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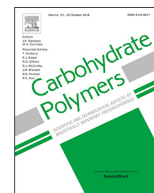

Extracellular depolymerisation triggers fermentation of tamarind xyloglucan and wheat arabinoxylan by a porcine faecal inoculum

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ABSTRACT

Arabinoxylan (AX) and xyloglucan (XG) are important components of primary cell walls of cereal grains and vegetables/fruits, respectively. Despite the established health benefits of these non-starch polysaccharides, the mechanisms of their utilisation by the gut microbiota are poorly understood. In this study, the mechanisms of solubilised wheat AX and tamarind XG degradation were investigated under *in vitro* fermentation conditions using a porcine faecal inoculum. Through structural analysis of the polymers, we demonstrate that depolymerisation by microbial surface accessible *endo*-degrading enzymes occurs prior to active fermentation of AX or XG. Breakdown products are released into the medium and potentially utilised cooperatively by other microbes. Acetate and propionate are the main fermentation products and are produced concurrently with polysaccharide depletion. Butyrate, however, is produced more slowly consistent with it being a secondary metabolite.

1. Introduction

Plant cell walls are rich in polysaccharides and are the main structural components of plant-based foods such as grains, fruits and vegetables. They are not digested or absorbed in the human small intestine, and therefore pass to the large intestine where they are available for fermentation by the resident microbiota (Gidley, 2013; Harris & Smith, 2006; Mikkelsen, Gidley, & Williams, 2011). The fermentation of these polymers is not only critical in maintaining colonic health (Neyrinck et al., 2011), but also has profound effects on host metabolism, and the immune system (Mendis, Leclerc, & Simsek, 2016).

Xyloglucan (XG) is found in the cell walls of almost all land plants. It is abundant in the primary walls of vegetables and fruits (Larsbrink, Rogers et al., 2014), as well as in the seeds of some species, e.g. tamarind, as a storage polymer providing energy for germination (Schultink, Liu, Zhu, & Pauly, 2014). The structure of XG consists of a β-1,4-glucopyranose (Glc*p*) backbone partially substituted with α-D-xylopyranose (α-D-Xyl*p*) at C(O)6. The α-D-Xyl*p* residues can be further substituted with other monosaccharides, including galactose (such as in tamarind seed), fucose (such as in eudicotyledons) and/or arabinose (such as in Solanaceae) (Hsieh & Harris, 2009). The structure of tamarind XG (Fig. 1) consists of four motifs, a heptasaccharide (XXXG, Glc_4Xyl_3), two octasaccharides (XXLG and XLXG, Glc_4Xyl_3Gal) and a nonasaccharide (XLLG, $Glc_4Xyl_3Gal_2$), with the ratio of hepta-/octa-/nona-saccharide being 13:39:48 (Yamatoya, Shirakawa, Kuwano, Suzuki, & Mitamura, 1996).

Arabinoxylan (AX) is especially abundant in the primary cell walls of many cereal grains. The backbone of wheat or rye AX consists of β-(1,4)-linked D-xylopyranosyl units which are doubly substituted with arabinofuranosyl moieties at C(O)2 and C(O)3 ($A^{2+3}X$), or singly substituted at C(O)3 (A^3X) (Fig. 1).

The large intestinal microbiota produce various enzymes which degrade XG/AX into their monosaccharide components, which are then metabolised into smaller molecules, such as short chain fatty acids (SCFA) (Larsbrink, Rogers et al., 2014; Rogowski et al., 2015). The complete degradation of XG into monosaccharides (glucose, xylose and galactose in the case of tamarind XG) requires the concerted action of *endo*-β-1,4-xyloglucanase, *exo*-β-glucosidase, *exo*-α-xylosidase and *exo*-β-galactosidase. Similarly, to degrade AX into xylose and arabinose, *endo*-β-xylanase, *exo*-α-arabinofuranosidases and *exo*-β-xylosidase are needed. Genomic and metagenomic analyses identify both extracellular and intracellular XG/AX-degrading enzymes, and putative degradation pathways within single microbes have been proposed (Larsbrink, Rogers et al., 2014; Ravachol et al., 2016; Rogowski et al., 2015).

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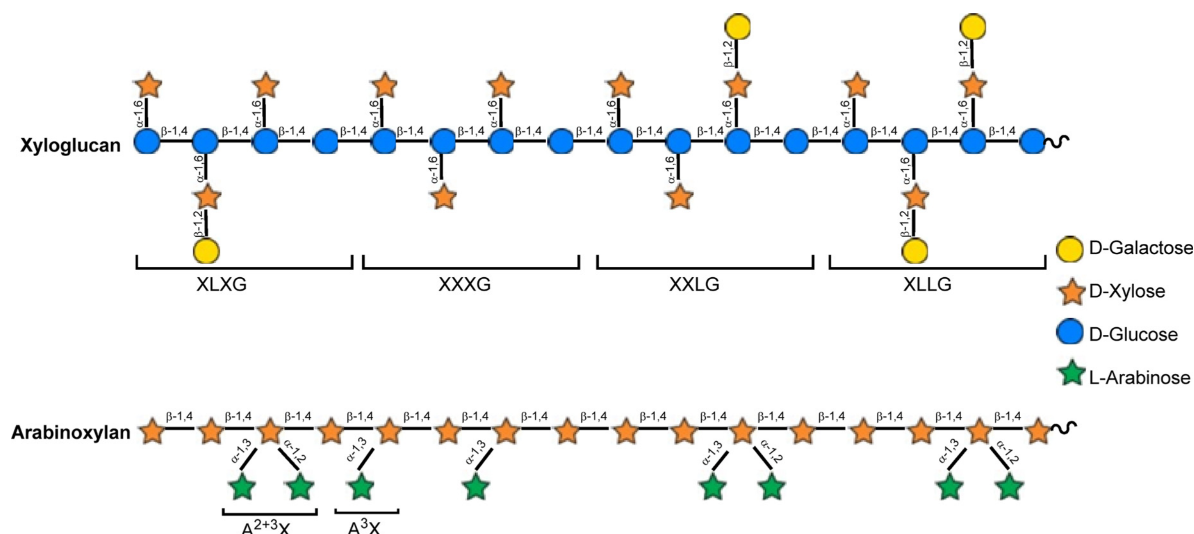

Fig. 1. Structural features present in tamarind xyloglucan and wheat arabinoxylan. Note that the sequence of these features is not defined.

However, systems using single microbes are limited, as the gut microbiota contains hundreds of microbial species in a highly dynamic and competitive gut environment. Therefore, it is useful to include the entire microbial community to understand how specific polysaccharides are degraded.

In our previous study, general features of AX utilisation by a porcine faecal inoculum were deduced using AX in a powdered state (Feng et al., 2018). However, when powdered XG was used, gel lumps were apparent in the medium, which inhibited utilisation by the microbes to a variable extent. Therefore, in order to examine the mechanisms of XG utilisation, XG was pre-dissolved. Pre-dissolved AX was also included in this study to compare with powdered AX. A porcine faecal inoculum was used as a model for colonic microbiota (Miller & Ullrey, 1987; Roura et al., 2016), and a semi-defined medium was used in which the XG/AX polymers were the only carbon sources available for energy. AX and XG are large polymers and are not expected to be able to pass through the microbial cell walls (Demchick & Koch, 1995). Therefore, the hypothesis was that both XG and AX in solution would be depolymerised and debranched by microbial surface-accessible enzymes with release of degradation products back into the medium during active fermentation.

2. Materials and methods

2.1. Materials

XG (product code: P-XYGLN) and AX (product code: P-WAXYM) were purchased from Megazyme (Bray, Ireland). Dimethyl sulfoxide-d$_6$ (DMSO-d_6, 99.9 atom % D, 151874), deuterium oxide (D_2O, 99.9 atom % D, 151882) and 3-(trimethylsily) propionic-2,2,3,3-d$_4$ acid sodium salt (TSP, 98 atom % D, 269913) were purchased from Sigma-Aldrich (Castle Hill, Australia).

2.2. In vitro fermentation of XG and AX

2.2.1. Preparation of the substrate solutions

For each polymer, XG or AX, 21 g was dissolved overnight at 25 °C in boiled Milli-Q water (2.1 L) with constant stirring and bubbling with a stream of oxygen-free carbon dioxide, and then dispensed into serum bottles (38 mL). The bottles were sealed with butyl rubber stoppers, aluminium caps crimped, and autoclaved (15 min, 121 °C).

2.2.2. Preparation of the medium

The medium was modified from Lowe et al. (Lowe, Theodorou, Trinci, & Hespell, 1985) and Williams et al. (Williams, Bosch, Boer, Verstegen, & Tamminga, 2005). In brief, the concentration of the 'basal solution' was double that of the basal solution described by Williams et al. (Williams et al., 2005), and each serum bottle contained 38 mL of the concentrated basal solution. The basal solution contained 1.189 g/L trypticase as a source of peptides and amino acids, as well as 0.642 g/L NH_4Cl as an addition source of nitrogen in the form of ammonium. The vitamin/phosphate solution, bicarbonate solution, and reducing agent were prepared according to methods described by Williams et al. (Williams et al., 2005).

2.2.3. Preparation of the inoculum

The inoculum was prepared based on the method described by Williams et al. (Williams et al., 2005). Faeces were collected from five pigs fed on a standard semi-defined diet for ten days prior to collection. The diet, based on readily digestible maize starch and fishmeal (Feng et al., 2018), was formulated to be as free as possible of XG or AX to avoid adaptation of the microbiota. The faeces were diluted five times (w/v) with pre-warmed (39 °C), sterile, saline solution (9 g/L NaCl). The inoculum was obtained after homogenisation of the faeces with a hand mixer for 60 s, and filtration through four layers of muslin cloth.

2.2.4. Fermentation

The 'substrate solution' and the 'basal solution' were combined under a constant flow of CO_2. Then, 1 mL of the 'vitamin/phosphate solution', 4 mL of the 'bicarbonate solution' and 1 mL of the 'reducing agent' were added (Williams et al., 2005). Following inoculation, fermentation proceeded for up to 72 h at 39 °C. Two blanks were included. One blank contained the substrate and the medium but the inoculum was substituted by 5 ml saline solution (AX_Med or XG_Med), for which there were two replicates each at 0 h and at 72 h. The other blank contained the medium and the inoculum but no substrates (Inoc_Med, 38 mL of autoclaved Millipore water was used), for which two bottles were taken for each time removal point (0 h, 2 h, 4 h, 6 h, 8 h, 10 h, 12 h, 18 h, 24 h, 48 h, and 72 h).

At each time removal point, the microbial activity was retarded by plunging the bottles into ice water for 20 min, and samples were taken for various analyses according to the methods described by Feng et al (Feng et al., 2018).

2.2.5. Cumulative gas production

Cumulative gas production was calculated according to the method described by Williams et al. (Williams et al., 2005). In brief, at regular time intervals, the fermentation bottles were connected to a pressure

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