Research article

Molecular insights of a xyloglucan endo-transglycosylase/hydrolase of radiata pine (PrXTH1) expressed in response to inclination: Kinetics and computational study

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ABSTRACT

Xyloglucan endotransglycosylase/hydrolases (XTH) may have endotransglycosylase (XET) and/or hydrolase (XEH) activities. Previous studies confirmed XET activity for PrXTH1 protein from radiata pine. XTHs could interact with many hemicellulose substrates, but the favorite substrate of PrXTH1 is still unknown. The prediction of union type and energy stability of the complexes formed between PrXTH1 and different substrates (XXXGXXXG, XXFGXXFG, XLFGXLFG and cellulose) were determined using bioinformatics tools. Molecular Docking, Molecular Dynamics, MM-GBSA and Electrostatic Potential Calculations were employed to predict the binding modes, free energies of interaction and the distribution of electrostatic charge. The results suggest that the enzyme formed more stable complexes with hemicellulose substrates than cellulose, and the best ligand was the xyloglucan XLFGXLFG (free energy of $-58.83 \pm 0.8$ kcal mol$^{-1}$). During molecular dynamics trajectories, hemicellulose fibers showed greater stability than cellulose. Additionally, the kinetic properties of PrXTH1 enzyme were determined. The recombinant protein was active and showed an optimal pH 5.0 and optimal temperature of 37 °C. A Km value of 20.9 mM was determined for xyloglucan oligomer. PrXTH1 is able to interact with different xyloglycans structures but no activity was observed for cellulose as substrate, remodeling cell wall structure in response to inclination.

1. Introduction

The loss of vertical growth in trees triggers molecular and biochemical events, which leads morphological changes to restore verticality (Muday, 2001; Ramos and Herrera, 2013). These events involve the perception of gravity and its subsequent transduction into physiological signals inducing differential growth along the stem or affected organ. It promotes cell wall loosening, due to cell wall modifications orchestrated by a battery of enzymes. In pine tree, in order to understand the molecular gravitropic response the enzymes involved in these modifications have been studied\textsuperscript{3−6}. Several genes were differentially expressed in inclined radiata pine seedlings depending on time of inclination or the stem side (Ramos et al., 2012, 2016; Valenzuela et al., 2014; Mateluna et al., 2017; Gomez et al., 2018). Among them, PrXTH1 was identified to be highly expressed in the lower side of inclined stems (Valenzuela et al., 2014), suggesting an active participation of this enzyme in cell wall reorganization.

Plant cell walls are a complex supra-molecular assembly that displays diverse structures. Thus, the crystalline cellulose microfibrils are embedded in a matrix of amorphous non-cellulosic polysaccharides such as pectins and hemicelluloses, such as heteroxylans, xyloglucans (XG), (1,3; 1,4)-β-D-glucans, galactomannans/glucomannans, and other polysaccharides, as well as inorganic molecules and proteins (Carptia et al., 2000). In higher plants, the most common XG are polymers of the following repetitive units: XXXG, XXLG, XLXG, XXFG, XLXLG and XLFG (Hoffman et al., 2005; Hsieh and Harris, 2009; Peña et al., 2008), where each letter represents a type of substitution at C (O) 6 in the backbone of glucosyl residues, so the α-D-xylopyranosyl residues are indicated with X letter, α-D-xylopyranosyl-β-D-galactopyranosyl are indicated with L letter or α-D-xylopyranosyl-β-D-galactopyranosyl-α-L-fucopyranosyl oligosaccharides are indicated with F letter (Fry et al., 1993; Tuomivaara et al., 2015).

Xyloglucan endotransglycosylase/hydrolase (XTH) enzymes hold transglycosylase (XET), hydrolase (XEH), or both activities (Campbell
XET Activity was assayed as Sulová et al. (1995) at pH 5.5. Values correspond to mean ± SE of four replicates.

**Table 2**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (mL)</th>
<th>Protein (mg/mL)</th>
<th>XET activity (a.u. µg⁻¹ h⁻¹)</th>
<th>XEH activity (a.u. µg⁻¹ h⁻¹)</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentrated crude extract</td>
<td>10</td>
<td>7.06</td>
<td>2.9 ± 1.1</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td>Anion exchange chromatography</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eluted protein (0.5 M NaCl)</td>
<td>10</td>
<td>6.08</td>
<td>20.03 ± 2.4</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Affinity chromatography</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eluted purified protein (25 mM imidazole)</td>
<td>2</td>
<td>5.01</td>
<td>37.86 ± 3.3</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Cellulase from A. niger a</td>
<td>–</td>
<td>–</td>
<td>0.0 ± 1.1</td>
<td>65.7 ± 4.0</td>
<td>–</td>
</tr>
<tr>
<td>Empty vector pPICZaA</td>
<td>–</td>
<td>–</td>
<td>4.24 ± 2.6</td>
<td>7.4 ± 2.7</td>
<td>–</td>
</tr>
</tbody>
</table>

**Table 3**

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Affinity energy (local ads –1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>XLFGXLFG</td>
<td>–11.1</td>
</tr>
<tr>
<td>XXXGXXXG</td>
<td>–10.7</td>
</tr>
<tr>
<td>XFGXXXXG</td>
<td>–10.0</td>
</tr>
<tr>
<td>cellodextrin 8-mer</td>
<td>–6.9</td>
</tr>
</tbody>
</table>

**Table 1**

Purification of recombinant PrXTH1 protein expressed in _P. pastoris._

**2. Methods**

**2.1. PrXTH1 protein production**

The cloning of _PrXTH1_ was previously reported by Valenzuela et al. (2014) (Valenzuela et al., 2014). The transformed _E.coli_ was used for the production of recombinant PrXTH1 protein, which was carried out according to the method employed by Mendez-Yañez et al. (2017) (Mendez-Yañez et al., 2017). Briefly, the gene was expressed in _P. pastoris_ and the production of protein was induces by methanol. The PrXTH1 protein was purified in two steps. First, by using a cation exchange chromatography (CMC, carboxymethyl cellulose resin, Sigma) in 50 mM sodium phosphate buffer (pH 6.0), and elution with 0.5 M NaCl; then, desalting through PD-10 columns (Sephadex™ G-25
Medium) to remove salt and remaining CMC. The second step considers the use of an affinity chromatography (Talon Metal Affinity column, BD Biosciences) in the presence of 50 mM sodium phosphate buffer (equilibrium buffer at pH 7.4, washing buffer at pH 6.8), and finally protein was eluted with 0.025 M imidazole (pH 6.0). Protein concentration was determined according to Bradford method using bovine serum albumin as standard (Bradford, 1976). FcXTH1 protein expressed in yeasts was used as positive control, and the protein was purified using the same protocol (Mendez-Yañez et al., 2017).

2.2. Colorimetric XET assay

Enzyme activity was assayed by the colorimetric method described previously (Bradford, 1976), which is based on the overall XG-degrading activity (XDA) in the presence (XDAOS) or absence (XDAO) of XG oligosaccharides (XGOs). The XDAO activity was measured in a reaction mixture of 200 μL total volume consisting of 0.2 mg tamarind xyloglucan (Megazyme) in 200 mM sodium acetate buffer (pH 5.5). XDAOS was measured in the presence of 0.10 mg mL⁻¹ XGOs (heptasaccharide X₇Glc₄, molecular weight 1062.9 g mol⁻¹, Megazyme). The reaction was initiated by the addition of 50 μg protein extract. The mixture was incubated during 2 h at 37 °C with gentle shaking. Aliquots (30 μL) were removed at the end of the incubation period. To each aliquot, 300 μL of 20% (w/v) Na₂SO₄ and 30 μL of I₂–KI solution (0.5% I₂ + 1% KI) were added, and after 30 min in the dark at room temperature changes in the absorbance at 620 nm were determined in an Epoch 2 microplate spectrophotometer (BioTek). The XG-transglycosylating (XET) activity was expressed as arbitrary units (a.u.) ug protein⁻¹ h⁻¹ as described by Sulová et al. (1995) (Sulová et al., 1995). In our assay conditions, the reaction rate remains constant during the first 4 h of incubation, and therefore incubation periods of 2 h were adopted. Determinations were performed in four replicates and values correspond to means ± SE. Additionally, PrXTH1 inactivated enzyme by temperature (100 °C by 10 min) was used as negative control.

2.3. XEH activity assay

A sensitive reducing sugar assay was recently implemented to determine XTH activity (Arnal et al., 2017). The increment of the reducing sugars by BCA was followed through changes in absorbance at 562 nm determined in an Epoch 2 microplate spectrophotometer. Glucose was used as standard (0–50 μM). All measurements were performed in four replicates. PrXTH1 inactivated enzyme (by temperature, 100 °C by 10 min) was used as negative control.

2.4. pH profile and optimal temperature

To determine optimum assay conditions, pH was modified from 4 to 7, and the temperature ranged from 30 to 42 °C according to Mendez-Yañez et al. (2017) (Mendez-Yañez et al., 2017). XET activity was expressed in relative terms as % of the highest activity.

2.5. Kinetic parameters: $K_{mo}$, $V_{max}$ and $k_{cat}$

To determine kinetic parameters Michaelis–Menten saturation curves were built using XGOs as substrate (from 0.0 to 0.20 mg mL⁻¹) under standard assay conditions. Kinetic parameters were calculated from nonlinear least-squares data fitting (NLSF) in GraphPad Prism 6, using a Hanes-Woolf plot. Determinations were performed in quadruplicates and expressed as mean ± SE.

2.6. Protein-ligand interactions

Docking studies were performed to predict the putative binding between the PrXTH1 protein model previously described by Valenzuela et al. (2014) (Valenzuela et al., 2014) and different ligands: three hemicellulose octasaccharides (XXXGXXXG, XXFGXXFG, and XLFGXLFG) and one cellodextrin 8-mer that resembles a water-soluble cellulose molecule employed as negative control. The four ligands were built using GlyCAM-Web software (http://glycam.ccr.columbia.edu/), and ParmaChem (Vanommeslaeghe et al., 1992) was used to provide and check the force field parameters required for the ligands. Autodock Vina v1 program (http://vina.scripps.edu/)(Trott and Olson, 2010) was used for the analysis.

Molecular Dynamics Simulation (MDS) of each substrate inside the active site of PrXTH1 was performed using NAnoscale Molecular Dynamics (NAMD) 2.12 (Phillips et al., 2005) with Chemistry at HARvard Macromolecular Mechanics (CHARMM) version 27 (MacKerell et al., 1992) force field for the protein and CHARMM version 36 (Guvench et al., 2009) for the carbohydrates, along with the TIP3P model for water (Jorgensen et al., 1983). The initial coordinates for MDS calculations were taken from the docking experiments. All simulations were performed during 50 ns according to the method employed by Gaete-Eastman et al. (2015) (Gaete-Eastman et al., 2015). During MDS, motion equations were integrated with a 2 fs time step in the canonical ensemble (NVT), with constant and pre-defined value of
the number of molecules (N), box size or volume (V) and temperature (T). The data were collected every 10 ps. Two replicates were used for each system. MM-GBSA (MM, Molecular Mechanics; GB, Generalized Born; SA, Surface Area) method (Homeyer and Gohlke, 2012) was employed according to Valenzuela-Riff et al. (2015) to estimate the relative free binding energy of protein-ligand complexes. Visualization of protein-ligand complexes and MD trajectory analyses were carried out with Visual Molecular Dynamics (VMD) v1.9.1 software (Humphrey et al., 1996).

2.7. Electrostatic potential

Using the adaptive Poisson–Boltzmann solver (APBS) (Baker et al., 2001) the electrostatic surfaces of PrXTH1 in complex with each of the four ligands was analyzed. A potential scale from −5 to +5 KT/e was employed in order to use the APBS software within VMD software.

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**Fig. 3. MDS analysis of the PrXTH1 with the three hemicelluloses and cellulose as ligand.** In A) to D) a graph indicating the percentage of time that a hydrogen bond between particular residue and the ligand is established during the MDS; the catalytic residues are shown in black, only values greater or equal of 15% frequency are shown in the graph. In E) to H) The number of the hydrogen bonds established during MDS between the ligand and different amino acid residues located in the open groove of PrXTH1.
3. Results and discussion

3.1. Characterization of PrXTH1 recombinant protein

*P. pastoris* yeast strains previously transformed with PrXTH1 gene (Valenzuela et al., 2014) were induced with methanol for the expression of the recombinant protein. The authors purified the enzyme through an affinity chromatography (His-tag) and PrXTH1 protein displayed strict XET activity (Valenzuela et al., 2014). In here, the enzyme was further purified (13-fold purification) by the incorporation of an extra purification step: a cation exchange chromatography, and the analysis confirmed that PrXTH1 protein is effectively a strict XET enzyme (Table 1). This is coincident with the properties described for DkXTH1, DkXTH2 (Han et al., 2015), DkXTH6, DkXTH7 (Han et al., 2016), and DkXTH8 (Han et al., 2017) recombinant proteins from persimmon (*Diospyros kaki* L.) fruit, AtXTH15 from *Arabidopsis thaliana* (Shi et al., 2015), FcXTH1 from *Fragaria chiloensis* (Baumann et al., 2007), and PttXET16A from (*Populus tremula x tremuloides*) (Kallas et al., 2005). All of them showed significant XET activity without any detectable XEH activity.

As shown in Fig. 1a, the recombinant purified PrXTH1 protein exhibited a bell-shaped pH profile, with an optimum pH interval between pH 5.0 and 5.5. Furthermore, a rapid loss of XET activity was shown below pH 5; only 45% of activity remains at pH 4.5, and only 35% of activity at pH 4. This pH dependence is within the range reported for other XTHs previously described (Bradford, 1976; Han et al., 2015, 2016, 2017; Kallas et al., 2005). In addition, XET activity increases with temperature, and the optimum activity was obtained at 37 °C (Fig. 1b). Two different XTH have been described in term of optimal temperature. Some recombinant XTH proteins showed optimal temperature between 37°-40 °C (Bradford, 1976; Han et al., 2017). The dependence of the XET activity on the concentration of the added XGOs was analyzed (Fig. 1c). A saturation of the XET activity was observed at 140 μM (Fig. 1c).

3.2. Kinetic parameters of PrXTH1

The kinetic parameters (KM and Kcat/KM) of PrXTH1 enzyme were determined (Table 2). The KM value determined was 20.9 μM, while the specificity (Kcat/KM ratio) was 0.049 S⁻¹ μM⁻¹ (Table 2). The data obtained for PrXTH1 is similar to that of previously described XET enzymes (Bradford, 1976; Hrmova et al., 2009), however, PrXTH1 enzyme is apparently less specific than FcXTH1 or HvXET6 enzymes (Kcat/KM ratios of 0.078 S⁻¹ μM⁻¹ and 0.087 S⁻¹ μM⁻¹, respectively (Bradford, 1976; Hrmova et al., 2009)), but significantly more specific than HvXET5 enzyme (Kcat/KM ratio of 0.0021 S⁻¹ μM⁻¹) (Hrmova et al., 2009).

3.3. Substrate specificity of PrXTH1

A protein-ligand interaction study was carried out using the refined PrXTH1 model obtained previously by Valenzuela et al. (2014) (Valenzuela et al., 2014). Firstly, the protein-ligand conformation was evaluated using an automatic docking analysis. As shown in Table 3, favorable binding energies were obtained for the four ligands evaluated, nevertheless the interaction of PrXTH1 with the three hemicellulose octasaccharides was highly favored compared to the cellulose...
octasaccharide (cellodextrin 8-mer) (Table 3). Fig. 2 displays the orientation of the ligands obtained from the different MD simulation analyses. In general, it can be observed that the different hemicellulose octasaccharides are positioned along the open groove of the protein, in contrast to cellulose that showed perpendicular orientation (Fig. 2).

One of the main differences observed among the different protein-ligand complexes was the type and number of residues within the open groove that interact with each ligand. For example, the three hemicellulose ligands interact with the three catalytic residues (Glu79, Asp81, and Glu83) with occupancies over 60% during the MD trajectory. The other catalytic residue (Asp81) does not interact with cellulose (occupancy time of 2.6%, data not shown). On the other hand, important differences were found in the number of hydrogen bonds formed between the different ligands and PrXTH1 model (Fig. 3a–h). In the case of PrXTH1 with XXXGXXXG, XLFGXLFG and XXFGXXFG as ligands more than three H-bonds were established during MD simulations (Fig. 3a–g), while only two H-bonds were established between PrXTH1 protein and cellodextrin (Fig. 3h).

This trend is similar to a previous report (Mendez-Yañez et al., 2017) where a ranking of a different MD simulation was with XLFGXLFG (Table 4). Additionally, when PrXTH1 and PrXTH1-XXXG complex showed a RMSD value around 2 Å, demonstrating the conformational stability of the different protein-ligand structures (Fig. 4).

3.4. Electrostatic potential of PrXTH1

The electrostatic potential on the surface of PrXTH1 protein before and after the MD simulation was analyzed to further explain the differences in protein-ligand interaction between the protein and different ligands (Fig. 5). Interestingly, the four systems are mainly electro-negatively charged in the open groove region before MD simulation (Fig. 5), whereas after MD simulations, the open groove of PrXTH1 changes to electro-positive form after the interaction with the three hemicellulose ligands (XXXG, XXFG and XLFG) (Fig. 5). However, it remains electronegative in the PrXTH1-cellulose complex (Fig. 5). This could explain in essence why the ligand is oriented differently on this complex respect to the other three ligands (Fig. 2).

3.5. Free binding energy estimated by MM-GBSA

To obtain more reliable estimates of binding energy, MM-GBSA calculations for each protein-ligand complex were performed (Table 4). The analysis confirmed docking experiments as the affinity for cellodextrin is considerably lower than for the three hemicelluloses tested. There is a major contribution of van der Waals forces in the stability of the hemicellulosic complexes. The total binding energy indicates that the complex between PrXTH1 and XLFGXLFG is the most stable, followed by XXXGXXXG (Table 4). These results may be compared to those published for other XTHs. XET activity from AtXTH15 has better preference for XXXG as substrate than other XGOs (Shi et al., 2015), although the study in AtXTH15 was performed in vitro, these results are like the in silico data reported for FcXTH1 (Bradford, 1976). Albeit, a different ligand was determined for PrXTH1 because the higher ΔGbind was with XLFGXLFG (Table 4). Additionally, when PrXTH1 and FcXTH1 ligand-complexes were compared, dramatic differences among the systems were determined for the same ligand. For example, in the case of XXXGXXXG as ligand, FcXTH1 binding energy was ∼95.3 kcal mol⁻¹ (Bradford, 1976) compared to ∼42.3 kcal mol⁻¹ for PrXTH1 (Table 4). On the other hand, the contribution of solvation (ΔGsol-pol + ΔGpol-apo) in MM-GBSA analysis of PrXTH1 did not show dramatic differences among the systems and did not affect the final ranking of affinities of the complexes (Table 4).

4. Conclusions

In conclusion, PrXTH1 recombinant enzyme has an optimal pH value between 5 and 5.5. PrXTH1 displays strict XET activity, which confirm previous studies. XET activity was saturated at 140 μM of XGOs concentrations (Fig. 1c). Additionally, our studies using the PrXTH1 structural model showed a better binding interactions with xyloglucans than cellulose, and in term of the differences among the systems and did not affect the final ranking of affinities of the complexes (Table 4).

Author contributions

DB, LM-Q, MAM-L and RH designed and performed the biochemical experiment. CC, LM-Q and RH designed and developed the structural bioinformatic analysis. LM-Q, RH and MAM-L wrote the manuscript.

Competing financial interests

The authors declare no competing financial interests.

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