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Glucuronoxylan recognition by GH 30 xylanases: A study with enzyme and substrate variants

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ABSTRACT

XynA from *Erwinia chrysanthemi* (*Ec*Xyn30A), belonging to glycoside hydrolase family 30 subfamily 8, is specialized for hydrolysis of 4-*O*-methylglucuronoxylan (GX). Carboxyl group of 4-*O*-methylglucuronic acid serves as a substrate recognition element interacting ionically with positively charged Arg293 of the enzyme. We determined kinetic parameters of *Ec*Xyn30A on GX, its methyl ester (GXE) and 4-*O*-methylglucoxylan (GXR) and compared them with behavior of the enzyme variant in which Arg293 was replaced by Ala. The modifications of the substrate carboxyl groups resulted in several thousand-fold decrease in catalytic efficiency of *Ec*Xyn30A. In contrast, the R293A replacement reduced catalytic efficiency on GX only 18-times. The main difference was in catalytic rate (k_{cat}) which was much lower for *Ec*Xyn30A acting on the modified substrates than for the variant which exhibited similar k_{cat} values on all three polymers. The R293A variant cleaved GX, GXE and GXR on the second glycosidic bond from branch towards the reducing end, similarly to *Ec*Xyn30A. The R293A replacement caused 15–times decrease in specific activity on MeGlcA^{3}Xyl$_4$, but it did not influence low activity on linear xylooligosaccharides. Docking experiments showed that MeGlcA^{3}Xyl$_4$ and its esterified and reduced forms were bound to both enzymes in analogous way but with different binding energies.

1. Introduction

The most abundant hemicellulose on the Earth is β-1,4-xylan. Its depolymerization is performed by endo-β-1,4-xylanases (xylanases; **EC 3.2.1.8**) that are crucial enzymes in the xylan degradation. They are produced by various microorganisms, including bacteria, fungi and yeasts [1,2]. During the evolution the microorganisms have developed different strategies for xylan degradation what is reflected in a production of several types of xylanases. Glucuronoxylan xylanohydrolases (**EC 3.2.1.136**) are highly specialized xylanases which require for their action D-glucuronic or 4-*O*-methyl-D-glucuronic acid (MeGlcA) attached to the xylan backbone [3]. They hydrolyze the glucuronoxylan main chain at the second glycosidic linkage from the MeGlcA residue towards the reducing end and they do not attack, or attack extremely slowly, xylan or xylooligosaccharides without MeGlcA substitution [4–8]. The glucuronoxylanases are classified in glycoside hydrolase (GH) family 30, subfamily 8 (http://www.cazy.org/) [9,10].

The GH30 family includes also xylanases which are not MeGlcA-dependent. Most of them are grouped to GH30_7 subfamily that includes XynIV from *Trichoderma reesei* showing exo- and endo-xylanase activity [11] and XYLD from *Bispora* sp. MEY-1 [12] having activity towards glucuronoxylan and arabinoxylan, releasing Xyl, Xyl$_2$ and Xyl$_3$ as main hydrolysis products. However, classification of xylanases to GH30_7 and GH30_8 subfamilies does not precisely determine their catalytic properties. XynVI of *T. reesei* belonging to GH30_7 subfamily is MeGlcA-dependent xylanase [13] while *Cp*Xyn30A from *Clostridium papyrosolvens* belonging to GH30_8 subfamily is not appendage-dependent [14]. There is no doubt that the differences in catalytic properties arise from the topology of the substrate binding site which is not conserved among all GH30 members.

The most studied member of GH30_8 subfamily is xylanase A of a phytopathogenic bacterium *Erwinia chrysanthemi* (*Ec*Xyn30A). *Ec*Xyn30A was first isolated by Braun and Rodrigues [15]. The corresponding gene cloning [16] was later used for its high-level expression,

Abbreviations: GH, glycoside hydrolase; *Ec*Xyn30A, XynA from *Erwinia chrysanthemi*; GX, 4-*O*-methylglucuronoxylan; GXE, methyl ester of 4-*O*-methylglucuronoxylan; GXR, 4-*O*-methylglucoxylan; MeGlcA, 4-*O*-methylglucuronic acid; Me–MeGlcA, methyl ester of MeGlcA; MeGlc, 4-*O*-methylglucose; Xyl$_n$, linear xylooligosaccharide composed of n D-xylopyranosyl residues linked by β-1,4-linkages; MeGlcA^{z}Xyl$_n$, Me–MeGlcA^{z}Xyl$_n$, MeGlc^{z}Xyl$_n$, Xyl$_n$ wherein zth xylopyranosyl residue counted from the reducing end is α-glycosylated at position 2 by MeGlcA, Me–MeGlcA and MeGlc

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crystallization and determination of the 3-D structure [17]. The catalytic properties have also been reported [5,18]. Solution of the crystal structure of *Ec*Xyn30A complex with aldotetraouronic acid (MeGlcA^{2}Xyl$_3$) as a ligand has revealed the structural features responsible for its unique mode of action [19]. It was found that the MeGlcA residue is tightly coordinated in -2b subsite through several hydrogen bonds involving Trp289, Tyr295, Tyr255 and Ser258 residues. Moreover, Arg293 was shown to form a strong bidentate ionic interaction with the carboxyl group of the ligand. It was calculated that this ionic interaction corresponds to about 36% of the total binding energy of MeGlcA^{2}Xyl$_3$ and can be responsible for a proper positioning of a substrate for its effective binding [19]. The topology of glycone subsites was also confirmed by ligand-bound crystal structure of another GH30_8 glucuronoxylan xylanohydrolase, XynC from *Bacillus subtilis* [20]. Based on the reduced number of protein–ligand contacts in the −1 and −2a subsites of XynC and the precise coordination of the MeGlcA residue in -2b subsite provided by Arg272, Trp268, Tyr274, Tyr231 and Ser235, the authors suggested the MeGlcA side residue binding mechanism which forces xylan main chain into a proximity of catalytic glutamates for hydrolytic cleavage.

The bidentate ionic bond between the carboxyl group of the ligand and Arg293 of the enzyme was found to be the strongest interaction in the *Ec*Xyn30A–ligand complex. Therefore, in this study we decided to construct a variant wherein the Arg293 was substituted for Ala. Kinetic constants of the R293A variant on glucuronoxylan and its derivatives missing the free carboxyl group (4-*O*-methylglucuronoxylan methyl ester and 4-*O*-methylglucoxylan) were determined and the product formation from polymeric and oligomeric substrates was studied and compared to the wild-type enzyme.

2. Materials and methods

2.1. Substrates and standards

4-*O*-Methylglucuronoxylan (GX), 4-*O*-methylglucuronoxylan methyl ester (GXE), 4-*O*-methylglucoxylan (GXR) and aldopentaouronic acid MeGlcA^{3}Xyl$_4$ were prepared as described earlier [21–23]. We should emphasize that to guarantee the same branching pattern in all three xylan derivatives the GXR and GX were prepared from the same batch of soluble GXE fraction by a reduction and alkaline deesterification, respectively [21]. Standards of linear β-1,4-xylooligosaccharides (Xyl$_2$-Xyl$_6$) were purchased from Megazyme International (Bray, Wicklow, Ireland).

2.2. Enzymes

GH3 β-xylosidase was a recombinant *Aspergillus niger* enzyme expressed in *Saccharomyces cerevisiae* [24]. *Ec*Xyn30A was prepared according to Urbániková et al. [19]. The variant of the *xynA* gene from *Erwinia chrysanthemi* was obtained by ordering a synthetic gene encoding the R293A modification. The gene sequence was the same as described in Urbániková et al. [19] except for the mutation, where the CGT codon coding for R293 was changed to a GCC codon coding for A. The gene was generated by the company DNA2.0 (Menlo Park, CA, USA) and delivered as a cloned fragment in their standard cloning vector (kanamycin-resistant). The cloning into the expression vector pDG268neo and transformation into *E. coli* and *Bacillus subtilis* was done as for the WT gene described in Urbániková et al. [19]. The variant was also expressed with a signal peptide from Savinase replacing the native secretion signal. All enzymes were stored at −20 °C.

2.3. Determination of kinetic constants

Different concentrations of GX, GXE and GXR (2.5–40 mg ml^{-1}) in 0.05 M sodium acetate buffer, pH 5.5, were incubated with an appropriate amount of *Ec*Xyn30A or the R293A variant (0.05 μM for GX and 0.5 μM for GXE and GXR) at 35 °C. At three different time points (5–30 min) 50 μl aliquots were taken and reducing sugars were determined by Somogyi-Nelson procedure [25] using calibration with xylose. The initial reaction rates were calculated and used for determination of kinetic constants by non-linear regression using Origin 6.0 program (OriginLab Corp., Northampton, MA, USA). The experiments were done in triplicate. One unit of enzyme activity is defined as the amount of enzyme liberating 1 μmol of xylose equivalents in 1 min under these conditions.

2.4. Hydrolysis of oligosaccharides and polysaccharides

5.3 mM solutions of Xyl$_5$ or MeGlcA^{3}Xyl$_4$ in 0.05 M sodium acetate buffer, pH 5.5, were incubated with different concentrations (0.001 μM, 0.01 μM, 0.1 μM, 1 μM, 10 μM, 100 μM) of *Ec*Xyn30A or R293A variant at 35 °C. Aliquots of 1 μl were spotted on silica gel coated aluminium sheets (Merck, Darmstadt, Germany) after 3 h (MeGlcA^{3}Xyl$_4$) or 24 h (Xyl$_5$) of hydrolysis. TLC plate was developed in the solvent system ethyl acetate/acetic acid/2-propanol/formic acid/water 25:10:5:1:15 (v/v) and visualized with orcinol reagent (0.5% orcinol in 5% sulphuric acid in ethanol). For the determination of specific activity on MeGlcA^{3}Xyl$_4$, the 5.3 mM substrate was incubated with 0.2 μM *Ec*Xyn30A or 5 μM R293A variant. For the determination of specific activity on Xyl$_5$ (5.3 mM), 50 μM concentration of both enzymes was used. At time intervals (10–40 min) 20 μl aliquots were taken and the reducing sugars were determined by Somogyi-Nelson procedure as mentioned above. Polysaccharides (10 mg ml^{-1} solutions of GX, GXE and GXR) in 0.05 M acetate buffer, pH 5.5, were mixed with *Ec*Xyn30A or the R293A variant (0.05 μM for GX and 0.5 μM for GXE and GXR) and incubated at 35 °C for 24 h. The reaction was terminated by 5 min boiling and the hydrolysates were analyzed by TLC (as described above) and MALDI ToF MS. Treatment with β-xylosidase (1 U ml^{-1}) was done overnight at 35 °C, after adjusting pH of the hydrolysates to 4.0 with 4 M acetic acid (due to a lower pH optimum of β-xylosidase).

2.5. MALDI ToF MS

The hydrolysates of GX, GXE and GXR were decationized by Dowex 50 (H^+ form) and after separation from the ion-exchanger mixed with one tenth of volume of 0.5 M NaCl. 1 μl of the sample was mixed with 1 μl of the matrix (1% solution of 2,5-dihydroxybenzoic acid in 30% acetonitrile) directly on MS target plate. After drying, the samples were analyzed by UltraflexXtreme MALDI ToF/ToF mass spectrometer (Bruker Daltonics, Bremen, Germany) operating in reflectron positive mode.

2.6. Molecular modeling

The 3-D structure of the *Ec*Xyn30A-MeGlcA^{2}Xyl$_3$ complex (PDB ID: 2Y24) was used for docking. The tetrasaccharide ligand was extracted from the complex and extended at the reducing end by one xylosyl residue having the same Φ and Ψ angle values as the xylosyl residues of the complex bound in negative enzyme subsites. The carboxyl group of the pentasaccharide MeGlcA^{3}Xyl$_4$ was *in silico* methyl esterified or replaced with hydroxymethyl group to give Me-MeGlcA^{3}Xyl$_4$ and MeGlc^{3}Xyl$_4$, respectively. All the pentasaccharides were optimized at the DFT level employing the B3LYP functional and 6-31 + G* basis set implemented in the Jaguar software (Schrödinger Release 2017–1: MS Jaguar, Schrödinger, LLC, New York, NY, 2017). Simultaneously, electrostatic potential charges on all atom centers were calculated and later used for docking. *Ec*Xyn30A was prepared using the Protein Preparation Wizard implemented in the Schrodinger Suite 2017.01 (Schrödinger Suite 2017–1, Schrödinger, LLC, New York, NY, 2017). The protein was protonated to its pH optimum 5.5. Catalytic acid Glu165 was modeled in a protonated form to properly simulate the pre-reaction complex while glucuronic acid in MeGlcA^{3}Xyl$_4$ was treated in