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Xyloglucan xyloglucosyl transferases from barley (*Hordeum vulgare* L.) bind oligomeric and polymeric xyloglucan molecules in their acceptor binding sites

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ABSTRACT

Background: Xyloglucan xyloglucosyl transferases (EC 2.4.1.207), known as xyloglucan endotransglycosylases (XETs) use a disproportionation reaction mechanism and modulate molecular masses of xyloglucans. However, it is not known precisely how these size modulations and transfer reactions occur with polymeric acceptor substrates.
Methods: cDNAs encoding three barley HvXETs were expressed in *Pichia pastoris* and reaction mechanism and molecular properties of HvXETs were investigated.
Results: Significant differences in catalytic efficiencies ($k_{cat}\cdot K_m^{-1}$) were observed and these values were 0.01, 0.02 and 0.2 $s^{-1}\cdot mg^{-1}\cdot ml$ for HvXET3, HvXET4 and HvXET6, respectively, using tamarind xyloglucan as a donor substrate. HPLC analyses of the reaction mixtures showed that HvXET6 followed a stochastic reaction mechanism with fluorescently or radioactively labelled tamarind xyloglucans and xyloglucan-derived oligosaccharides. The analyses from two successive reaction cycles revealed that HvXET6 could increase or decrease molecular masses of xyloglucans. In the first reaction cycle equilibrium was reached under limiting donor substrate concentrations, while xyloglucan mass modulations occurred during the second reaction cycle and depended on the molecular masses of incoming acceptors. Deglycosylation experiments indicated that occupancy of a singular N-glycosylation site was required for activity of HvXET6. Experiments with organic solvents demonstrated that HvXET6 tolerated DMSO, glycerol, methanol and 1,4-butanediol in 20% (v/v) concentrations.
Conclusions: The two-phase experiments demonstrated that large xyloglucan molecules can bind in the acceptor sites of HvXETs.
General significance: The results characterise donor and acceptor binding sites in plant XET, report that HvXETs act on xyloglucan donor substrates adsorbed onto nanocrystals and that HvXETs tolerate the presence of organic solvents.

1. Introduction

Plant cell walls are complex and dynamic supra-molecular assemblies that contain crystalline cellulose microfibrils embedded in a matrix of amorphous non-cellulosic polysaccharides such as pectins, heteroxylans, xyloglucans, (1,3;1,4)-β-D-glucans and other polysaccharides, as well as proteins and inorganic molecules [1]. Xyloglucans are a diverse group of polysaccharides composed of a backbone of (1,4)-β-linked glucosyl residues, which are substituted at C(O)6 with α-D-xylopyranosyl residues or with short oligosaccharide chains of α-D-xylopyranosyl, β-D-galactopyranosyl and occasionally with α-L-fucopyranosyl residues [2]. The tamarind xyloglucan main chain has a ribbon-like shape and fibre diffraction data indicate that it adopts a twofold helical conformation, although its conformation in aqueous media has not been determined [3].

The molecular masses of xyloglucan molecules can be altered during their depositions in cell walls [4,5], and this process is thought to be mediated by enzymes known as xyloglucan endotransglycosylases/hydrolases (XTHs). These widely distributed plant enzymes are classified in the glycoside hydrolases GH16 group [6], and can have either xyloglucan endotransglycosylase (XET) activity, or both XET and xyloglucan endohydrolase (XEH) activities [7,8].

Models of plant cell walls postulate that cellulose microfibrils and xyloglucans interact by non-covalent interactions that constitute the

Abbreviations: a.u., arbitrary units; DMBA, dimethylamine borane complex; DMSO, dimethyl sulfoxide; DPM, Disintegration per minute; ELSD, evaporative light scattering detection (detector); GH16, family 16 glycoside hydrolase; IMAC, Immobilized Metal Affinity Chromatography; MS, mass spectrometry; PABA, *p*-aminobenzoic acid; PDB, Protein Data Bank; SE, size-exclusion; TXG, tamarind xyloglucan; XEH, xyloglucan hydrolase; XET, xyloglucan endotransglycosylase; XGOs, xyloglucan-derived oligosaccharides; XTH, xyloglucan transglycosylase/hydrolase; XXXG, xyloglucan-derived heptasaccharide [Xyl*p*(α1,6)]-Glc*p*(β1,4)-[Xyl*p*(α1,6)]-Glc*p*(β1,4)-[Xyl*p*(α1,6)]-Glc*p*(β1,4)-Glc*p*; XXXGol, reduced XXXG

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major load-bearing force in the primary cell walls [9]. Computational models suggest that these interactions occur through hydrogen bonds, van der Waals and electrostatic interactions, and are formed between flat, outstretched sugar residues of xyloglucan and cellulosic chains [10]. However, recent evidence [11] supports earlier suggestions that xyloglucans might also be covalently linked to pectins and other polysaccharides [9]. To this end, Hrmova et al. [12] have shown that a highly purified barley xyloglucan endotransglycosylase (XET) or xyloglucan xyloglucosyl transferase (EC 2.4.1.207) can catalyse the *in vitro* formation of covalent linkages between xyloglucan oligosaccharides and cellulosic polysaccharides. More recently, Fry et al. [13] reported that a transglycosylase from the horsetail *Equisetum* showed a marked preference for (1,3;1,4)-β-D-glucans as a donor substrate, as compared with xyloglucans, although the preferred acceptor substrate was a xyloglucan-derived oligosaccharide (XGO). However, at this stage the functional relevance of these observations is hypothetical with respect to the function of the XETs *in muro*, and the existence of covalently linked cellulose and xyloglucan has yet to be demonstrated [13].

The reaction mechanism of XETs proceeds in two steps, where XETs first cleave the (1,4)-β-D-glucan backbone of xyloglucan. The non-reducing terminal product remains covalently linked with the enzyme's surface and during the 2nd step XETs transfer the non-reducing portion of the original xyloglucan substrate onto another xyloglucan molecule [7,15]. The xyloglucan molecule that is initially cleaved by the enzyme is referred to as the donor substrate, while the incoming xyloglucan molecule to which the cleaved product is transferred, is known as the acceptor substrate. It has been generally accepted that during transglycosylation reactions, XETs use a disproportionation reaction mechanism and modulate molecular masses of xyloglucans, such that some will increase in mass, while others will decrease in mass [2,15,16]. However, it is not known precisely how these size modulations are mediated and what parameters control it, as well as it has not been clearly demonstrated how transfer reactions occur, particularly with polymeric acceptor substrates. There is also uncertainty as to how the xyloglucan polymer is cleaved by typical XETs in terms of polymer topology. In other words, do XETs cleave xyloglucan molecules through a random or stochastic reaction mechanism along the chain [2,15], or could XETs preferentially recognize reducing or non-reducing termini of xyloglucans [17], at least during the initial catalytic stages? One would anticipate that if the role of XETs was to increase the actual size of xyloglucans during their deposition in the cell walls, then the enzyme would have to cleave the donor xyloglucan polymer close to its reducing terminus and if it did not then the net effect would only be to broaden the molecular mass distributions of the xyloglucans in the reaction mixture. The other way of producing an increase in xyloglucan molecular mass would be if xyloglucans were cleaved randomly, but preferentially transferred onto larger acceptors.

In the current work, the molecular properties of three xyloglucan xyloglucosyl transferases HvXET3, HvXET4 and HvXET6 from barley (*Hordeum vulgare* L.), expressed heterologously in *Pichia pastoris*, are described. We report a detailed characterization of the reaction mechanism of HvXET6, which represents one of the most abundant XETs in young barley seedlings [14], using chemically labelled xyloglucans. We describe the reaction mechanism of HvXET6 during two successive reaction cycles and show that HvXET6 can modulate molecular masses of xyloglucans, after equilibrium is established during the first reaction cycle. Thus, we show that average molecular masses of xyloglucans can be increased and that this net increase resulted from accepting larger xyloglucan acceptor substrates during transglycosylation reactions. Further, we report that HvXETs catalyse transglycosylation reactions with xylooligosaccharides and xyloglucan donor substrates adsorbed onto nanocrystals. Finally, through experiments using a range of organic solvents, it is shown that HvXET6 was active in the presence of 20% (v/v) DMSO, glycerol, methanol and 1,4-butanediol, and that the enzyme activity of HvXET6 was increased in the presence of 20% (v/v) methanol.

2. Materials and methods

2.1. Materials

Tamarind xyloglucan (TXG), xyloglucan-derived heptasaccharide XXXG, its reduced form XXXGol, barley (1,3;1,4)-β-D-glucan (medium viscosity) and (1,3;1,4)-β-D-glucan endohydrolase from *Bacillus subtilis* were obtained from Megazyme (Bray, Ireland). Xyloglucan-derived octasaccharides XXLG and XLXG and nonasaccharide XLLG, and their reduced [^{3}H]-labelled forms were prepared as described previously [18]. *p*-Aminobenzoic acid (PABA), dimethylamine borane complex (DMAB) and cellulose were obtained from Sigma Chemical Company (St. Louis, MO, USA). A suspension of cellulose nanocrystals from parenchyma cellulose of sugar beet pulp was provided by Dr Laurent Heux (CNRS, Grenoble, France).

2.2. Cloning, expression and purification of HvXET3, HvXET4 and HvXET6

The cDNAs of HvXET3 (GenBank accession number P93671) and HvXET4 (P93672) were cloned using standard techniques, as described previously for HvXET6 [14]. The pPICZα constructs containing cDNAs of the HvXETs, lacking signal peptides, were obtained from Dr Andrew Harvey (University of Adelaide) and were expressed in *Pichia pastoris* [14]. Secreted HvXET enzymes were dialysed in 50 mM ammonium acetate buffer, pH 7.8, and affinity purified by Immobilized Metal Affinity Chromatography (IMAC) on the Co^{2+}-charged Talon resin (Clontech, Mountain View, CA, USA) as follows. The dialysed HvXET preparations were applied onto the column matrix, and bound proteins were washed with five column volumes of ammonium acetate buffer, pH 7.8, followed by elution of proteins with a 0–200 mM imidazole gradient in 50 mM ammonium acetate buffer, pH 7.8. The fractions (0.5 ml) were collected and their enzyme activities were measured using a colorimetric assay [18]. The active fractions were pooled and concentrated using Amicon centrifugal filter units from Millipore (Bedford, MA, USA), followed by 16 h dialysis in 50 mM ammonium acetate buffer at pH 6.0. All purification steps were conducted at 4 °C. The dialysed proteins were filtered through 0.2 μm filters (Millipore) and sampled into sterile test tubes. Purified HvXETs were stored at 4 °C and were stable for at least for 6 months (data not shown).

2.3. Enzyme assays, protein determination and SDS-PAGE

Xyloglucan transglycosylase activity was measured by a radiometric method, which quantified the incorporation of the radioactive [1-^{3}H]-labelled xyloglucan-derived heptaitol XXXGol into TXG. The incorporation of [1-^{3}H]-XXXGol was measured by scintillation counting [12]. Hydrolytic activity of a barley β-D-glucan glucohydrolase was measured as described by Hrmova et al. [19] and that of a *B. subtilis* (1,3;1,4)-β-D-glucan endohydrolase with 0.2% (w/v) barley (1,3;1,4)-β-D-glucan [20]. Enzyme activity measurements were performed in duplicate at 30 °C. The effects of organic solvents on the activities of the two hydrolases were measured at 20% (v/v) final concentrations of dimethyl sulphoxide (DMSO), glycerol, methanol, 1,4 butanediol and acetone. Protein concentrations were estimated using the Coomassie G-250 detection reagent from Thermo Scientific (Rockford, IL, USA) and bovine serum albumin (Sigma) as the standard. SDS-PAGE and protein staining were performed as described by Hrmova et al. [12].

2.4. Preparation of fluorescently labelled TXG with PABA

Reductive amination [21] was used for labelling TXG with *p*-aminobenzoic acid (PABA) in the presence of dimethylamine borane complex (DMAB). The PABA reagent was prepared by dissolving 14 mg PABA in 5 ml glacial acetic acid to yield a 20 mM solution, and