replacing traditional reducing sugar assays, a comparative analysis of GGGG-CNP, XXXG-CNP, and XLLG-CNP hydrolysis by a diversity of enzymes from GH5, GH12, and GH16 families was performed. Reducing sugar assays have previously indicated that *Tm*NXG1 from seeds is a poor *endo*-xyloglucanase relative to those from microbial

Table 2: Kinetics of Hydrolysis of GGGG-CNP, XXXG-CNP, and XLLG-CNP by Various *endo*-Xyloglucanases (EC 3.2.1.151) and Xyloglucan *endo*-Transglycosylases (EC 2.4.1.207)

		GGGG-CNP			XXXG-CNP			XLLG-CNP			
enzyme	mechanism, GH family	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} min^{-1})	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} min^{-1})	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} min^{-1})	ref
*Tm*NXG1	retaining, GH16	2.4	8.4	0.28	17	0.49	35	8.9	0.58	15.5	this work
*Pp*XG5	retaining, GH5	98	1.7	57	180	0.036	4900	250	0.015	16000	*20*
*Bl*XG12	retaining, GH12	45	0.86	52	9.3	1.1	8.7	8.5	3.6	2.3	*20*
*Ptt*XET16-34	retaining, GH16	nt[a]	–	–	nt[a]	–	–	nd[b]	–	–	*42*
*Bob*XET16A	retaining, GH16	nt[a]	–	–	nt[a]	–	–	nd[c]	–	–	this work

[a] Not tested. [b] Activity not detected if [*Ptt*XET16-34]$_t$ = 8 μM and [XLLG-CNP] = 1 and 2 mM in 20 mM NaOAc (pH 5.6) over 4 h. [c] Activity not detected if [*Bob*XET16A]$_t$ = 8 μM and [XLLG-CNP] = 1 mM in 20 mM NaOAc (pH 5.6) over 4 h.

sources, possibly reflecting different physiological requirements for metered versus rapid substrate saccharification. At high, near-saturating xyloglucan concentrations (1 g/L), *Tm*NXG1 had a $v_o/[E]_t$ value of 5.5 ± 0.5 min^{-1} at the pH optimum (*16*). Under similar conditions, *endo*-xyloglucanases from *Paenibacillus pabuli* (GH5, retaining) and *Bacillus licheniformis* (GH12, retaining) have $v_o/[E]_t$ values of 8700 ± 220 and 54 ± 3 min^{-1}, respectively (*20*). Similarly, the inverting microbial xyloglucanases from the GH74 family have catalytic rates 5−8000 times greater than that of *Tm*NXG1 at saturation (*10, 18, 19, 24, 53*).

The enzymes represented in Table 2 all cleave xyloglucan at unsubstituted Glc residues either to yield Glc$_4$-based xylogluco-oligosaccharides as limit digestion products [*Tm*NXG1 (*16*), *Pp*XG5 (*20*), and *Bl*XG12 (*20*)] or to catalyze xyloglucan *endo*-transglycosylation without hydrolysis [*Bob*XET16A (*44*) and *Ptt*XET16−34 (*16, 43*)]. CNP (xylo)glucosides were indeed substrates for the anomeric configuration-retaining *endo*-xyloglucanases from GH5, GH12, and GH16 families, and the k_{cat}/K_m values are indicative of both the catalytic ability and substrate specificity of these enzymes toward the natural polysaccharide. The proficient microbial xyloglucanase *Pp*XG5, which exhibited the greatest rate of xyloglucan hydrolysis, was the fastest XXXG-CNP and XLLG-CNP degrader. This enzyme slightly favored the bis-galactosylated substrate and is notably impotent toward GGGG-CNP. Analogous soluble $\beta(1\rightarrow4)$ glucans (hydroxyethyl- and carboxymethylcellulose) are not cleaved by *Pp*XG5 (*20*). In constrast, the other retaining microbial enzyme tested, *Bl*XG12, was previously shown to have little selectivity for xyloglucan over unbranched glucans and glucomannan (*20*). This catalytic flexibility was likewise reflected in the k_{cat}/K_m values for GGGG-CNP and xylosylated homologues, with a slight, but significant, inverse relationship between the degree of side chain extension and selectivity (Table 2). The xyloglucan specificity of the plant *Tm*NXG1, which hydrolyzes hydroxyethylcellulose only slowly and has no activity toward carboxymethylcellulose (*15, 16*), was confirmed by the CNP oligosaccharide series, with the k_{cat}/K_m for GGGG-CNP being ca. 2 orders of magnitude lower than for the xylosylated and galactoxylosylated congeners. Despite this specificity, the weak xyloglucanase activity of *Tm*NXG1 is nonetheless confirmed by our analysis with artificial substrates; *Tm*NXG1 is only marginally more active toward XXXG-CNP and XLLG-CNP than the nonspecific *Bl*XG12 and 2−3 orders of magnitude less active toward these substrates than *Pp*XG5 (Table 2).

Surprisingly, XXXG-CNP and XLLG-CNP were not donor substrates for the strict xyloglucan *endo*-transglycosylases (XETs) from hybrid aspen and cauliflower (Table 2). However, both enzymes are capable of using these oligosaccharides as glycosyl acceptors in the breakdown of the glycosyl−enzyme intermediate to produce transglycosylation products (ref *42* and data not shown). *Ptt*XET16-34 and *Bob*XET16A have previously been shown to be devoid of xyloglucanase activity using highly sensitive reducing sugar assays (*43, 44*). While it is at present unclear whether some form of nonproductive binding or a requirement for saccharide binding in both the positive and negative subsites is responsible for the lack of activity of true XETs toward XGO-Ar substrates (*42*), these compounds may in fact prove to be useful for delineating xyloglucanase and XET activity in the diverse subfamily of plant xyloglucan-active enzymes in GH16 (*16*).

Aryl glycosides of xylogluco-oligosaccharides are nonetheless excellent substrates for retaining xyloglucanases that are conveniently prepared in a minimal number of synthetic steps (four or five, including one or two enzymatic steps) from readily available tamarind xyloglucan, an agricultural byproduct (*8, 9*). We envision that the straightforward method of obtaining these complex substrates described here will facilitate their wider use for the screening and detailed kinetic characterization of xyloglucanase activity across carbohydrate-active enzyme families.

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SUPPORTING INFORMATION AVAILABLE

Characterization data for compounds **1**−**8**, the corresponding per-*O*-acetyl derivatives, and 2-chloro-4-nitrophenyl cellotetraose, GGGG-CNP. This material is available free of charge via the Internet at http://pubs.acs.org.

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