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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%

beech xylan at 40 °C. One unit of xylanase activity was defined as the amount of enzyme required to produce 1 mmol of reducing sugar equivalent to xylose per min under the assay condition.

2.7. Hydrolysis of AGX by partially purified xylanase

AGX (3 g) was suspended in 100 ml of water and heated at 100 °C for 15 min in a water bath giving a suspension. This solution was diluted with 100 ml of 0.1 M sodium acetate buffer, pH 5.5, and then incubated with the partially purified xylanase solution containing 13.8 U at 40 °C for 120 h. After removal of precipitate by centrifugation, the hydrolysis products were separated into neutral sugars and acidic sugars in the usual way (Shimizu, Ishihara, & Ishihara, 1976; Yamasaki et al., 2011).

2.8. Separation and identification of acidic sugars

The acidic sugars were fractionated by anion exchange chromatography on a preparative column of strongly acidic ion exchange resin Diaion CAO8Y (23–25 μm, AcO^-, 15 mm × 930 mm) by elution with 0.08 M sodium acetate (pH 5.9), giving seven groups. Each group was evaporated under reduced pressure to dryness and weighed after removal of sodium ion with a cation exchange resin Dowex 50E-X8 (H^+). Groups 1–7 were rechromatographed on preparative columns (10 mm × 849 mm and 5 mm × 436 mm) of Aminex A-27 (12–15 μm, AcO^-) by elution with 0.125–1.0 M acetic acid. The eluate was monitored by a differential refractometer (ERC Inc. Model 7515A). Volume distribution coefficients (*Dv*s) of each acidic sugar were calculated in the usual way (Samuelson, 1963).

Each acidic sugar was identified by *Dv*s, acid hydrolysis with 2 M TFA, subsequent identification of the hydrolysis products and ^1H and ^{13}C NMR spectroscopy. The anomeric configurations of the glycosyl residues were determined by means of ^1H and ^{13}C NMR spectroscopy. Assignments of signals were carried out using 2D NMR spectrometry.

3. Result and discussion

Xylanase fraction was isolated from the commercial cellulase preparation "Meicelase" from *T. viride* by absorbing on the insoluble beech glucuronoxylan. Three main proteins of different molecular weight (by SDS-PAGE) existed in the enzyme solution (Fig. 1). Through the process of xylanase isolation, xylan activity was recovered with 56.6% from the original solution.

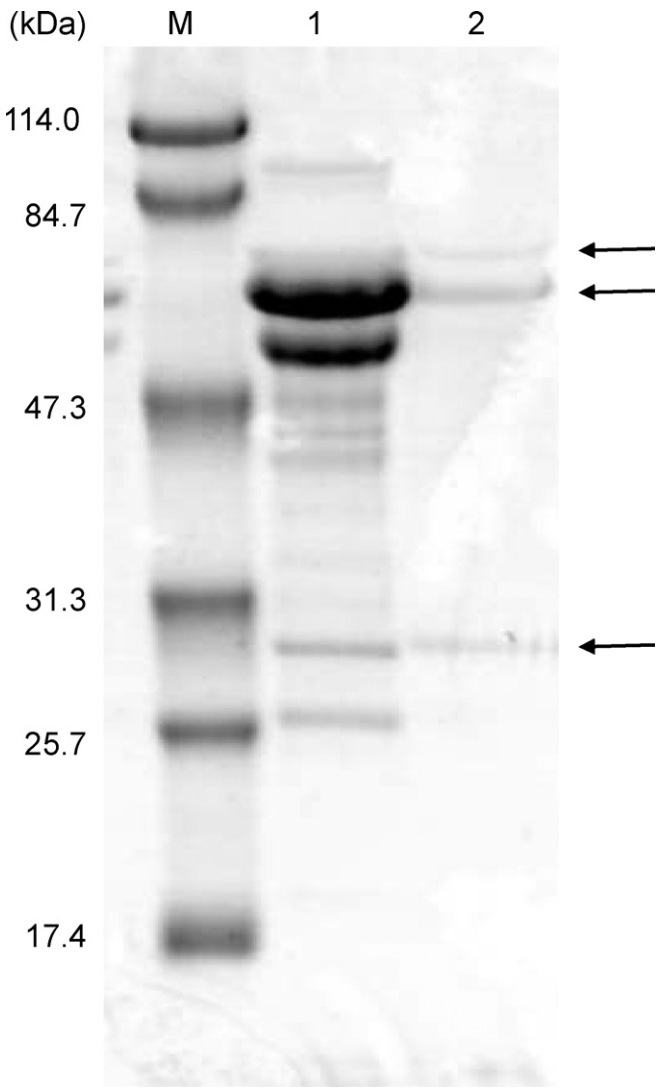

Fig. 1. SDS-PAGE of partially purified xylanases. Lane M contains molecular size standards (Sigma). Lane 1 contains the proteins in original cellulose preparation (Meicellase, Meiji Seika Co., Ltd.). Lane 2 contains the partially purified enzyme. The arrows indicate three main proteins.

AGX was extracted from the holocellulose of sugi (*C. japonica*) wood meal with 10% potassium hydroxide under the presence of barium hydroxide by the method of Brink and Pohlman (1972) as described previously (Yamasaki et al., 2011). The crude AGX preparation was contaminated with small amounts of other polysaccharides and an appreciable amount of lignin. The purified AGX contained one 4-*O*-Me-D-GlcA*p* residue per 6.2 D-Xyl*p* residues and one L-Ara*f* residue per 21.7 D-Xyl*p* residues, respectively (Yamasaki et al., 2011). It should be noted here that, in sugi AGX, the degree of substitution with 4-*O*-Me-D-GlcA*p* residue was ordinary level in softwood AGXs, but the substitution with L-Ara*f* residue was extraordinarily low, which was the same level as that of redwood AGX (Comtat & Joseleau, 1981).

The crude AGX preparation was subjected to enzymatic hydrolysis without further purification. Two percent solution of AGX (3 g) was incubated with the partially purified xylanase fraction. After two days incubation, the hydrolysis product was resolved into a neutral (74%) and an acidic (21%) portion with anion exchange resin in the usual way (Shimizu et al., 1976; Yamasaki et al., 2011).

The neutral sugar fraction was analyzed by SEC showing the presence of a homologous series of xylooligosaccharides from xylobiose (Xyl_2) to xylohexaose (Xyl_6) in addition to xylose (Fig. 2). Xyl_2 accumulated as an end product of hydrolysis.

Fig. 2 shows also large peaks at the void volume indicating that the presence of acidic sugars. The acidic sugars eluted at the void volume were separately collected and were analyzed by ESI-MS. The negative-ion mode ESI-MS spectrum (Fig. 3) of the sugars at void volume showed peaks at *m*/*z* 603.5, 735.7, 867.6, 999.6, 1131.6, corresponding to [$MeGlcAXyl_n$] (*n* = 3–7), respectively. This means that a part of aldouronic acids were eluted out from the strong anion-exchange resins column (OAc^- form) with water. On the other hand, no peaks corresponding to neutral xylooligosaccharides were found to be present at the void volume indicating that the longest xylooligosaccharide released was xylohexaose.

The acidic sugars were first separated into main seven groups (groups 1–7) with several minor groups which were neglected in the present study according to their molecular size by means of anion exchange chromatography on the preparative column (Diaion CAO8Y) using 0.08 M sodium acetate (pH 5.9) as eluent as shown in Fig. 4. Each group was rechromatographed and purified

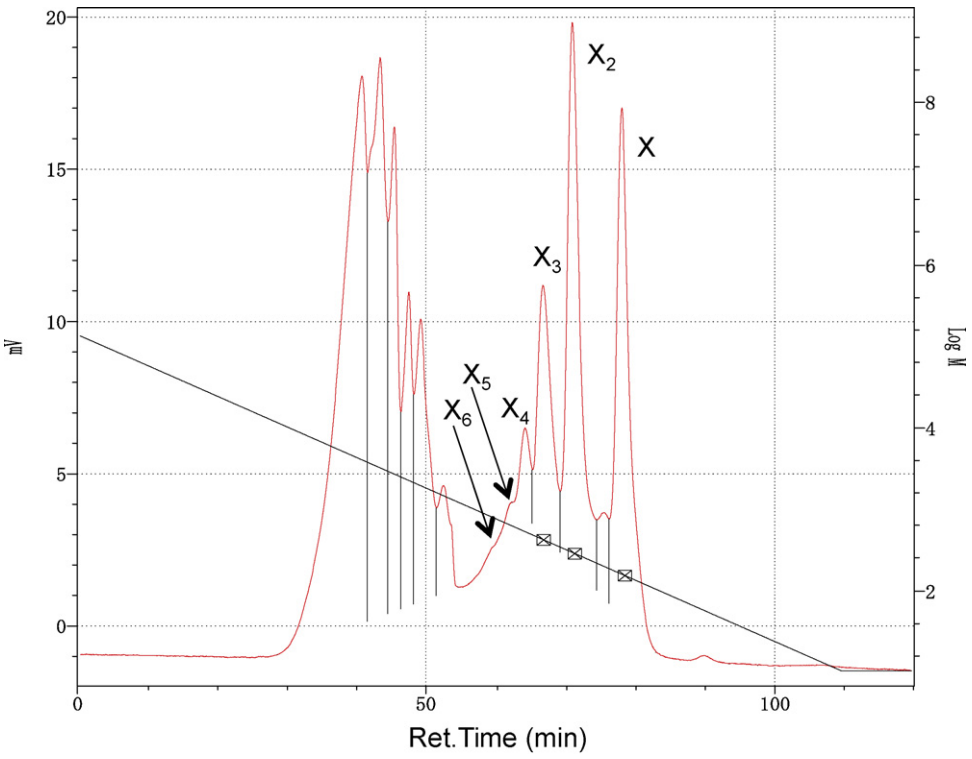

Fig. 2. Size exclusion chromatography of neutral sugars formed from arabinoglucuronoxylan of sugi (*C. japonica*) on hydrolysis with the partially purified xylanase fraction from *Trichoderma viride*; column: KS 802 (4.6 mm × 250 mm ×2, Shodex Co.) at 70 °C, eluent: distilled water at flow rate of 0.7 ml/min. X–X_6 = Xyl_n (n = 1–6).

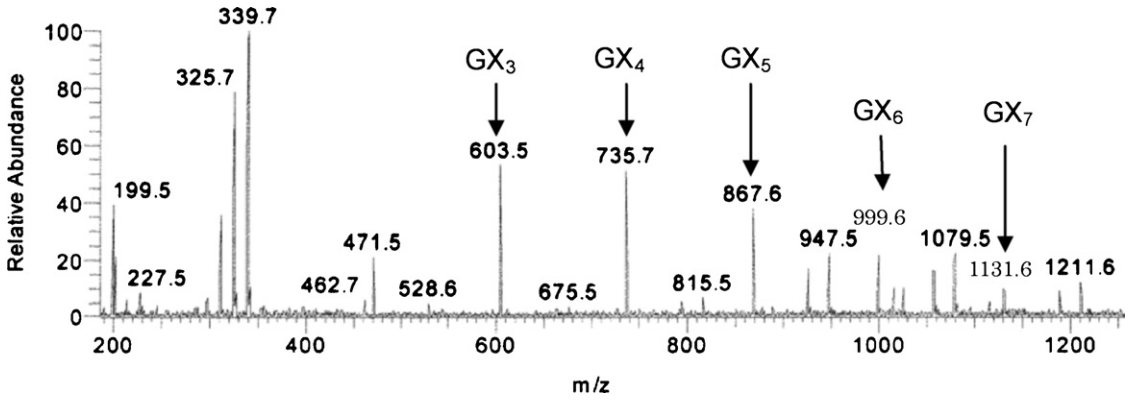

Fig. 3. Negative-ion mode ESI-MS spectrum of the acidic sugars remaining in the neutral sugar fraction obtained from the hydrolysate of sugi AGX by partially purified xylanase. GX_3–GX_7 = $[MeGlcA\text{-}Xyl_n]^-$ (n = 3–7).

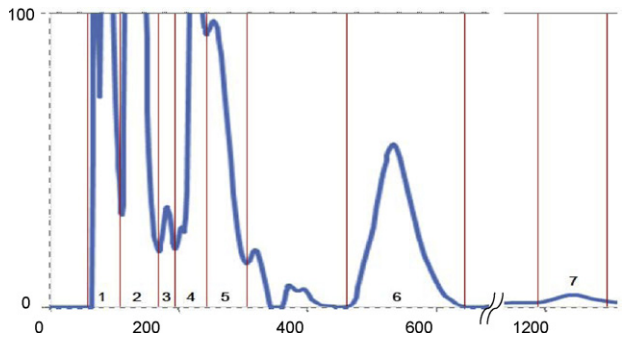

Fig. 4. Fractionation of aldouronic acids formed from arabinoglucuronoxylan of sugi (*C. japonica*) on hydrolysis with the partially purified xylanase fraction from *Trichoderma viride*; column: [15 mm × 930 mm, Diaion CAO8Y (AcO^-)], eluent: 0.08 M NaOAc at 1.5 ml/min.

by use of 0.125–1.0 M acetic acid, respectively. When acetic acid is used as eluent, the strength of the eluted acid has a predominant influence upon *Dv* (Havlicek & Samuelson, 1972).

Fraction 1 was rechromatographed on the preparative column (Aminex A-27) with 0.25 M acetic acid as eluent giving three fractions, Frs. 1-S1, 1-S2 and 1-S3 (Fig. 5).

Fr. 1-S1 was further purified by rechromatography with 0.125 M acetic acid as eluent. Fr. 1-S1 gave only Xyl as neutral sugar on hydrolysis and identified tentatively as *O*-β-Xyl*p*-(1 → 4)-*O*-β-D-Xyl*p*-(1 → 4)-[*O*-(4-*O*-Me-α-D-GlcA*p*)-(1 → 2)]-*O*-β-Xyl*p*-(1 → 4)-*O*-β-D-Xyl*p*-(1 → 4)-D-Xyl ($MeGlcA^3Xyl_5$). ^1H NMR and ^{13}C NMR spectra indicated that this acid was an aldohexaouronic acid, being either $MeGlcA^4Xyl_5$ or $MeGlcA^3Xyl_5$ (Debeire et al., 1990; John, Rice, & Preston, 2006; Kardošová, Matulová, & Maloviková, 1998; Komiyama, Kato, Aimi, Ogihara, & Shimizu, 2008; Yamasaki et al., 2011).

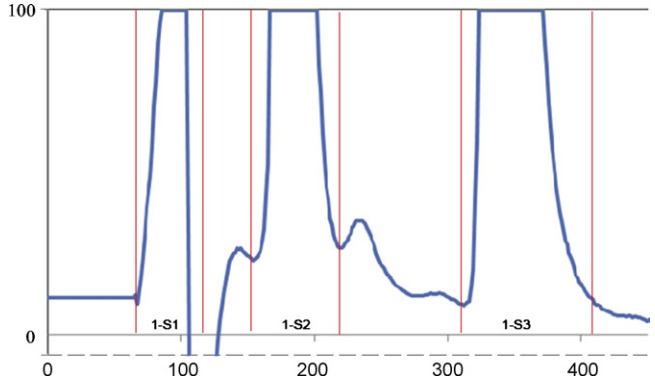

Fig. 5. Rechromatography of Fr. 1 in Fig. 1 on a column (10 mm × 830 mm) of Aminex A-27(AcO^-) with 0.25 M acetic acid at 0.7 ml/min.

It has been reported that $MeGlcA^3Xyl_5$ is produced from 4-*O*-methylglucuronoxylan by xylanase belonging to family 11 (Biely, Vršanská, Tenkanen, & Kluepfel, 1997; Debeire et al., 1990; Kolenová, Vršanská, & Biely, 2006; Kolenová, Ryabova, Vršanská, & Biely, 2010).

Fr. 1-S2 gave only xylose as neutral sugar on hydrolysis. Its *Dv*s were identical with those of the authentic sample *O*-β-Xyl*p*-(1 → 4)-[*O*-(4-*O*-Me-α-D-GlcA*p*)-(1 → 2)]-*O*-β-D-Xyl*p*-(1 → 4)-*O*-β-Xyl*p*-(1 → 4)-D-Xyl ($MeGlcA^3Xyl_4$) (Komiyama et al., 2009; Togashi, Kato, & Shimizu, 2009).

Fr. 1-S3 showed the same *Dv*s as a main component of Fraction 2.

When Fraction 2 was rechromatographed with 0.25 M acetic acid, it gave Fr. 2-S1 as a main fraction, with a few minor fractions which were neglected in this study. Fr. 2-S1 (identical with Fr. 1-S3) gave only xylose as neutral sugar on hydrolysis. Its *Dv*s were identical with those of the authentic sample *O*-(4-*O*-Me-α-D-GlcA*p*)-(1 → 2)-*O*-β-D-Xyl*p*-(1 → 4)-*O*-β-D-Xyl*p*-(1 → 4)-D-Xyl ($MeGlcA^3Xyl_3$) (Komiyama et al., 2009; Togashi et al., 2009).

Fraction 3 was rechromatographed with 0.5 M acetic acid giving three fractions, Frs. 3-S1, 3-S2 and 3-S3. Fr. 3-S1 showed the same *Dv*s and ^{13}C NMR spectrum as the authentic samples *O*-(α-D-GlcA*p*)-(1 → 2)-*O*-β-D-Xyl*p*-(1 → 4)-*O*-β-Xyl*p*-(1 → 4)-D-Xyl ($GlcA^3Xyl_3$) (Komiyama et al., 2008). Fr. 3-S1 was composed of D-Xyl and D-GlcA. Fr. 3-S2 was a main component of Fraction 4.

Fr. 3-S3 gave Rha, Gal, and Xyl as neutral sugars on hydrolysis. But this fraction was obtained in a very small amount and its ^{13}C NMR spectrum showed that it was still a mixture. Therefore, further study was not done.

Fraction 4 gave two fractions, Frs. 4-S1 and 4-S2 on rechromatography with M acetic acid with a few minor fractions which were neglected in this study. Fr. 4-S1 (identical with Fr. 3-S2) showed the same *Dv*s and ^{13}C NMR spectrum as the authentic sample *O*-(4-*O*-Me-α-D-GlcA*p*)-(1 → 2)-*O*-β-D-Xyl*p*-(1 → 4)-D-Xyl ($MeGlcA^2Xyl_2$) (Komiyama et al., 2008).

Fr. 4-S2 gave only Xyl as neutral sugar on hydrolysis. This acid was a new aldouronic acid containing two 4-O-Me-D-GlcA residues and identified as *O*-β-D-Xyl*p*-(1 → 4)-[*O*-(4-*O*-Me-α-D-GlcA*p*)]-(1 → 2)-*O*-β-D-Xyl*p*-(1 → 4)-[*O*-(4-*O*-Me-α-D-GlcA*p*)]-(1 → 2)-*O*-β-D-Xyl*p*-(1 → 4)-*O*-β-D-Xyl*p*-(1 → 4)-D-Xyl ($MeGlcA^4MeGlcA^3Xyl_5$) on the basis of 1D and 2D NMR spectra. The ^{13}C NMR spectrum is shown in Fig. 6 and the assignments of chemical shifts in the ^{13}C and ^1H spectra are shown in Table 1.

Fraction 5 overlapping with Fraction 4 gave two fractions, Frs. 5-S1 and 5-S2 on rechromatography with M acetic acid with a few minor fractions which were neglected in this study. Frs. 5-S1 and 5-S2 were identical with Frs. 4-S1 and 4-S2, respectively.

Fraction 6 gave one peak, Fr. 6-S1 on rechromatography with M acetic acid. The *Dv*s of this acid were identical with those of the authentic sample *O*-(4-*O*-Me-α-D-GlcA*p*)-(1 → 2)-*O*-β-D-Xyl*p*-(1 → 4)-*O*-[(4-*O*-Me-α-D-GlcA*p*)]-(1 → 2)-*O*-β-D-Xyl*p*-(1 → 4)-*O*-β-D-Xyl*p*-(1 → 4)-D-Xyl ($MeGlcA^4MeGlcA^3Xyl_4$) (Ishii et al., 2010;

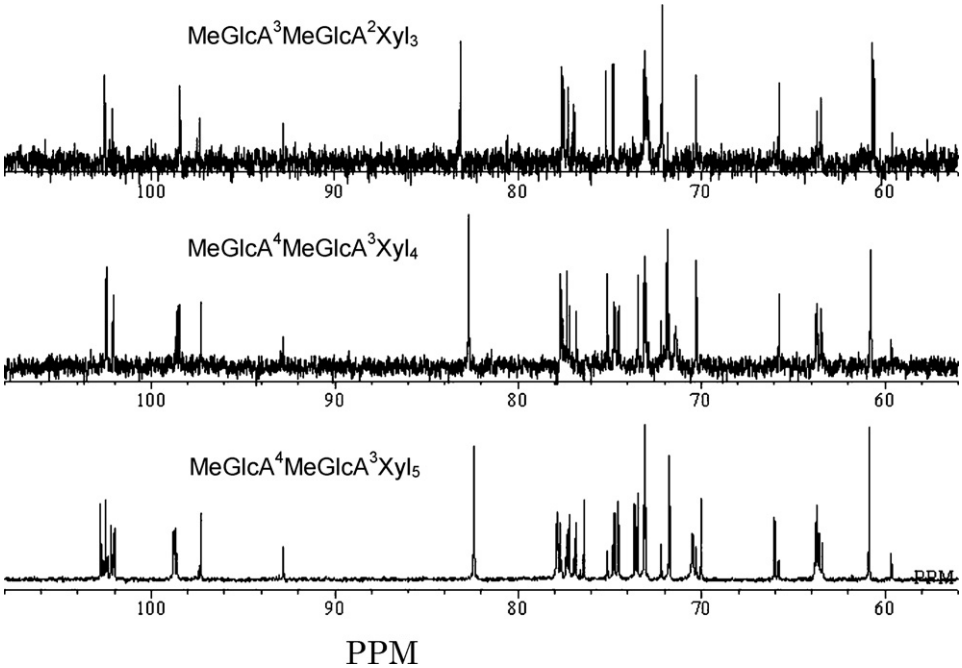

Fig. 6. ^{13}C NMR spectra of aldouronic acids containing two 4-O-Me-GlcA residues. MeGlcA3MeGlcA2Xyl3: *O*-(4-*O*-Me-α-D-GlcA*p*)-(1 → 2)-*O*-β-D-Xyl*p*-(1 → 4)-*O*-[(4-*O*-Me-α-D-GlcA*p*)]-(1 → 2)-*O*-β-D-Xyl*p*-(1 → 4)-D-Xyl MeGlcA4MeGlcA3Xyl4: *O*-(4-*O*-Me-α-D-GlcA*p*)-(1 → 2)-*O*-β-D-Xyl*p*-(1 → 4)-*O*-[(4-*O*-Me-α-D-GlcA*p*)]-(1 → 2)-*O*-β-D-Xyl*p*-(1 → 4)-*O*-β-D-Xyl*p*-(1 → 4)-D-Xyl MeGlcA4MeGlcA3Xyl5: *O*-β-D-Xyl*p*-(1 → 4)-[*O*-(4-*O*-Me-α-D-GlcA*p*)]-(1 → 2)-*O*-β-D-Xyl*p*-(1 → 4)-[*O*-(4-*O*-Me-α-D-GlcA*p*)]-(1 → 2)-*O*-β-D-Xyl*p*-(1 → 4)-*O*-β-D-Xyl*p*-(1 → 4)-D-Xyl.

Table 1
Assignments of chemical shifts in the ^{13}C and ^{1}H NMR spectra of aldouronic acids in the hydrolysate of AGX by xylanase.

$X^5 \rightarrow X^4 \rightarrow X^3 \rightarrow X^2 \rightarrow X^1$ $X^4 \rightarrow X^3 \rightarrow X^2 \rightarrow X^1$ $X^3 \rightarrow X^2 \rightarrow X^1$

$\uparrow \quad \uparrow$ $\uparrow \quad \uparrow$ $\uparrow \quad \uparrow$

$G^2 \quad G^1$ $G^2 \quad G^1$ $G^2 \quad G^1$.

		^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
	1	97.32	4.58	97.31	4.58	97.35	4.58
	2	74.81	3.24	74.79	3.24	74.81	3.23
$X^1\beta$	3	74.72	3.54	74.71	3.54	74.73	3.53
	4	77.22	3.77	77.19	3.78	77.22	3.77
	5	63.79	3.37, 4.05	63.79	3.37, 4.05	63.71	3.35, 4.04
	1	92.83	5.19	92.82	5.19	92.83	5.17
	2	72.18	3.54	72.17	3.54	72.18	3.54
$X^1\alpha$	3	71.76	3.74	71.75	3.75	71.80	3.73
	4	77.37	3.77	77.34	3.80	77.39	3.77
	5	59.65	3.78	59.64		59.65	3.78
	1	102.49	4.47	102.46	4.47	102.12	4.63
	2	73.48	3.28	73.48	3.28	77.50	3.39
X^2	3	74.54	3.56	74.52	3.57	72.99	3.65
	4	76.85	3.79	76.82	3.79	76.94	3.78
	5	63.69	3.43, 4.14	63.67	4.15	63.48	3.43, 4.15
	1	102.02	4.63	102.09	4.63	102.55	4.62
	2	77.85	3.38	77.62	3.42	77.60	3.37
X^3	3	73.14	3.65	73.13	3.66	75.13	3.50
	4	77.30	3.79	77.34		70.26	3.63
	5	63.41	3.43, 4.14	63.46		65.76	3.29, 3.97
	1	102.20	4.64	102.46	4.61		
	2	77.93	3.41	77.69	3.40		
X^4	3	73.14	3.63	75.12	3.50		
	4	77.69	3.79	70.26	3.64		
	5	63.59	3.37, 4.10	65.75	3.29, 3.97		
	1	102.78	4.46				
	2	73.64	3.25				
X^5	3	76.41	3.42				
	4	70.01	3.62				
	5	66.01	3.96, 3.30				
	1	98.73	5.30	98.52	5.31	98.45	5.31
	2	71.76	3.60	71.87	3.60	72.13	3.60
	3	73.07	3.80	73.02	3.79	73.12	3.80
G^1	4	82.40	3.31	82.70	3.29	83.16	3.31
	5	70.50	4.65	71.42	4.52	71.80	4.53
	6	174.60		175.70		174.50	
	OCH_3	60.85	3.51	60.78		60.65	3.50
	1	98.80	5.28	98.63	5.27	98.64	5.27
	2	71.76	3.59	71.87	3.59	72.13	3.59
	3	73.07	3.80	73.13	3.79	73.12	3.80
G^2	4	82.40	3.31	82.76	3.28	83.16	3.31
	5	70.50	4.65	71.42	4.55	71.80	4.53
	6	174.60		175.70		174.50	
	OCH_3	60.85	3.51	60.74	3.50	60.55	3.50

X, xylose residue; G, 4-*O*-methylglucuronic acid residue.

Shimizu et al., 1978; Yamasaki et al., 2011). The ^{13}C NMR spectrum is shown in Fig. 6 and the assignments of chemical shifts in the ^{13}C and ^{1}H spectra are shown in Table 1.

Fraction 7 gave one peak, Fr. 7-S1 on rechromatography with M acetic acid. The *Dv*s of this acid were identical with those of the authentic sample *O*-(4-*O*-Me-α-D-GlcA*p*)-(1 → 2)-*O*-β-D-Xyl*p*-(1 → 4)-*O*-[(4-*O*-Me-α-D-GlcA*p*)]-(1 → 2)-*O*-β-D-Xyl *p*-(1 → 4)-D-Xyl ($MeGlcA^3MeGlcA^2Xyl_3$) (Ishii et al., 2010; Shimizu et al., 1978; Yamasaki et al., 2011). The ^{13}C NMR spectrum is shown in Fig. 6 and the assignments of chemical shifts in the ^{13}C and ^{1}H spectra are shown in Table 1.

Finally, eight acidic sugars were isolated in pure states. Their yields were summarized in Table 2. These acidic oligosaccharides were classified into three groups.

The first group was the aldouronic acids, $MeGlcA^3Xyl_5$ (Fr. 1-S1), $MeGlcA^3Xyl_4$ (Fr. 1-S2), $MeGlcA^3Xyl_3$ (Fr. 2-S1), and $MeGlcA^2Xyl_2$ (Fr. 4-S1) from aldotrio- to aldohexaouronic acids composed of D-Xyl residues and one 4-*O*-Me-D-GlcA residue. $MeGlcA^3Xyl_3$ was the main product (Table 2).

The second group was the aldotetraouronic acid, $GlcA^3Xyl_3$ (Fr. 3-S1), consisting of D-Xyl and D-GlcA. This acid was obtained in a small amount (Table 2) showing that non-methylated glucuronic acid side chain residue was also present in the AGX.

The third group was the aldouronic acids, $MeGlcA^4MeGlcA^3Xyl_5$ (Fr. 4-S2), $MeGlcA^4MeGlcA^3Xyl_4$ (Fr. 6-S1) and $MeGlcA^3MeGlcA^2Xyl_3$ (Fr. 7-S1) consisting of 3–5 D-Xyl residues and two 4-*O*-Me-D-GlcA*p* residues which were located on two contiguous D-Xyl residues.

It is well known that *Trichoderma* spp. produce multiple xylanases with high xylanolytic activity as mentioned in Section 1 (Biely et al., 1991; Saloheimo et al., 2003;

Table 2
Yields of acidic oligosaccharides released from sugi AGX (3 g) by *Trichoderma viride* xylanase.

Fraction	Acidic oligasaccharides	Yield (mg)
Fr. 1-S1[a]	*O*-β-Xyl*p*-(1 → 4)-*O*-β-D-Xyl*p*-(1 → 4)-[*O*-(4-*O*-Me-α-D-GlcA*p*)-(1 → 2)]-*O*-β-Xyl*p*-(1 → 4)-*O*-β-D-Xyl*p*-D-Xyl ($MeGlcA^3Xyl_5$)	9.2
Fr. 1-S2	*O*-β-Xyl*p*-(1 → 4)-[*O*-(4-*O*-Me-α-D-GlcA*p*)-(1 → 2)]-*O*-β-D-Xyl*p*-(1 → 4)-*O*-β-Xyl*p*-(1 → 4)-D-Xyl ($MeGlcA^3Xyl_4$)	36.9
Fr. 2-S1	*O*-(4-*O*-Me-α-D-GlcA*p*)-(1 → 2)-*O*-β-D-Xyl*p*-(1 → 4)-*O*-β-D-Xyl*p*-(1 → 4)-D-Xyl ($MeGlcA^3Xyl_3$)	238.9
Fr. 3-S1	*O*-(α-D-GlcA*p*)-(1 → 2)-*O*-β-D-Xyl*p*-(1 → 4)-*O*-β-Xyl*p*-(1 → 4)-D-Xyl ($GlcA^3Xyl_3$)	1.5
Fr. 4-S1	*O*-(4-*O*-Me-α-D-GlcA*p*)-(1 → 2)-*O*-β-D-Xyl*p*-(1 → 4)-D-Xyl ($MeGlcA^2Xyl_2$)	45.0
Fr. 4-S2	*O*-β-D-Xyl*p*-(1 → 4)-*O*-(4-*O*-Me-α-D-GlcA*p*)-(1 → 2)-*O*-β-D-Xyl*p*-(1 → 4)-*O*-(4-*O*-Me-α-D-GlcA*p*)-(1 → 2)-*O*-β-D-Xyl*p*-(1 → 4)-*O*-β-D-Xyl*p*-(1 → 4)-D-Xyl ($MeGlcA^4MeGlcA^3Xyl_5$)	28.4
Fr. 6-S1	*O*-(4-*O*-Me-α-D-GlcA*p*)-(1 → 2)-*O*-β-D-Xyl*p*-(1 → 4)-*O*-(4-*O*-Me-α-D-GlcA*p*)-(1 → 2)-*O*-β-D-Xyl*p*-(1 → 4)-*O*-β-D-Xyl*p*-(1 → 4)-D-Xyl ($MeGlcA^4MeGlcA^3Xyl_4$)	17.7
Fr. 7-S1	*O*-(4-*O*-Me-α-D-GlcA*p*)-(1 → 2)-*O*-β-D-Xyl*p*-(1 → 4)-*O*-(4-*O*-Me-α-D-GlcA*p*)-(1 → 2)-*O*-β-D-Xyl*p*-(1 → 4)-D-Xyl ($MeGlcA^3MeGlcA^2Xyl_3$)	27.8
	Total yield	405.4

[a] Tentatively identified.

Tenkanen et al., 1992; Törrönen et al., 1992; Xu et al., 1998; Wong & Saddler, 1992). It can be considered that the AGX was synergetically hydrolyzed with these enzymes. The release of the acidic xylooligosaccharides described above can be explained by the specific mode of actions of the xylanases as discussed in the case of *Eucalyptus globulus* glucuronoxylan (Togashi et al., 2009). $MeGlcA^3Xyl_5$, $MeGlcA^3Xyl_4$ and $MeGlcA^4MeGlcA^3Xyl_5$ can be considered to be the end products released by the xylanase belonging to family 11 whereas $MeGlcA^3Xyl_3$, $GlcA^3Xyl_3$ and $MeGlcA^4MeGlcA^3Xyl_4$ by the xylanase belonging to family 10. $MeGlcA^2Xyl_2$ and $MeGlcA^3MeGlcA^2Xyl_3$ may possibly to be produced by the xylanase belonging to family 5 (Gallardo et al., 2010) which was isolated from *T. reesei* by Saloheimo et al. (2003). The formation of these acids also suggests the presence of unique xylanase "appendage-dependent xylanase" (John et al., 2006; Nishitani & Nevings, 1991; Vršanská et al., 2007) which is known to generate the aldotriouronic acid $MeGlcA^2Xyl_2$ from hardwood xylan together with β-xylosidase. But this enzyme has never been reported to be present in the enzymes secreted by *T. viride*.

As for neutral sugars, Xyl_6 was found to be present in the hydrolysate as the longest xylooligosaccharide. This indicated that there are hydrolysable regions having uronic acid groups which are at least eight xylosyl residues apart taking the mode of action of endo-xylanase into consideration. As reported in the previous paper (Yamasaki et al., 2011), Xyl_8 was detected to be present by ESI-MS as the longest xylooligosaccharide in the partial acid hydrolysate of sugi AGX.

The acidic xylooligosaccharides, $MeGlcA_2Xyl_{2-5}$, containing two 4-*O*-Me-D-GlcA residues which were located on the contiguous Xyl residues were isolated in addition to the aldouronic acids containing one the uronic acid residue, $MeGlcAXyl_{2-5}$. The distribution pattern of these vicinal uronic acid units along the D-xylan chain has not been cleared up. Jacobs et al. (2001) found that the vicinal uronic acid units are located, randomly or periodically, between larger domains composed of the major structural unit, which contains one 4-*O*-Me-D-GlcA residue per seven or eight xylose residues. The results in this study are compatible with the findings by Jacobs et al. (2001) as well as the results obtained by partial acid hydrolysis in the previous paper (Yamasaki et al., 2011).

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