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Arabinoxylanase from glycoside hydrolase family 5 is a selective enzyme for production of specific arabinoxylooligosaccharides

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ABSTRACT

An arabinose specific xylanase from glycoside hydrolase family 5 (GH5) was used to hydrolyse wheat and rye arabinoxylan, and the product profile showed that it produced arabinose substituted oligosaccharides (AXOS) having 2–10 xylose residues in the main chain but no unsubstituted xylooligosaccharides (XOS). Molecular modelling showed that the active site has an open structure and that the hydroxyl groups of all xylose residues in the active site are solvent exposed, indicating that arabinose substituents can be accommodated in the glycone as well as the aglycone subsites. The arabinoxylan hydrolysates obtained with the GH5 enzyme stimulated growth of *Bifidobacterium adolescentis* but not of *Lactobacillus brevis*. This arabinoxylanase is thus a good tool for the production of selective prebiotics.

1. Introduction

Increasing evidence shows that the gut microbiota has a profound influence on human health and that changes in its composition correlate with obesity, type 2 diabetes and other metabolic disorders, which constitute a rapidly increasing problem in large parts of the world (Nicholson et al., 2012). Furthermore, interplay between the gut microbiota and the brain has possible implications in conditions like autism, major depression and Parkinson's disease (Collins, Surette, & Bercik, 2012) (Jiang et al., 2015; Scheperjans et al., 2015). It is thus of considerable interest to influence the composition of the gut microbiota, to promote health. The most direct way is to supply living, beneficial microorganisms with the diet. These are called probiotics (Saulnier, Spinler, Gibson, & Versalovic, 2009), and bacteria belonging to *Lactobacillus* and *Bifidobacterium* are the most well-known examples. An alternative approach is to supply the diet with components, which promote the growth of the desirable microorganisms. These components are called prebiotics (Saulnier et al., 2009), and indigestible carbohydrates, like inulin and fructooligosaccharides, are the most well-known examples. More recently, similar effects have been demonstrated for arabinoxylan (AX), arabinoxylooligosaccharides (AXOS) and xylooligosaccharides (XOS) and thus these constitute a group of emerging prebiotics (Broekaert et al., 2011).

Arabinoxylan is a major type of hemicellulose in cereals, like wheat and rye. Its backbone consists of β-1,4-linked D-xylopyranoside residues and L-arabinofuranosyl substituents, linked to the main chain with α-1–2 and/or α-1,3 bonds are common. Additional substituents can be other hexoses or pentoses, uronic acids, acetic acid and ferulic acid (Andersson & Åman, 2008). It has been shown that the prebiotic effects of arabinoxylan-derived oligosaccharides is highly influenced by their size and structure (Broekaert et al., 2011) (Damen et al., 2011). Various types of enzymes are excellent tools for the conversion of AX to various oligosaccharides. Endo-xylanases are of special importance since they break the main chain and arabinofuranosidases are important for removing arabinose substituents.

To quantify prebiotic effects of dietary oligosaccharides, the “prebiotic index” has been defined (Palframan, Gibson, & Rastall, 2003). A positive prebiotic index indicates selective stimulation of bacteria belonging to *Bifidobacterium* and/or *Lactobacillus*, while *Bacteroides* and *Clostridium* have a negative influence on this index. When water-unextractable wheat AX was supplied to human fecal fermentation a positive prebiotic index was obtained and an even higher index was obtained after xylanase treatment of AX (Vardakou et al., 2008).

It has been shown that different prebiotic oligosaccharides promote the growth of different gut bacteria. In one *in vitro* fermentation study, fructooligosaccharides were efficient in promoting lactobacilli, while XOS and lactulose produced the highest increases in numbers of bifidobacteria (Rycroft, Jones, Gibson, & Rastall, 2001). It is not known exactly which bacterial strains are the most health-promoting ones. Use of selective prebiotics is a possible way to achieve stimulation of just part of the microorganisms, which are generally considered health-promoting. Xylanases and other AX-degrading enzymes having various

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substrate specificities can be useful tools to prepare such prebiotics.

Xylanases have different tolerance for substituents in AX. The most commonly used xylanases belong to glycoside hydrolase families 10 or 11. They are highly active on unsubstituted parts of the xylan chain and have limited tolerance for arabinose substituents. On the other hand, the enzyme from *Clostridium thermocellum*, belonging to glycoside hydrolase family 5, requires arabinose substituents to be active (Correia et al., 2011). Recently, other enzyme candidates in the same family, all classified under subfamily 34 (www.cazy.org), have been shown to have similar substrate specificity (Labourel et al., 2016) and they are therefore called arabinoxylanases. It has been pointed out that this substrate specificity can be useful for the complete degradation of highly substituted, normally highly recalcitrant, arabinoxylan materials and thus valuable in the production of biofuels (Correia et al., 2011). Here, we explore the possibilities to use the arabinoxylanase to produce AXOS, without significant production of XOS, and thereby achieve a prebiotic product, which selectively promotes the growth of microorganisms able to metabolise AXOS. Two different AXOS ligands were also docked into the active site of the *C. thermocellum* enzyme, to explain the structural implications of the arabinoxylan specificity.

2. Materials and methods

2.1. Materials

Arabinoxylanase from *Clostridium thermocellum* (*Ct*Xyl5A) was purchased from Nzytec (Lisboa, Portugal). The catalytic module of a xylanase from *Rhodothermus marinus* (*Rm*Xyn10ACM) was prepared as described by Falck et al. (2013). Pentopan Mono BG, a commercial family 11 xylanase was kindly provided by Novozymes (Bagsvaerd, Denmark). High purity recombinant α-L-arabinofuranosidase (product code E-ABFCJ, classified under GH51) from *Cellvibrio japonicus* and endosperm arabinoxylans from wheat (P-EDWAX30) and rye (P-RAXY) were purchased from Megazyme (Wicklow, Ireland).

2.2. Enzymatic conversions

Arabinoxylan substrates were dissolved in MQ water according to the manufacturer's instructions and pH was adjusted to 7 with 8 M HCl. The final substrate concentration was 10 g/l. To reaction mixtures involving *Ct*Xyl5A, $CaCl_2$ was added to a concentration of 2 mM to stabilise the enzyme. Xylanases were added (1 mg/g substrate, unless otherwise stated) and the reaction mixtures were incubated at 50 °C for 24 h. Upon retrieval of samples, the enzymes were inactivated by incubation at 95 °C for 30 min.

2.3. Acid catalysed debranching of AXOS

Arabinoxylan hydrolyzate obtained using *Ct*Xyl5A was treated with a weak acid of 1.6 mM hydrochloric acid at 90 °C for 24 h to release the majority of bound arabinose on the oligosaccharides according to a method by Swennen, Courtin, Van der Bruggen, Vandecasteele, and Delcour (2005). The sample containing the de-branched xylose backbones was neutralized using aqueous NaOH solution and analysed by HPAEC-PAD.

2.4. Analysis by HPAEC-PAD

The oligosaccharides produced by xylanase incubation were analysed by HPAEC-PAD (ICS-5000) using a CarboPac PA200 column (250 mm × 3 mm, 5.5 μm) and a guard column (50 mm × 3 mm) of the same material, as described previously (Falck et al., 2014). Monosaccharide and xylooligosaccharide standards used were as follows: arabinose (Sigma), xylose (Sigma), xylobiose (X_2), xylotriose (X_3), xylotetraose (X_4), xylopentaose (X_5), and xylohexaose (X_6) (Megazyme). AXOS standards (1,3-arabinosyl-xylobiose (A^3X) and 1,2-arabinosyl-xylotriose (A^2XX)) were kindly supplied by Megazyme.

2.5. Docking of a DP6 AXOS into the glycone and aglycone subsites of *Ct*Xyl5A

The crystallographic structure of *Ct*Xyl5A in complex with the arabinoxylooligosaccharide ligand Xyl*p*-β-1,4-Xyl*p*-β-1,4-Xyl*p*-[α-1,3-Ara*f*]-β-1,4-Xyl*p* (PDB 5LA2) in the glycone subsites −4 to −1 and −2* (Labourel et al., 2016), was used to build a longer ligand by adding two xylose units in the potential aglycone subsites +1 and +2. The xylose residues of the oligosaccharide were placed manually in the corresponding subsites, in accordance with the surface hydrophobicity in the respective subsite. Surface hydrophobicity was obtained using the software UCSF Chimera v 1.11.2 (Pettersen et al., 2004). The atomic coordinates of the xylose residues were constructed using the *building tools* from YASARA v15.10.18 (Krieger & Vriend, 2014) and then, loaded into the program Chimera for manual docking. Thus, each sugar was placed in the corresponding subsite with the hydroxyl groups pointing away from the hydrophobic areas, after finding the best potential interactions between the xylose residues and the amino acids of the protein. The glycosidic bonds were then created to connect the individual sugars to each other as well as to the co-crystallized ligand in the glycone subsites.

In addition, the mutated catalytic amino-acid Ser279 in the crystallographic structure was restituted to Glu279. This initial structure was energetically minimized using the AMBER14 force field (Duan, Wu, Chowdhury, & Lee, 2003) implemented in YASARA. The resulting structure was a model of the complex between *wild type Ct*Xyl5A and the ligand Xyl*p*-β-1,4-Xyl*p*-β1,4-Xyl*p*-[α-1,3-Ara*f*]-β-1,4-Xyl*p*-β-1,4-Xyl*p*-β-1,4-Xyl*p*.

2.6. Molecular dynamics simulations

Three structures were simulated by molecular dynamics in water: (1) *Ct*Xyl5A free of ligands (PDB 2Y8K), (2) *Ct*Xyl5A in complex with Xyl*p*-β-1,4-Xyl*p*-β-1,4-Xyl*p*-[α-1,3-Ara*f*]-β-1,4-Xyl*p* (PDB 5LA2, only chain A) and (3) *Ct*Xyl5A with the ligand Xyl*p*-β-1,4-Xyl*p*-β-1,4-Xyl*p*-[α-1,3-Ara*f*]-β-1,4-Xyl*p*-β-1,4-Xyl*p*-β-1,4-Xyl*p*. The first two are entirely crystallographic structures, while the third contains the ligand, partially modeled by manual docking as described above. Each structure was simulated into a cubic cell, with periodic boundaries, 20 Å larger than the protein containing explicit molecules of water as solvent and 2 mM $CaCl_2$. The pH and temperature were 7 and 323 K, and the simulation time 10 ns. The calculations were done using AMBER14 force field (Duan et al., 2003) via the YASARA (Krieger & Vriend, 2014) program. Snapshots were collected and analysed every 250 ps. Molecular dynamic trajectories were analysed using VMD program v1.9.2 (Humphrey, Dalke, & Schulten, 1996).

2.7. Bacterial growth

Bifidobacterium adolescentis (ATCC 15703) and *Lactobacillus brevis* (DSMZ 1269) were grown under anaerobic conditions as described previously (Falck et al., 2013). When evaluating growth on arabinoxylan hydrolysates (mainly oligosaccharides with the monosaccharide composition: arabinose: xylose ratio of 30: 70; glucose, galactose and mannose < 2%), these were filter sterilized through a 0.45 μm filter and added to the media at a final concentration of 5 g/l and a total volume of 5 ml. The fermentation experiments started from precultures using 2% (v/v) inoculum and samples were withdrawn after 24 and 48 h. Optical density (at 620 nm) and pH was measured after 0, 24 and 48 h, while oligosaccharides were analysed after 0 and 48 h using HPAEC-PAD as described above.

3. Results and discussion

3.1. Enzymatic oligosaccharide production from wheat and rye arabinoxylan

The ability of *Ct*Xyl5A, a GH5 arabinoxylanase (subfamily 34) from *Clostridium thermocellum*, (Correia et al., 2011) to produce