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Structural insights of *Rm*Xyn10A – A prebiotic-producing GH10 xylanase with a non-conserved aglycone binding region

Anna Aronsson[a,*], Fatma Güler[a,b,c], Maxim V. Petoukhov[d,e,f], Susan J. Crennell[g], Dmitri I. Svergun[d], Javier A. Linares-Pastén[a], Eva Nordberg Karlsson[a]

[a] Biotechnology, Department of Chemistry, Lund University, PO Box 124, SE-221 00 Lund, Sweden
[b] Department of Food Engineering, Faculty of Engineering, Ankara University, 06830 Gölbaşı, Ankara, Turkey
[c] Department of Food Engineering, Faculty of Engineering, Bülent Ecevit University, 67900 Çaycuma, Zonguldak, Turkey
[d] European Molecular Biology Laboratory Hamburg Outstation, EMBL c/o DESY Notkestrasse 85, 22607 Hamburg, Germany
[e] A.V. Shubnikov Institute of Crystallography of Federal Scientific Research Centre "Crystallography and Photonics" of Russian Academy of Sciences, Leninsky prospect 59, 119333 Moscow, Russia
[f] A.N. Frumkin Institute of Physical chemistry and Electrochemistry of Russian Academy of Sciences, Leninsky prospect 31, 119071 Moscow, Russia
[g] Department of Biology & Biochemistry, University of Bath, Claverton Down, Bath BA2 7AY, United Kingdom

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ABSTRACT

Hydrolysis of arabinoxylan (AX) by glycoside hydrolase family 10 (GH10) xylanases produces xylo- and arabinoxylo-oligosaccharides ((A)XOS) which have shown prebiotic effects. The thermostable GH10 xylanase *Rm*Xyn10A has shown great potential to produce (A)XOS. In this study, the structure of *Rm*Xyn10A was investigated, the catalytic module by homology modelling and site-directed mutagenesis and the arrangement of its five domains by small-angle X-ray scattering (SAXS). Substrate specificity was explored *in silico* by manual docking and molecular dynamic simulations. It has been shown in the literature that the glycone subsites of GH10 xylanases are well conserved and our results suggest that *Rm*Xyn10A is no exception. The aglycone subsites are less investigated, and the modelled structure of *Rm*Xyn10A suggests that loop $\beta_6\alpha_6$ in the aglycone part of the active site contains a non-conserved α-helix, which blocks the otherwise conserved space of subsite +2. This structural feature has only been observed for one other GH10 xylanase. In *Rm*Xyn10A, docking revealed two alternative binding regions, one on either side of the α-helix. However, only one was able to accommodate arabinose-substitutions and the mutation study suggests that the same region is responsible for binding XOS. Several non-conserved structural features are most likely to be responsible for providing affinity for arabinose-substitutions in subsites +1 and +2. The SAXS rigid model of the modular arrangement of *Rm*Xyn10A displays the catalytic module close to the cell-anchoring domain while the carbohydrate binding modules are further away, likely explaining the observed lack of contribution of the CBMs to activity.

1. Introduction

Arabinoxylan (AX) is the second largest carbohydrate component after cellulose in cereal bran, a by-product from the production of flour. With an estimated annual world production of 2.0 million tonnes of cereal grains [1], cereal bran makes up a large source of AX with the potential to be utilised in value-adding applications. AX in cereal crops such as wheat and rye is present in the cell walls and there tightly associated with cellulose and lignin. The bran of these cereals contains around 50 to 250 mg AX per g dry matter, and the more easily-accessible water extractable AX makes up 2 to 10, and in the case of rye up to 14, mg per g dry matter [2–4]. AX consists of a backbone of 1,4 linked ß-D-xylopyranosyl units decorated with α-L-arabinofuranose units linked by an α-1,3 and/or α-1,2 bond with a degree of substitution (DS) between 0.4 and 0.8. Additional substitutions found in AX are ferulic and *p*-coumaric acid linked by an ester linkage to the O5-position of the arabinofuranose-unit as well as α-1,2 linked units of glucuronopyranosyl and its 4-*O*-methyl ether [3,5]. The two latter substitutions are

Abbreviations: X_1, D-xylose; X_2, 1,4-β-D-xylobiose; X_3, 1,4-β-D-xylotriose; X_4, 1,4-β-D-xylotetraose; X_5, 1,4-β-D-xylopentaose; X_6, 1,4-β-D-xylohexaose; A^3X, $1,3^2$-α-L-arabinofuranosyl-1,4-β-D-xylobiose; A^2XX, $1,2^3$-α-L-arabinofuranosyl-1,4-β-D-xylotriose; A^3XX, $1,3^3$-α-L-arabinofuranosyl-1,4-β-D-xylotriose; XA^3X, $1,3^2$-α-L-arabinofuranosyl-1,4-β-D-xylotriose; XA^2XX, $1,2^3$-α-L-arabinofuranosyl-1,4-β-D-xylotetraose; XA^3XX, $1,3^3$-α-L-arabinofuranosyl-1,4-β-D-xylotetraose; $A^{2+3}XX$, $1,2^3,1,3^3$-di-α-L-arabinofuranosyl-1,4-β-D-xylotetraose; $XA^{2+3}XX$, $1,2^3,1,3^3$-di-α-L-arabinofuranosyl-1,4-β-D-xylotetriose; $U^{4m2}XX$, $1,2^3$-α-D-(4-*O*-methyl-glucuronyl)-1,4-β-D-xylotriose

* Corresponding author.
E-mail address: anna.aronsson@biotek.lu.se (A. Aronsson).

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common in the vegetable part of the plant but not in the AX present in the grain [6]. The hydrolysis products of AX from cereal grain are xylose, xylooligosaccharides (XOS) and arabinoxylooligosaccharides (AXOS), the latter two collectively termed AX-oligosaccharides ((A)XOS). XOS are abbreviated X_n where n is the degree of polymerisation (DP). For the nomenclature of the more complex AXOS, in 2009 Fauré and colleagues proposed a system for oligosaccharides derived from heteroxylans. The system is based on a one-letter code starting in the non-reducing end and where substitutions of the backbone chain xylose units are given unique letters associated with uppercase letters and numbers describing non-glycoside substitutions and position of substituents [7]. For example, an unsubstituted, a 1,3-arabinofuranosyl-substituted and a 1,2-(4-*O*-methyl-glucuronopyransyl)-substituted xylose unit are abbreviated X, A^3 and U^{4m2}, respectively.

(A)XOS have been shown to have prebiotic effects [8]. Reported health improvements resulting from ingestion of such prebiotics are reduced gut infections, better adsorption of minerals, and suppression of colon cancer [8]. Prebiotics are compounds that are neither degraded by gastric acid or host enzymes, nor adsorbed in the gastrointestinal tract, but fermented by intestinal bacteria that contribute to the health and well-being of the host, known as probiotic intestinal bacteria [9]. Stimulation of probiotic bacteria by (A)XOS has mainly been ascribed to strains of *Bifidobacterium* and a few strains of *Lactobacillus*, *e.g. brevis* [10–12]. Utilisation of (A)XOS varies between strains, even within the same genus, and depends on the length of the xylose chain and presence of arabinose-substitutions. AXOS can only be utilised by a limited number of species in the gut, mainly from *Bifidobacterium* but also a few from *Bacteroides* and *Lactobacillus*.

The hydrolysis of cereal bran AX into prebiotic (A)XOS can be achieved by endo-β-1,4-xylanases (often only called xylanases), which hydrolyse the internal glycosidic linkages between xylose units in the AX backbone. Endo-β-1,4-xylanases belong mainly to glycoside hydrolase (GH) family 10 and GH11, but can also be found in GH families 5, 8, 30, 43, 51 and 98 in the Carbohydrate Active Enzyme database (CAZy, www.cazy.org) [13]. Enzymes from the same family share a catalytic mechanism and overall fold. However, what compounds can be hydrolysed by an enzyme and products that can be formed from a given substrate, the substrate specificity, is not necessarily conserved among enzymes within one family [14]. Production of prebiotic (A)XOS has been performed by xylanases belonging to GH10, GH11 and GH30 [15]. In general, xylanases from GH11, which have a relative narrow active site, are less tolerant to arabinose-substitutions than xylanases from GH10 while most xylanases from GH30 have been shown to require a methyl glucuronic acid-substitution for hydrolysis to occur [16–18]. GH10 is thus a good choice for hydrolysis of cereal bran AX.

For the production of prebiotic (A)XOS from AX, understanding of how the active site residues of GH10 xylanases bind AX and (A)XOS is crucial. Improvement of purity and yield of the production by rational engineering is possible when the structure of the enzyme and its substrate binding interactions are known. In the active site of glycoside hydrolases, each sugar unit is bound into a subsite. According to the nomenclature of the field [19], the two subsites on either side of the cleavage point are named −1 and +1 respectively, and the number increases by one integer for each subsite away from the cleavage point. The subsites towards the reducing end of the substrate are the plus- or aglycone subsites and the subsites towards the non-reducing are consequently the minus- or glycone subsites. GH10 xylanases produce small oligosaccharides which can be explained by the strong binding of the well-conserved subsites −1, −2 and +1 [20]. More than two glycone subsites exist in the family but are not conserved [21]. The more distant aglycone subsites are not conserved, neither the interacting amino acids, nor the substrate specificity. Up to four aglycone subsites have been reported [22,23] and mainly hydrophobic interactions are observed [24]. The ability to bind arabinose-substituted substrates also varies within the family. Arabinose-substitutions in subsite −2 are permitted while substitutions in the aglycone subsites are reported but not conserved [20]. Considering the low conservation of the aglycone subsites and substrate specificity in the family, the xylanase for a given application must be carefully chosen based on structural and biochemical information.

To date, only three studies have presented crystallographic structures of GH10 xylanases in complex with AXOS [25–27]. The arabinose substitution is located at subsite −2 in all structures. This position is highly interesting since arabinose-substitutions at this position can have a role in substrate recognition as discovered by Xie and co-workers. They found four GH10 xylanases that had higher activity on A^3XX than X_3 [28]. However, structures of complexes with arabinose-substitutions in other positions are necessary for the prediction of hydrolysis products and as a base for tailor-made prebiotic (A)XOS.

A modular GH10 xylanase with valuable properties, *Rm*Xyn10A, has been cloned from the thermophilic bacterium *Rhodothermus marinus*, which was isolated from a hot spring in Iceland [29,30]. *Rm*Xyn10A comprises one signal peptide, two tandem carbohydrate binding modules (CBMs) from family 4, one domain with unknown function, one catalytic module and one putative cell anchoring domain [31]. Characterisation of the catalytic module revealed thermostability, activity remained for 24 h in 70 °C [32], which makes it suitable for industrial applications. The non-catalytic modules have no impact on the specific activity. However, the full-length enzyme has a higher stability than the catalytic module alone, indicating thermostabilising interactions between the domains [33]. *Rm*Xyn10A has been used for production of (A)XOS from various xylan-containing sources, *e.g.* rye bran, wheat bran, birchwood and quinoa stalks [11,12,34,35]. The produced oligosaccharides are mainly X_2, X_3 and A^3X, but also unidentified AXOS have been detected. Efforts have been made to determine the three-dimensional structure of the catalytic module in order to study substrate binding but several attempts have failed, showing that the module is difficult to crystallise.

In this paper we use a different approach by applying computational methods to provide deeper insight into substrate specificity and binding. The structure of the catalytic module of *Rm*Xyn10A is described through a homology model. Subsite affinities and substrate preferences are explored by molecular dynamics and docking techniques. Interesting structural features in the active site are further explored by site-directed mutagenesis. Valuable insights in the modularity of the full-length enzyme are gathered by combining structural data from small-angle X-ray scattering (SAXS) of the full-length enzyme with NMR data of the structure of CBM4-2 [36] and the homology models of the catalytic module and CBM4-1. The results are evaluated and compared to existing biochemical data of *Rm*Xyn10A as well as to the entire GH10.

2. Material and methods

2.1. Homology modelling

Homology modelling of the catalytic module of *Rm*Xyn10A (*Rm*Xyn10A_CM), residues 549–870 according to the full-length numbering, was carried out using the YASARA program [37,38]. Parameters used for the homology modelling are presented in Table S1.

Three crystal structures of XynB from *Xanthomonas axonopodis* pv. citri (Xac), PDB ID: 4PMZ-B (no ligand), 4PN2-A (X_2) and 4PMX-A (X_3), were identified as templates after running the PSI-BLAST algorithm [39] to extract a position specific scoring matrix, PSSM, from UniRef90 [40] and then searching for a match in the Protein Data Bank (PDB) [41]. The matches were ranked based on a total score defined as the product of the BLAST alignment score, the WHAT_CHECK [42] quality score obtained from the PDBFinder2 database [43] and the target coverage, see Table 1. The BLAST E-values are identical as the sequences are the same.

The following procedure was applied to generate alignments between the target and the identified templates. A secondary structure