Active-site Mapping of a Populus Xyloglucan endo-Transglycosylase with a Library of Xylogluco-oligosaccharides

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Restructuring the network of xyloglucan (XG) and cellulose during plant cell wall morphogenesis involves the action of xyloglucan endo-transglycosylases (XETs). They cleave the XG chains and transfer the enzyme-bound XG fragment to another XG molecule, thus allowing transient loosening of the cell wall and also incorporation of nascent XG during expansion. The substrate specificity of a XET from Populus (PtxXET16–34) has been analyzed by mapping the enzyme binding site with a library of xyloglucano-oligosaccharides as donor substrates using a labeled heptasaccharide as acceptor. The extended binding cleft of the enzyme is composed of four negative and three positive subsites (with the catalytic residues between subsites −1 and +1). Donor binding is dominated by the higher affinity of the XXXG moiety (G = Glcβ(1→4) and X = Xylo(1→6)Glcβ(1→4)) of the substrate for positive subsites, whereas negative subsites have a more relaxed specificity, able to bind (and transfer to the acceptor) a cello-oligosaccharidyl moiety of hybrid substrates such as GGGGXXG. Subsite mapping with $k_{cat}/K_m$ values for the donor substrates showed that a GG-unit on negative and -XXG on positive subsites are the minimal requirements for activity. Subsites −2 and −3 (for backbone Glc residues) and +2’ (for Xyl substitution at Glc in subsite +2) have the largest contribution to transition state stabilization. GalGXXGXXG (Gal = Galβ(1→4)) is the best donor substrate with a “blocked” nonreducing end that prevents polymerization reactions and yields a single transglycosylation product. Its kinetics have unambiguously established that the enzyme operates by a ping-pong mechanism with competitive inhibition by the acceptor. The plant cell wall, a composite structure of cellulose, hemicelluloses, pectin, lignin, and structural proteins, is continually modified during cell growth and differentiation. Xyloglucan (XG) is one of the key structural polysaccharides in dicot and certain monocot cell walls and also sometimes serves as a plant energy reserve (1–3). It forms hydrogen bonds with cellulose microfibrils and provides a molecular tether between adjacent microfibrils by forming a three-dimensional cellulose-XG network (4). Loosening of these tethers from the microfibrils provides a physical control of cell expansion (5, 6). Xyloglucan consists of a cellulose-like main chain of β(1→4)-linked β-glucosyl residues, some of which are substituted at C-6 with α-D-xyllosyl and β-D-galactosyl-(1→2)-α-D-xyllosyl residues. Some of the 2-hydroxyl groups of the β-galactosyl units are α-L-fucosylated (1, 7). High molecular mass xyloglucans are hydrolyzed by microbial and plant endo-β-glucanases to xyloglucan oligosaccharides (XGOs), which constitute the repetitive structural units of the XG polysaccharide (8). The main XGOs from tamarind seed XG depolymerization are XXXG, XXLG, XLXG, and XLLG (using the established one-letter code nomenclature) (9): $G = \alpha-D-Glc, X = \alpha-D-Xyl(1→6)\beta-D-Glc, and L = \beta-D-Gal(1→2)\alpha-D-Xyl(1→6)\beta-D-Glc$. Xyloglucan endo-transglycosylases (XETs; EC 2.4.1.207) are key enzymes involved in the restructuring of the cell wall during morphogenesis (10–14). They cleave the xyloglucan chains and transfer the enzyme-bound XG fragment to another XG molecule, thus allowing transient loosening of the cell wall and also incorporation of nascent XG during expansion. XETs and xyloglucan endo-hydrolases (XEHs), encoded by the $XTH$ (xyloglucan endo-transglycosylase/hydrolase) gene family (15), have been classified in three phylogenetic groups (16), but recently, the classification has been revisited to account for the different transferase/hydrolase ratios found among $XTH$ gene products (17). XETs and a few XEHs belong to glycoside hydrolase family GH16 (18, 19), acting by the canonical double-displacement mechanism of retaining glycosidases (20, 21). It involves the participation of two catalytic carboxylic amino acid residues, a general acid/base, and a nucleophile. First, the catalytic nucleo-

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phile attacks the anomeric carbon of the substrate at the scissile glycosidic bond assisted by proton transfer from the general acid residue to form a covalent glycosyl-enzyme intermediate. In a second inverting step, an acceptor XG or XGO (in XET enzymes) or an acceptor water molecule (in XEH enzymes) attacks the intermediate after activation by general base catalysis, leading to the transglycosylation or hydrolysis product with overall retention of configuration. Formation of a glycosyl-enzyme intermediate is supported by the observation that XET from nasturtium seeds is able to form a relatively stable syl-enzyme intermediate with XG from which the enzyme can be released to be a good donor substrate and was used to kinetically characterize PttXET16–34 (35).

To undertake a detailed analysis of substrate specificity, we embarked on the preparation of a xyloglucosyl-oligosaccharide library using a chemoenzymatic approach (34) and the development of an activity assay for kinetic evaluation of low molecular mass donors (35). Here, we report the detailed kinetic analysis of donor substrates to define the specificity of PttXET16–34 by active-site mapping. Considering the extended binding site cleft comprised of positive and negative subsites, with the site of bond cleavage and transfer between subsites −1 and +1 (Scheme 1A), the donor substrates span the entire active-site cleft. All were assayed with the same acceptor compound (XXG-8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS)), so by systematically altering the sugar moieties binding the positive and negative subsites, respectively, a detailed description of substrate specificity and the contribution of subsites to binding and catalysis was obtained.

**MATERIALS AND METHODS**

**Chemicals and Enzyme—PttXET16–34** (formerly PttXET16A (36)) from *Populus tremula × tremuloides* was recombinantly expressed in *Pichia pastoris* and purified to homogeneity as described previously (36). Enzyme concentration was determined spectrophotometrically at 280 nm using a calculated ε of 72,970 M⁻¹ cm⁻¹. ANTS and sodium cyano borohydride were from Fluka. The donor library and labeled XXXG-ANTS acceptor were obtained by chemoenzymatic syntheses as reported (34, 35). Compounds 17 and 18 were obtained following the same methodology. The chemical structures of the donors and synthetic details are provided in the supplemental material.

**High Performance Capillary Electrophoresis (HPCE)—** The HPCE method was developed and validated previously (35). Capillary electrophoresis was performed on a Hewlett-Packard HP3D CE G1600 AX system with a fused silica capillary of 72-cm effective length and 50-μm internal diameter with an extended light path bubble (150 μm) in the detection window.
Electrophoretic separation was performed at −30 kV at a constant temperature of 30 °C under inverted-electro-osmotic flow conditions with 50 mM phosphoric acid adjusted to pH 2.5 with triethylamine as running buffer and UV detection at 270 nm (20-nm slit).

ANTS-derivatized Xylogluco-oligosaccharide Standards—Each donor substrate (10 nmol) was dissolved in a solution of ANTS (0.15 M) in AcOH/water (10 μL, 3:17, v/v). A solution of NaCNBH3 (1 M) in freshly purified tetrahydrofuran (10 μL) was then added, and the mixture was incubated for 7 h at 55 °C. The crude reaction was freeze-dried. Derivatized products were redissolved in water and used as HPCE standards without further purification.

Donor Concentrations—Lyophilized donor compounds purified by silica gel chromatography (34) were dissolved in water to an approximate concentration of 20 mM. The final concentration of the stock solutions to be used in enzyme kinetics was determined by HPCE. An aliquot of each stock solution was derivatized with ANTS as described above, and the concentration was determined by interpolation of peak areas to a standard curve (area versus concentration) prepared with highly pure XXXG-ANTS as used previously for HPCE method validation (35).

Enzyme Kinetics—Kinetic analysis was performed in citrate (50 mM)/phosphate (50 mM) buffer (I = 0.5 M) with added KCl at pH 5.5 and 30 °C. For $k_{cat}/K_m$ determinations, donor concentrations were between 0.3 and 1 mM, and enzyme concentrations were between 0.3 and 8.4 μM. For GalGXGGXXXG (18) kinetics, the donor was varied from 0.5 to 7 mM, the acceptor (XXXG-ANTS) from 0.3 to 7 mM, and PttXET16–34 from 0.05 to 0.2 μM. For reaction monitoring, product identification, and yields at 24 h, 1 mM donor, 5 mM acceptor (XXXG-ANTS), and 3 μM PttXET16–34 were used. In all cases, aliquots of the reaction mixture (20 μL) were taken at different time intervals, diluted with 20 μL of 2 mM Man-ANTS as internal standard, and boiled for 10 min to stop the enzymatic reaction. Samples were analyzed by HPCE, and product concentrations were calculated from the relative areas to the internal standard for each peak assuming the same response factor for all compounds because all share the same chromophore. Initial rates were calculated as the slope of product concentration versus time. Yields (%) after 24-h reactions were expressed as the concentration of each product detected relative to the maximal concentration that would be obtained for that product considering all transfer reactions leading to the same product.

RESULTS

Library of Donor Substrates and HPCE Activity Assay

A library of xylogluco-oligosaccharides has been synthesized previously (34) for donor specificity studies of XET enzymes. Here, kinetics with PttXET16–34 were analyzed to define the substrate requirements for catalysis and to map the binding site of the enzyme. Donors have been grouped in two families to probe separately the acceptor and donor subunits of the binding site cleft (Scheme 1B). Family I donors have a common nonreducing part (XXXG-) and differ on the reducing end substructure (for mapping positive subsites), whereas Family II donors differ on the nonreducing end and share a common reducing end substructure (for mapping negative subsites). Compounds 16–18 with a Gal residue on the nonreducing end are also included in Family II donors. All of these compounds were assayed as donor substrates for PttXET16–34 using a heptasaccharide derivatized with ANTS as acceptor (XXXG-ANTS). Reactions were monitored by the HPCE method developed for the separation and quantitation of ANTS-labeled transferase products (35).

All donor compounds of the library were derivatized by reductive amination with ANTS to obtain standards for HPCE analysis. Because all ANTS-derivatized xyloglucan-oligosaccharides have the same negative charge, their separation on capillary electrophoresis is based on size and shape. Under inverted electro-osmotic flow conditions at pH 2.5, the electrophoretic mobility correlates well with the log of molecular mass as shown in Fig. 1.

Donor Reactivity and Product Identification

First, enzymatic reactions at 1 mM donor and 5 mM acceptor were monitored under standard conditions (pH 5.5, 30 °C) to detect reaction products after 24 h by HPCE. Products were identified by co-injection with standard compounds (ANTS-derivatized xylogluco-oligosaccharides), and yields were calculated from the electropherograms by the relative peak areas using Man-ANTS as an internal standard (35). Results are summarized in Table 1, in which the detected products and their molar yields after 24 h are given for each donor substrate. For analysis of the transferase specificity, donors were grouped on the basis of their variable substructure on either the reducing or nonreducing end moiety.

Family I donors (compounds 1–6) share the nonreducing end substructure XXXG-, which is transferred to the acceptor. When the reducing end moiety occupies two subsites (with
-GG in compound 1 and -XG in compound 3) or has an unbranched backbone (-GGG in compound 2), a single product was detected (XXXG-XXG-ANTS) in very low yield (<8%). Donors XXXGXXG (4) and XXXGXXGXXG (5) were more reactive and yielded multiple transfer products, which arose from sequential transfer of the XXXG unit. The major product in both cases was XXXGXXG-ANTS, and higher oligomers resulted from further transfers from the donor to products as acceptors and/or donor self-transglycosylation followed by transfer to the XXXG-ANTS acceptor (Scheme 2a for compound 5). For XXXGXXGXXGXXG (6), the same XXXGXXGXXG-ANTS transglycosylation product was formed, but the higher oligomer (XXXG)₂XXG-ANTS was also produced at an initial reaction time, presumably from direct transfer of XXXGXXGXXG from donor 6 instead of (or in addition to) product elongation (see below).

A second family of donors in the library shares the -XXXG substructure on the reducing end and were designed to map the negative subsites of the enzyme binding cleft. Three subfamilies can be distinguished with a linear glucosyl chain, a branched chain, and a terminal galactosyl residue on the nonreducing end moiety of the molecules, respectively.

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### TABLE 1

Product formation and substrate reactivity for PtxXET16–34 catalyzed transglycosylations of donors 1–18 and XXXG-ANTS as acceptor.

| Donor Products | Yield at 24 h | $k_{cat}/K_m$ |
|---------------|--------------|---------------|
| XXXG-GG (1)   | XXXG-XXG-ANTS 1.4 | <0.1 |
| XXXG-GGGG (2)  | XXXG-XXG-ANTS 7.8 | <0.1 |
| XXXG-GG (3)   | XXXG-XXG-ANTS 1.1 | <0.1 |
| XXXG-XXG (4)  | XXXG-XXG-ANTS 84.1 | 57.8 ± 4.3 |
| XXXG-XXG (5)  | XXXG-XXG-ANTS 16.2 | 58.2 ± 2.9 |
| XXXG-XXG (6)  | XXXG-XXG-ANTS 29.6 | 142 ± 7.1 |
| XXXG-XXG (7)  | XXXG-XXG-ANTS 3.6 | 221 ± 16 |
| G-XGXG (8)    | XXXG-XXG-ANTS 12.0 | 344 ± 20 |
| GGG-XXXXG (9) | GXXG-XXXG-ANTS 20.8 | 0.16 ± 0.02 |
| GXXG-XXXXG (10)| GXXG-XXXG-ANTS 48.1 | 40.9 ± 2.6 |
| XG-XXXXG (11) | GXXG-XXXG-ANTS 8.9 | 1.7 |
| XG-XXXXG (12) | XG-XXG-ANTS 8.9 | 17.0 |
| XXXG-XXXG (13)| XXXG-ANT 17.6 | 37.6 |
| GXXG-XXXG (14)| GXXG-XXG-ANTS 12.0 | 41.4 |
| GXXG-XXXXG (15)| GXXG-XXG-ANTS 32.0 | 51.7 ± 6.2 |
| GXXG-XXXXG (16)| GXXG-XXG-ANTS 49.5 | 203 ± 17 |
| GaXXG-XXXXG (17)| GaXXG-XXG-ANTS 83.8 | 58.2 ± 5.3 |
| GaXXG-XXXXG (18)| GaXXG-XXG-ANTS 31.5 | 1.66 ± 0.07 |

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**Linear Glucosyl Chain—Compounds 7–10 contain one to four β(1→4)-glucosyl units on the nonreducing end. Compound 7 with only one Glc unit was a poor donor, yielding only traces of a single transfer product identified as GXXG-ANTS. Reactivity increased from two to four Glc units. For GXXGXXG (8), single transfer of the G- moiety was obtained, whereas compounds 9 and 10 showed multiple transfer products. The major transglycosylation product from GXXGXGXG (9) was that expected from GGG- transfer, but also two minor products were detected after a long incubation time, identified as GXXGXGXG-ANTS and GXXGXGXG-ANTS. Whereas the first may arise from direct transfer of the G- moiety to the acceptor, the second is unlikely to come from G- transfer, but rather is a by-product of a second transfer from the major transglycosylation product (Scheme 2b).**

For the longer GXXGXGXG (10) donor, GXXGXGXG-ANTS was formed first at the initial reaction time, but two other major compounds were obtained later, GXXGXGXG-ANTS and GXXGXGXG-ANTS, plus one minor product identified as GXXGXGXG-ANTS. Reaction monitoring (Fig. 2) showed that the first product formed partially disappeared after a prolonged incubation, whereas the others increased in concentration. Therefore, the products obtained can be explained by the sequence of transfer reactions shown in Scheme 2c.

**Branched Chain on the Nonreducing End—Compounds 11–15 have xylosyl substitutions on the β(1→4)-glucosyl backbone on the nonreducing end of the molecules. Donors**

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**SCHEME 2. Transglycosylation products of PtxXET16–34 reaction with selected donors and XXXG-ANTS as acceptor (see "Results"). The arrows indicate the glycosidic bond cleaved for transfer.**
Under initial rate conditions, a single transglycosylation event was observed for all donors, before other products were detectable at longer reaction times as discussed above (Table 1), except for XXXGCGGCGCG (6), which rendered two products even at short reaction times. Initial rates at varying donor concentrations (0.3–1 mM) and at a fixed acceptor concentration (5 mM) were determined at pH 5.5 and 30 °C. Apparent $k_{cat}/K_m$ (donor) values for the reactions leading to the first transglycosylation product were calculated as the slope of the linear dependence of the initial rate versus donor concentration and are given in Table 1.

When comparing the relative values of $k_{cat}/K_m$ with reaction yields for the major transglycosylation product after 24-h reactions (Table 1), discrepancies are noticeable for those donors that gave multiple products after long reaction times (i.e. 48% versus 69% yield (24 h) for major products from GGGGCGG (10) and XGGCGGCGG (15) when both donors have similar $k_{cat}/K_m$ values calculated from the initial rates). This is due to the consumption of the first transglycosylation product when it is involved in further transfer reactions as discussed above, resulting in a lower yield after 24 h, whereas $k_{cat}/K_m$ values reflect donor reactivity at the initial reaction time to form the first transglycosylation product.

Apparent $k_{cat}/K_m$ values were used to evaluate the contribution of individual residues in the donor substrates by applying a subsite mapping methodology (37–39). The contribution of single subsites or a combination of subsites to transition state stabilization can be calculated from the second-order rate constants $k_{cat}/K_m$ and can be expressed by the difference in transition state activation energy ($\Delta \Delta G^\ddagger$) between two homologous substrates according to Equation 1,

$$\Delta \Delta G^\ddagger = \Delta G_{n+1}^\ddagger - \Delta G_n^\ddagger = -RT \ln \left(\frac{(k_{cat}/K_m)_{n+1}}{(k_{cat}/K_m)_n}\right)$$  (Eq. 1)

where $n+1$ and $n$ indicate two substrates differing in one substituent, and $\Delta \Delta G^\ddagger$ is then the contribution of these subsites differentially occupied by both substrates to transition state stabilization or, equivalently, the effect of the substituent that differentiates both substrates with regard to transition state stabilization. For instance, compounds 5 and 15 share the -XXXG substructure binding on the positive subsites and differ only in the Xyl substituent at the Glc residue in subsite $-3$; therefore, $\Delta \Delta G^\ddagger$ calculated from their $k_{cat}/K_m$ values is the contribution of this Xyl residue to transition state (TS) stabilization (or the contribution of subsite $-3'$ to TS stabilization). Errors in $\Delta \Delta G^\ddagger$ values were estimated to be $\pm 0.1$ kcal/mol by error transmission from the standard errors of $k_{cat}/K_m$ values. Schemes 4 and 5 summarize the calculated $\Delta \Delta G^\ddagger$ values, the first for the series of substrates differing on the reducing end substructure (Family I) and the second for Family II substrates with variable non-reducing end substructures (see “Discussion”).

**Kinetics with the GalGCGGCGCG Substrate**

In a previous work (35), the kinetics of the PttXET16–34-catalyzed reaction between XXXGCGG (5) as donor substrate and XXXG-ANTS as acceptor substrate were analyzed to establish a kinetic model describing the reaction and to determine the steady-state kinetic parameters. A ping-pong mechanism with competitive inhibition by the acceptor upon binding to the free enzyme and inhibition by the donor due to binding to the acceptor subsites gave the best results. However, deviations from the model were apparent at low acceptor concentrations because of donor self-transglycosylation (5 mM) at pH 5.5 and 30 °C. Apparent $k_{cat}/K_m$ values for the reactions leading to the first transglycosylation product were calculated as the slope of the linear dependence of the initial rate versus donor concentration and are given in Table 1.

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Active-site Mapping of XET

Indeed, the 4-OH group of the Glc residue on the nonreducing end of donor 5 has the acceptor’s stereocheristry and acted as an acceptor, leading to self-condensation and elongation reactions (Scheme 2a). To prevent these side reactions and to characterize the enzyme by a simpler kinetic model, a “blocked” donor was used in this study. Donors containing a Gal unit of their nonreducing end (16 and 17) did not show oligomerization reactions (Table 1) and were good candidates for kinetic characterization. However, they were less reactive that the standard substrate XXXGXXXG (5) used in the previous work, and a new compound was designed and synthesized combining both high reactivity and blocked self-transglycosylation: GalGXXXGXXXG (18).

Compound 18 was synthesized by the chemoenzymatic methodology used to prepare the library of donor substrates (34) using the recently developed practical concept of lactosyl protection/deprotection at the 4″-OH group of donors (34, 40, 41). Starting from XXXG, a lactosyl group (GalG−) was introduced on the nonreducing end by the glycosynthase HiCel7B E197A. The resulting nonasaccharide was fully acetylated and activated as the corresponding α-glycosyl fluoride, followed by de-O-acetylation (34). It was condensed with XXXG using the HiCel7B E197A glycosynthase at 92% yield and afforded the target compound 18 in 10 steps, from lactose and tamarind seed xyloglucan, with an overall yield of 15%.

Time course monitoring of the PttXET16–34 reaction with GalGXXXGXXXG (18) and XXXGXXXG (5; reference compound) as donors and XXXG-ANTS as acceptor showed the different behavior of both donor substrates (Fig. 3). Whereas compound 5 yielded oligomers from donor self-transglycosylation and product elongation at initial reaction times, the new donor 18 gave a single transglycosylation product (GalGXXXGXXXG-ANTS) at short reaction times, reaching up to 90% yield. Only after a long incubation was partially consumed to afford two new products identified as GalGXXXG-ANTS and XXXGXXXG-ANTS (Table 1). These come from transfer of GalG- from the first transglycosylation product to the acceptor, resulting in equimolar formation of both compounds.

Because donor 18 does not undergo self-transglycosylation at short reaction times, steady-state kinetics under initial rate conditions were determined. Kinetic analyses varying both donor (0.5–7 mM) and acceptor (0.3–7 mM) concentrations were performed at pH 5.5 and 30 °C. Double-reciprocal plots (1/v versus 1/[S]) for each substrate (donor and acceptor) at different constant concentrations of the other are presented in Fig. 4 (A and B). The data fit well to Equation 2, which describes a Ping Pong Bi Bi mechanism with competitive inhibition by the acceptor upon binding to the free enzyme (Scheme 3),

where AB is the donor, C is the acceptor, \( K_{m,AB} \) is the \( K_m \) for the donor, \( K_{m,C} \) is the \( K_m \) for the acceptor, and \( K_{IC} \) is the inhibition constant for the acceptor. In contrast to the reference substrate XXXGXXXG (5), no donor inhibition was observed (Fig. 4A).

Kinetic parameters are given in Table 2, in which they are compared with those obtained for donor 5 under the same experimental conditions (35).

**DISCUSSION**

**Background**

Previously, no systematic studies of substrate specificity had been conducted for XET enzymes, mainly because of the difficulty of synthesizing substrates with sufficient structural diversity. However, early work by Fanetti et al. (28, 29) analyzed the transglycosylation and hydrolytic activities of a mixed-function XG endo-transglycosylase/endo-hydrolase from germinated nasturtium seeds with different oligosaccharides from xylogluccan hydrolysis, concluding that (a) transfer occurs from an unsubstituted Glc residue in subsite −1; (b) because GXXGXXXG underwent transglycosylation acting as a donor and an acceptor, Xyl substitutions at subsites −4 (when acting as donor) and +1 (when acting as acceptor) are not required; (c) Xyl substitution at subsite −2 is also not required because donor GXXGXXXGXXXG was hydrolyzed at the underlined Glc residue; (d) Xyl substitutions at subsites +2 and/or +3

![Figure 3. Time course monitoring of the PttXET16–34 reactions of donors XXXGXXXG (A) and GalGXXXGXXXG (B) with XXXG-ANTS as acceptor. Reaction conditions were as follows: 1 mM donor, 5 mM acceptor, and 3 μM enzyme in citrate-phosphate buffer (pH 5.5) at 30 °C.](image)

![Figure 4. Kinetics of PttXET16–34-catalyzed transglycosylation of donor 18 and the XXXG-ANTS acceptor.](image)
seem to be essential because GXXG acted as an acceptor; and (e) based on the conformation of xyloglucan by molecular modeling studies, it was hypothesized that Xyl substitutions at subsites −2 and +3 were not essential. Altogether, the initial hypothesis was that the minimal structural requirements of the substrate were a β(1→4)-Glc backbone with Xyl substitutions at subsites −3 and +2.

On the other hand, Fry et al. (11) proposed XG to be the simplest structural unit for a molecule to be an acceptor with cauliflower and mung bean enzymes. To determine which of the Xyl substitutions of XG is required, GXG and XGG were tested as acceptors but did not show any activity (42). It was concluded that both Xyl substitutions at subsites +1 and +2 were necessary, but this result was inconsistent with the hypothesis of Fanutti et al. (29), which proposed that subsite +1 did not require a Xyl substitution.

Linear oligosaccharides (cello-oligosaccharides) are not efficient acceptors; some branching is required, as seen for XETs from adzuki beans (10), pea (11, 42), and nasturtium (28) and for PttXET16–34 (17, 36). However, crude nasturtium XET extracts have been demonstrated to use cello-oligosaccharides as alternative donor and acceptor substrates together with XGOs and polysaccharides (43). These hetero-transglycosylations are likely to be due to NXG1 (nasturtium xyloglucanase 1) activity present in the nasturtium extracts (17). A barley XET isozyme was shown recently to have very low hetero-transglycosylation activity using XG/β-glucan and β-glucan/XG donor/acceptor pairs in vitro (44). Therefore, the ability to catalyze hetero-transglycosylations may be a feature of some XETs and xyloglucanases.

**Donor Library**

Here, a new library of xylogluco-oligosaccharides synthesized by chemoenzymatic approaches (34), together with a sensitive activity assay for low molecular mass donors based on HPCE (35), has allowed for a more systematic and quantitative analysis of enzyme specificity. Taking the tetradecasaccharide XXXGXXXG (5) as the reference donor substrate (35), the library of potential donors was divided in two sublibraries to analyze separately the negative and positive subsites of the binding cleft of PttXET16–34 (Scheme 1B).

**Enzyme Specificity**

Monitoring the PttXET16–34-catalyzed reactions between each donor and XXXG-ANTS as acceptor, ANTS-labeled transglycosylation products were identified, and yields after a 24-h reaction were determined. As described under “Results,” the structures of the reaction products (Table 1) defined the productive binding modes of the donors for transglycosylation, from which the details of substrate specificity were inferred.

(a) The first conclusion from the specificity analysis was that the scissile bond for transfer to the acceptor was always the glycosidic bond at an unsubstituted glucosyl unit for all donors. This is in keeping with the original conclusion by Fanutti et al. (28, 29). Moreover, our study showed that if more than one unsubstituted glucosyl unit exists in the donor, several transfer products may be detected, depending on the size and binding preferences to form multiple productive enzyme-donor complexes, but again, cleavage and transfer occurred from an unsubstituted glucosyl residue.

(b) When comparing the reactivity of donors with the same molecular mass, donors that have a xyloglucan substructure (i.e., XXXG) on the positive subsites were better substrates than those that have it on the negative subsites. This is clearly seen when comparing the yields after 24 h (as well as $k_{cat}/K_m$ values) (Table 1) between XXXG-GG (1.4%) and GG-XXXG (20.8%), XXXG-GGGG (7.8%) and GGGG-XXXG (48%), and XXXG-XG (1.1%) and XG-XXXG (30.4%). This indicates that the -XGG- moiety has a preference for the positive substrates. Also consistent with this is the observation that GalGXXXG (17) gave only GalGXXXG-ANTS as the reaction product, where -XXXG- occupied subsites +1 to +4 and the nonreducing end GalG-moiety was transferred to the XXXG-ANTS acceptor. Alternative binding in which -GG was placed on the positive substrates did not occur because GalGXXXG transfer was not detected. These results explain the observation that the x-ray structure solved for the complex of PttXET16–34 with XLLG contained the ligand on the acceptor subsites and the donor subsites were empty (30). Altogether, it is concluded that donor binding is dominated by a higher affinity of the XXXG moiety for the positive subsites than for the negative subsites.

(c) Donor substrates that have consecutive unsubstituted glucosyl units on the nonreducing end rendered different products arising from bond cleavage at each unsubstituted glucosyl unit (i.e., donor GGGGXXXG (10) in Scheme 2c), except for the terminal glucosyl unit on the nonreducing end, as shown by the poor reactivity, if any, of compound GXXXG (7). The enzyme requires occupancy of subsite −2 with either an unsubstituted or substituted Glc residue (compounds GGXXXG (8) and XGXXXG (11), respectively) for activity.

(d) Oligomerization reactions arising from multiple transfers from the donor were significant for good substrates because the initial transglycosylation product was accumulated in enough concentration to compete with XXXG-ANTS as acceptor. Moreover, the transglycosylation product may be a better acceptor because of its larger size and location of the ANTS label at a longer distance away from the binding site.

### Active-site Mapping of XET

Table 2. Steady-state parameters for GalGXXXGXXXG donor 18

| Kinetic parameter | GalGXXXGXXXG (18) | XXXGXXXXG (5) |
|------------------|------------------|--------------|
| $k_{cat}$ (s⁻¹)  | 4.8 ± 0.3        | 0.45 ± 0.04  |
| $K_{cat}K_m$ (mM) | 2.8 ± 0.2        | 0.37 ± 0.09  |
| $K_{cat}$ (mM)   | 1.1 ± 0.1        | 1.9 ± 0.3    |
| $K_m$ (mM)       | 1.7 ± 0.2        | 1.5 ± 0.4    |

* Kinetic parameters calculated by fitting initial rates to Equation 2 describing a ping-pong kinetic mechanism with competitive inhibition by the acceptor substrate (Scheme 3).

a Data from Ref. 35.
transfer product and reflect the affinity and reactivity of the donor bound to these specific subsites of the enzyme. As commonly used for glycoside hydrolases acting on polymeric substrates, subsite mapping methodologies allow the evaluation of the contribution of each individual subsite in the enzyme binding site to binding and catalysis (37–39). \(\Delta G^*\) values between pairs of substrates were calculated according to Equation 1 and are given in Schemes 4 and 5.

**Mapping Positive Subsites**—All Family I donors transfer the common XXXG- substructure to the acceptor, thus XXXG- occupies subsites \(-4\) to \(-1\). From analysis of the \(\Delta G^*\) values, the contribution of individual residues of the substrate bound to the positive subsites to TS stabilization was obtained (Scheme 4).

Substrates with a linear glucosyl chain on the reducing end (compounds 1 and 2) were poor donors, indicating that some branching on the positive subsites is required. This is in keeping with the initial observations that cello-oligosaccharides are poor donors or acceptors for XETs (10, 11, 17, 28, 42–44). The low reactivity of compounds 1 and 2, together with the conclusion that the XXXG moiety has higher affinity for the positive subsites (see above), strongly suggests that these compounds bind preferentially in the acceptor subsites and behave as inhibitors.

Compound XXXGGG (1) was a poor substrate, and addition of a Xyl substitution at subsite \(+1\) (XXXGXG (3)) did not increase reactivity. The minimal structural requirement for positive subsites was the -XXG structure because compound 4 was the smallest Family I substrate for which \(k_{cat}/K_m\) could be determined. These results are in agreement with the proposal of XGG as the minimal acceptor for the cauliflower and mung bean XETs (11). A large increase in reactivity was observed in going from XXXGXG (3) to XXXGXXG (4) (\(\Delta G^*(3\rightarrow4) = -3.8\) kcal/mol), indicating that occupancy of subsites \(+3\) and/or \(+2\) is required in the acceptor subsites. Intermediate substrates such as XXXG-XX and XXX-GXXG have not been tested to separate both contributions. However, taking into account the results of Fry et al. (11) that XGG and GXG are not acceptors (although with different XETs), it can be proposed that both Glc(\(+3\)) and Xyl(\(+2\)) are required. Nevertheless, a larger contribution can be assigned to Xyl(\(+2\)) because compound XXXGGGGG (2) with unbranched Glc units was a poor substrate. On going from XXXGXGG (4) to XXXGXXGG (5), a slight increase in reactivity was observed (\(\Delta G^* = -0.5\) kcal/mol). Therefore, Xyl(\(+3\)) and Glc(\(+4\)) have a moderate contribution to TS stabilization.

**Mapping Negative Subsites**—Similarly, analysis of \(\Delta G^*\) values for Family II donors (Scheme 5) provided information on negative subsites. Donors 7–16 share the -XXXG moiety binding to the positive sub-

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**Scheme 4.** Mapping acceptor (positive) subsites. \(\Delta G^*\) values between each pair of compounds were calculated from \(k_{cat}/K_m\) values (Equation 1) and are given in kcal/mol.

**Scheme 5.** Mapping donor (negative) subsites. \(\Delta G^*\) values between each pair of compounds were calculated from \(k_{cat}/K_m\) values (Equation 1) and are given in kcal/mol.
sites in the productive binding mode, whereas donor Gal-GXXXGGG (17) has two extra glycosyl residues on the reducing end.

The series of compounds 7-10 locate unsubstituted Glc units in the negative subsites to yield (G)_{n}XXXG-ANTS transfer products. A cellobiose moiety is the minimal requirement for activity, and reactivity increases with the length of the Glc chain on the nonreducing end. The main contribution to TS stabilization comes from the glucosyl units at subsites -2 and -3 (with $\Delta \Delta G^\ddagger \leq -1.5$ and $-2.1$ kcal/mol, respectively); Glc(-4) has a lower contribution, with $\Delta \Delta G^\ddagger(9\rightarrow10) = -0.5$ kcal/mol. The contribution of this subsite can also be obtained from the 13/14 substrate pair, which results in a slightly larger effect of $-0.8$ kcal/mol. This difference illustrates that subsites are not independent and that energy contributions are not fully additive.

Xylosyl substituents in the negative subsites have a lower contribution than the main chain Glc residues, with $\Delta \Delta G^\ddagger$ values ranging from $-0.3$ to $+0.3$ kcal/mol. Xyl(-2') has a small effect, with $\Delta \Delta G^\ddagger = -0.2$ or $+0.1$ kcal/mol, calculated from the 8/11 or 12/13 substrate pair, respectively (values close to experimental error). The largest contribution comes from Xyl(-3'), with $\Delta \Delta G^\ddagger = -0.3$ kcal/mol, the same value obtained for the 9/12 or 15/5 substrate pair. Although it has a significant contribution, the xylosyl residue in subsite -3' is not essential for activity, which contradicts an initial hypothesis of substrate specificity (28, 29), emphasizing the importance of Xyl substitutions at subsites -3 and +2. On the other hand, Xyl(-4') seems to elicit some destabilizing effect because compound GXXGXXXXG (14) has a higher $k_{cat}/K_m$ than the reference compound XXXGXXXXG (5), which results in $\Delta \Delta G^\ddagger = +0.2$ kcal/mol. Moreover, the first transglycosylation product from compound 14 accumulates faster and reacts as an acceptor, leading to secondary products in high yields; it results in an apparent yield for the first transglycosylation product after 24 h that is lower than that obtained for compound 5.

The inclusion of a Gal residue on the nonreducing end prevents the donor from acting as an acceptor due to the axial C-4 hydroxyl group. Compounds GalGXXG (16) and Gal-GXXGGGG (17) yielded a single transglycosylation product even after long incubation times. For both compounds, the Gal residue in the productive complex is located in subsite -2. Compared with the homologous GXXGXXXG (8) donor, (Scheme 5B), the Gal-for-Glc substitution has a small effect ($\Delta \Delta G^\ddagger(8\rightarrow16) = -0.2$ kcal/mol). Addition of a cellobiose unit on the reducing end has no further effect ($\Delta \Delta G^\ddagger(16\rightarrow17) = 0$) because the Glc residues lay outside of the binding site cleft, which is composed of four negative and three positive subsites as observed in the x-ray crystal structure of PttXET16-34 in complex with the XLLG ligand (30).

The limits of the binding site cleft are often not well defined because other surface interactions with the substrate at the edges of the binding cavity may contribute to binding. This is notably observed in endo-depolymerases, in which longer oligomers are often better substrates due to additional binding interactions at specific surface regions of the enzyme (21, 45). Compound XXXGXXGXXGXXG (6) shows two productive binding modes corresponding to transfer of XXXG- and XXXGXXXG- at initial reaction times (Table 1). The second transfer occurs with a higher $k_{cat}/K_m$ corresponding to $\Delta \Delta G^\ddagger(5\rightarrow6) = -0.5$ kcal/mol (Scheme 5C), which reflects some additional interactions between enzyme and substrate after subsite -4. Likewise, compound GalGXXGXXGXXG (18) also shows a significant stabilizing effect of the lactosyl moiety after subsite -4 ($\Delta \Delta G^\ddagger(5\rightarrow8) = -0.6$ kcal/mol). In contrast, other interactions contributing to binding and TS stabilization on the reducing end (after subsite +3) are less significant, as observed for transfer of XXXG from compound 6 compared with compound 5 ($\Delta \Delta G^\ddagger(5\rightarrow6) = -0.2$ kcal/mol) (Scheme 5C) and the same reactivity for compounds 16 and 17 (Scheme 5B). Therefore, the enzyme takes advantage of additional interactions outside the binding cleft on the nonreducing end to improve the catalytic efficiency with polymeric substrates.

Although it has been repeatedly stated that donor substrates for most XETs must have molecular masses $>10$ kDa (15, 46) based on data for one isoform (10, 47), the mapping data reported here clearly show that low molecular mass xyloglucosyloligosaccharides are good donor substrates for PttXET16-34. Similarly, nasturtium NXG1 also efficiently uses Glc$_8$-based XGO donors (17, 28, 29). Considering the available structural and functional data, this is likely to be a general feature for GH16 XETs and XEHs; the binding site cleft (composed of seven subsites in the case of PttXET16-34) recognizes short oligosaccharide fragments, whereas the rest of the chain in polymeric substrates extends past the enzyme, where some additional interactions on the enzyme surface may potentially contribute to catalytic efficiency.

**Simplified Ping-Pong Kinetics with the GalGXXGXXGXXG Donor Substrate**

A kinetic ping-pong mechanism was established for PttXET16-34 from steady-state kinetics with XXXGXXXXG (5) and XXXG-ANTS as donor and acceptor substrates, respectively (35). The kinetic scheme accounted for donor and acceptor inhibition, which was significant even at low substrate concentrations. Because PttXET16-34 has no detectable hydrolase activity (36), the scheme did not include a hydrolysis branch, as was the case for the mixed-function nasturtium XET/XEH, for which kinetics with xyloglucan rendered a rather complex modified ping-pong mechanism (17, 48). The use of defined low molecular mass substrates provides a better understanding of the kinetic mechanism because simple processes take place as opposed to reactions with xyloglucan, where the heterogeneity of the donor, multiple transglycosylation events, oligomerizations, and hydrolysis (in the case of the nasturtium enzyme) render complex behaviors from which only apparent kinetic parameters can be derived.

Even though the donor XXXGXXXXG (5) gave kinetics that supported the ping-pong mechanisms for PttXET16-34, data at high donor and low acceptor concentrations deviate from the model because of significant donor self-transglycosylation (35). With the blocked donor GalGXXGXXGXXG (18), a simplified mechanism was obtained. Although other side reactions took place after long reaction times (Fig. 3B), by-products were formed only after accumulation of the first transglycosylation product, which reached up to 90% yield. Therefore, side reac-
tions did not take place (or were negligible) under initial rate conditions in which <10% of the substrate was consumed.

A kinetic model that better explains the experimental data is a Ping Pong Bi Bi mechanism with competitive inhibition by the acceptor (Scheme 3). Notably, no donor inhibition was observed as in the case of substrate XXXGXXXG (5), which arose from binding to the acceptor subsites, leading to self-transglycosylation. Comparing the steady-state parameters of both donors with the XXXG-ANTS acceptor (Table 2), the $K_m$ for the acceptor and the inhibition constant ($K_i$) for the acceptor binding to the free enzyme are equal, as expected. The 10-fold higher $k_{cat}$ value for compound 18 reflects both the higher reactivity of compound 18 (as seen from the $k_{cat}/K_m$ values) and the lack of self-transglycosylation and oligomerization reactions with the Gal substrate; for substrate 5, a fraction of the glycosyl-enzyme intermediate is consumed in donor self-transglycosylation (observed as donor inhibition by binding to the acceptor subsites), decreasing the rate of formation of the initial transglycosylation product from donor (5) to acceptor (XXXG-ANTS). Donor 18 has a higher $K_m$ than donor 5. Assuming a similar binding for both substrates (or even slightly better for compound 18 as discussed above), the higher $K_m$ value reflects a lower steady-state concentration of the glycosyl-enzyme intermediate, consistent with its higher reactivity.

Conclusions

A detailed picture of substrate specificity for PttXET16–34 is described, illustrating the requirements of branched xyloglucosidases for binding and catalysis. Donor binding is dominated by the higher affinity of the XXXG moiety for positive enzyme subsites than for negative subsites. Although cellobiose oligosaccharides are not donor or acceptor substrates, hybrid cello-xyloglucan oligosaccharides that contain the XXXG motif which binds to the positive subsites are able to transfer a cello-oligosaccharide moiety to an acceptor (e.g. GGGGXXXXG (10)). The rather relaxed specificity on the negative subsites, together with the preference for the xyloglucan substructure XXXG on the positive subsites, may have a physiological role because XETs may be able to select cleavage sites on the xyloglucan polymer depending on local fine structure. The contribution of the different subsites to TS stabilization is summarized in Scheme 6. The size of the binding site cleft observed in the x-ray structure of PttXET16–34 (30) suggests four subsites on the negative plus three subsites on the positive sites for the Glcβ(1→4) backbone of XG or XGOs. Kinetic analysis is consistent with at least four negative subsites and shows that a cellooligosaccharide is the minimal requirement for activity and that subsites −2 and −3 have the largest contribution to TS stabilization. On the acceptor side, a putative subsite +4 is not observed, and the main contribution comes from the Xyl substituent at subsite +2. In conclusion, it can be predicted that the minimal donor substrate for PttXET16–34 will be GG-XXG, with the dash being the bond cleaved for transfer to an acceptor.

GalGXXXGXXXG (18) is the best donor substrate studied here, with the highest $k_{cat}/K_m$ value leading to a single transglycosylation product that accumulates up to 90% yield. Because donor self-condensation and product elongation are prevented by the incorporation of a Gal residue on the nonreducing end, this donor substrate renders a simpler kinetic model for characterization of XET enzymes. In the absence of a saccharide acceptor, the glycosyl-enzyme formed by the action of PttXET16–34 on the blocked substrates 17 and 18 accumulates. As reported in the following article (23), this phenomenon provided a unique opportunity to demonstrate the covalent nature of this intermediate and to calculate the free energy of formation of the wild-type glycosyl-enzyme in an electronically unperturbed system.

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