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Enzymatically derived aldouronic acids from *Cryptomeria japonica* arabinoglucuronoxylan☆

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ABSTRACT

An arabinoglucuronoxylan was extracted from the holocellulose of sugi (*Cryptomeria japonica*) wood with 10% KOH and subjected to hydrolysis by partially purified xylanase fraction from a commercial cellulase preparation "Meicelase". Neutral sugars liberated were analyzed by size exclusion chromatography showing the presence of xylooligosaccharides up to xylohexaose. Aldouronic acids liberated were purified by preparative anion exchange chromatography. Their structures were identified by monosaccharide analysis, comparison of their volume distribution coefficients (*Dv*s) with those of the authentic samples in anion exchange chromatography and ^{1}H and ^{13}C NMR spectroscopy, resulting in the characterization of eight aldouronic acids including acids consisting of two 4-*O*-Me-α-D-Glc*A*p residues and 3–5 D-Xyl residues.

1. Fr. 1-S1: (aldohexaouronic acid, MeGlcA^3Xyl_5), *O*-β-Xyl*p*-(1 → 4)-*O*-β-D-Xyl*p*-(1 → 4)-[*O*-(4-*O*-Me-α-D-Glc*A*p)-(1 → 2)]-*O*-β-Xyl*p*-(1 → 4)-*O*-β-D-Xyl*p*-(1 → 4)-D-Xyl
2. Fr. 1-S2: (aldopentaouronic acid, MeGlcA^3Xyl_4), *O*-β-Xyl*p*-(1 → 4)-[*O*-(4-*O*-Me-α-D-Glc*A*p)-(1 → 2)]-*O*-β-D-Xyl*p*-(1 → 4)-*O*-β-Xyl*p*-(1 → 4)-D-Xyl
3. Fr. 2-S1: (aldotetraouronic acid, MeGlcA^3Xyl_3), *O*-(4-*O*-Me-α-D-Glc*A*p)-(1 → 2)-*O*-β-D-Xyl*p*-(1 → 4)-*O*-β-D-Xyl*p*-(1 → 4)-D-Xyl
4. Fr. 3-S1: (aldotetraouronic acid, GlcA^3Xyl_3), *O*-(α-D-Glc*A*p)-(1 → 2)-*O*-β-D-Xyl*p*-(1 → 4)-*O*-β-Xyl*p*-(1 → 4)-D-Xyl,
5. Fr. 4-S1: (aldotriouronic acid, GlcA^2Xyl_2), *O*-(4-*O*-Me-α-D-Glc*A*p)-(1 → 2)-*O*-β-D-Xyl*p*-(1 → 4)-D-Xyl
6. Fr. 4-S2: (MeGlc^{4}MeGlcA^3Xyl_5), *O*-β-D-Xyl*p*-(1 → 4)-[*O*-(4-*O*-Me-α-D-Glc*A*p)]-(1 → 2)-*O*-β-D-Xyl*p*-(1 → 4)-[*O*-(4-*O*-Me-α-D-Glc*A*p)]-(1 → 2)-*O*-β-D-Xyl*p*-(1 → 4)-*O*-β-D-Xyl*p*-(1 → 4)-D-Xyl
7. Fr. 6-S1: (MeGlcA^4MeGlcA^3Xyl_4), *O*-(4-*O*-Me-α-D-Glc*A*p)-(1 → 2)-*O*-β-D-Xyl*p*-(1 → 4)-*O*-[(4-*O*-Me-α-D-Glc*A*p)]-(1 → 2)-*O*-β-D-Xyl*p*-(1 → 4)-*O*-β-D-Xyl*p*-(1 → 4)-D-Xyl
8. Fr. 7-S1: (MeGlcA^3MeGlc$^2Xyl_3$), *O*-(4-*O*-Me-α-D-Glc*A*p)-(1 → 2)-*O*-β-D-Xyl*p*-(1 → 4)-*O*-[(4-*O*-Me-α-D-Glc*A*p)]-(1 → 2)-*O*-β-D-Xyl*p*-(1 → 4)-D-Xyl

Fr. 4-S2 was a new acidic oligosaccharide. The distribution pattern of these vicinal uronic acid units along the D-xylan chain was discussed.

1. Introduction

The chemical structure of AGX in the secondary wall of softwood has been well established (Shimizu, 1991). It has a linear backbone composed of (1 → 4) linked β-D-Xyl*p* residues, some of which are substituted at C-2 with a single 4-*O*-Me-α-D-Glc*A*p or α-D-Glc*A*p residue. The AGX also contains α-L-Ara*f* residues, directly linked to C-3 of the D-Xyl*p* residues. AGX contains generally one 4-*O*-Me-α-D-Glc*A*p residue per 5–6 D-Xyl*p* residues and one L-Ara*f* residue per 5–12 D-Xyl*p* residues, respectively.

However, the distribution of these side chains along the backbone of softwood AGX has long been an object of discussion. The distribution pattern of side chains in heteroxylan is an important feature affecting their solubility, interactions with other polymeric

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cell wall substances, degradability by enzymes, solution behavior and other functional properties (Ebringerová, Hromadkova, & Heinze, 2005).

We previously isolated the xylooligosaccharides [degree of polymerization (DP) 2–4] containing two 4-*O*-Me-α-D-GlcA*p* residues on the contiguous D-Xyl residues from the partial hydrolysates of the xylan precipitated from spruce neutral-sulfite liquor (Shimizu & Samuelson, 1973) and AGXs isolated from holocelluloses of Larch (*Larix leptolepis*) (Shimizu, Hashi, & Sakurai, 1978), sugi (*Cryptomeria japonica*) and hinoki (*Chamaecyparis obtusa*) (Yamasaki, Enomoto, Kato, Ishii, & Shimizu, 2011). Although the data were only semi-quantitative, it appeared that an appreciable amount of 4-*O*-Me-α-D-GlcA*p* residues were located on adjacent D-Xyl*p* residues in softwood AGXs.

No oligosaccharides containing more than one 4-*O*-Me-α-D-GlcA*p* residues were liberated on hydrolysis of AGXs from redwood (Comtat & Joseleau, 1981) and larch wood (Debeire, Priem, Strecker, & Vignon, 1990) by xylanases suggesting that the irregularity of the distribution of 4-*O*-Me-α-D-GlcA*p* residues on the xylosyl backbone and the presence of non-hydrolyzable blocks having a higher density of substituents. Vršanská, Kolenová, Puchart, and Biely (2007) also proved to be irregular distribution of the uronic acid side chains in AGX from larch as well as in the glucuronoxylan from hardwoods such as beech and birch by using appendage-dependent glycanase (Nishitani & Nevings, 1991).

On the other hand, Jacobs, Larsson, and Dahlman (2001) also reported on the basis of MALDI spectra of the oligosaccharides produced by partial acid hydrolysis that the 4-*O*-Me-D-GlcA*p* side chains are irregularly distributed in hardwood GX. In contrast, in softwood AGX, the major portion of the side chains were found to be distributed regularly on every seventh or eighth D-Xyl residue, while a minor portion of the uronic acids are attached to adjacent D-Xyl residues located, randomly or periodically, between larger domain.

It is well known that *Trichoderma* spp. produce multiple xylanases with xylanolytic activity (Wong & Saddler, 1992). In particular, five specific xylanases, XYL-I, XYL-II, XXL-III, XYL-IV, and EG, have been identified in *Trichoderma reesei* (Biely, Kršanskš, & Claeyssens, 1991; Saloheimo et al., 2003; Tenkanen, Puls, & Poutanen, 1992; Törrönen et al., 1992; Xu, Takakuwa, Nogawa, Okada, & Morikawa, 1998). The former two belong to glycosyl hydrolase family 11. The third and fourth belong to family 10 and family 5, respectively. The last is known to be a non-specific endoglucanase which can hydrolyze both cellulose and xylan. Xylosidic linkage of AGX will be synergetically hydrolyzed with these enzymes, and the oligosaccharides formed give some information on the distribution pattern of side chains because the endo-mechanism of xylanases is hindered by the side chains.

In this paper, we report on structural analysis of enzymatically derived oligosaccharides from AGX sugi (*C. japonica*). Based on the structure of oligosaccharides, the distribution pattern of 4-*O*-Me-α-D-GlcA*p* residues along the main chain of AGX is discussed.

2. Experimental

2.1. General methods

Relative sugar composition of poly- and oligo-saccharides was determined by means of partition chromatography on ion exchange resin after hydrolysis with 2 M trifluoroacetic acid (TFA) at 120 °C for 2 h. The partition chromatography was carried out by using a Shimadzu LC-10AT high performance liquid chromatograph following the procedure of Nakamura, Hatanaka, and Nagamatsu (2000). A mixture of monosaccharides was chromatographed in a TSK-gel SUGAR AX1 column (TOSOH Co.) with 0.5 M borate–1% ethanolamine–HCl buffer at pH 7.9. Relative percentage amounts were calculated electronically.

Size exclusion chromatography (SEC) for neutral xylooligosaccharides was performed on KS 802 [4.6 mm × 250 mm ×2 (in series), Shodex Co.] at 70 °C and a flow rate of 0.7 ml/min using distilled water as an eluent. The eluate was monitored by differential refractometer (TOSOH Co. Model RI-8010).

2.2. Electrospray-ionization mass spectroscopy

Electrospray-ionizaton mass spectroscopy (ESI-MS) analysis was performed with a Thermo-Quest LCQ DUO mass spectrometer (Thermoelectron, Waltham, MA, USA) operated in negative-ion mode with a spray voltage of 4.55 kV, a capillary voltage of 3.1 V, and a capillary temperature of 180 °C. Mass spectra were obtained between m/z 150 and 2000.

2.3. NMR spectrometry

NMR spectra of oligosaccharides were recorded at 25 °C by taking samples in D_2O with a JEOL ALPHA 500FT-NMR spectrometer ^1H and ^{13}C NMR spectra were obtained at 500.16 and 125.77 MHz, respectively. The chemical shifts are referred to acetone at 2.225 (^1H) and 31.07 ppm (^{13}C) as an internal standard and are reported relative to TSP. Standard pulse sequences were utilized to obtain COSY, HOHAHA, NOESY, HMQC and HMBC spectra.

2.4. Substrates

The preparation of AGX from sugi (*C. japonica*) was described in the previous paper (Yamasaki et al., 2011). Although AGX preparation was contaminated with other polysaccharides (12.0% by weight) consisting of mannose, glucose and galactose, and an appreciable amount (41%) of lignin, it was used as a starting sample without further purification. Purified AGX contained one 4-*O*-Me-D-GlcA residue per 6.2 D-Xyl residues and one L-Ara residue per 21.4 D-Xyl residues (Yamasaki et al., 2011).

2.5. Purification of xylanase

The source of enzyme was the commercial preparation "Meicelase" from *T. viride* which was kindly supplied by Meiji Seika Co., Ltd. Insoluble glucuronoxylan was prepared as follows. The glucuronoxylan (5 g) isolated from the holocellulose of beech (*Fagus crenate*) (Shimizu, Teratani, & Miyazaki, 1968) was suspended in 400 ml distilled water at room temperature for 30 min and then the insoluble portion of xylan was collected by centrifugation (4500 × *g*, 30 min). After three times repeating of this operation, the insoluble portion of xylan was freeze-dried. This insoluble xylan was added to the enzyme solution [0.1 M sodium acetate buffer (pH 5.7, 100 ml) dissolving "Meicelase" 100 mg] and stirred gently at 0 °C for 1 h. The xylan was collected by centrifugation (4500 × *g*, 30 min, 4 °C), suspended to 0.1 M sodium acetate buffer (20 ml), and incubated at 40 °C for 10 min to release xylanase from the insoluble xylan. Supernatant containing xylanase was prepared from the suspension by centrifugation (4500 × *g*, 20 min, 4 °C). This procedure was repeated 5 times.

Proteins were visualized by SDS-PAGE after staining with CBB reagent.

2.6. Assay of enzyme activity

Xylanase activity was assayed by measuring the reducing sugars released from the beech xylan using Somogyi–Nelson method. The enzyme [300 μl (5 mg/30 ml) diluted with 200 μl H_2O] was incubated 1/20 M sodium acetate buffer (500 μl, pH 5.5) containing 1.0%