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Enzymatically derived aldouronic acids from *Eucalyptus globulus* glucuronoxylan

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ABSTRACT

A glucuronoxylan was extracted from the holocellulose of *Eucalyputus globulus* wood with 10% KOH and subjected to hydrolysis by a commercial cellulase preparation "Meicelase". Neutral xylooligosaccharides liberated were analyzed by size exclusion chromatography. Aldouronic acids liberated were purified by preparative anion exchange chromatography. Their structures were studied by monosaccharide analysis, comparison of volume distribution coefficients (*Dv*s) in anion exchange chromatography with those of the authentic samples, and ^{1}H and ^{13}C NMR spectroscopy, resulting in the characterization of seven aldouronic acids including a novel one containing galactose residue.

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
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
O-(4-*O*-Me-α-D-GlcA*p*)-(1 → 2)-*O*-β-D-Xyl*p*-(1 → 4)-D-Xyl
O-β-D-Xyl*p*-(1 → 4)-*O*-β-D-Xyl*p*-(1→3)-*O*-α-L-Rha*p*-(1 → 2)-*O*-α-L-GalA*p*-(1 → 4)-D-Xyl
O-β-D-Xyl*p*-(1 → 4)-*O*-β-D-Xyl*p*-(1 → 3)-*O*-α-L-Rha*p*-(1 → 2)-D-GalA
O-β-D-Xyl*p*-(1 → 3)-*O*-α-L-Rha*p*-(1 → 2)-*O*-α-D-GalA*p*-(1 → 4)-D-Xyl
O-β-D-Gal*p*-(1 → 2)-*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.

The oligosaccharides liberated provide information on multiplicity of xylanases secreted by *Trichoderma viride*. The presence of the last aldouronic acid shows a structural feature of *E. globulus* xylan.

1. Introduction

The chemical structure of 4-*O*-methylglucuronoxylan from hardwoods has been well established. It consists of a backbone of about 200 (1 → 4)-linked D-xylopyranose (DXyl*p*) residues. Every tenth Xyl residue, on average, is substituted at C-2 by a 4-*O*-methylglucopyranosyl uronic acid (4-*O*-Me-D-GlcA*p*) residue.

About 30 years ago, we isolated an acidic oligosaccharide "*O*-β-D-Xyl*p*-(1 → 4)-*O*-β-D-Xyl*p*-(1 → 3)-*O*-α-L-Rha*p*-(1 → 2)-*O*-α-L-GalA*p*-(1 → 4)-D-Xyl" from the enzymatic hydrolyzate of birch xylan (Shimizu, Ishihara, & Ishihara, 1976). Recently, we isolated this acidic sugar from enzymatic hydrolyzate of kenaf xylan (Komiyama et al., 2009). Evtuguin, Tomás, Silva, and Pascoal-Neto (2003) also found this glycosyl sequence to be present in the heteroxylan isolated from *Eucalyptus globulus* Labill wood by extraction of peracetic acid delignified holocellulose with dimethyl sulfoxide.

Enzymatic hydrolysis of the holocellulose from spruce meal reduced with borohydride liberated *O*-β-D-Xyl*p*-(1 → 3)-*O*-α-L-Rha*p*-(1 → 2)-*O*-α-D-GalA*p*-(1 → 4)-D-xylitol (Andersson, Samuelson, Ishihara, & Shimizu, 1983). From these facts with other evidences (Johansson & Samuelson, 1977), we concluded that the glycosyl sequence is located on the reducing end of xylans from hardwood and softwood. It has been shown that this glycosyl sequence contributes markedly to the stability of xylan towards endwise alkaline degradation (peeling) during alkaline cooking of wood (Johansson & Samuelson, 1977).

Peña et al. (2007) showed by ^{1}H NMR spectroscopy that the reducing end of *Arabidopsis* xylan contains this glycosyl sequence and demonstrated that this sequence is required for normal xylan synthesis in secondary walls of dicots. Recently, various researchers (Brown et al., 2007; Lee et al., 2007; Peña et al., 2007) revealed that this glycosyl sequence plays very important role as a primer and/or regulator for chain length in normal glucuronoxylan synthesis by plants and they suggested that genetic manipulation of glucuronoxylan biosynthesis leads to the more efficient use of plant secondary walls in energy production, wood and paper production, and nutrition (York & O'Neill, 2008).

On the other hand, the xylan isolated from the holocellulose of *E. globulus* Labill with 2 M KOH and/or dimethyl sulfoxide has been shown to have a chemical structure different from that normally found in xylans of angiosperms by Shatalov, Evtuguin, and Pascoal-Neto (1999) and Evtuguin et al. (2003). Besides (1 → 4)-linked β-D-xylopyranosyl units of the backbone and short side chains of terminal (1 → 2)-linked 4-*O*-Me-α-D-GlcA*p* residue in a 1:10 molar

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ratio, this hemicellulose contains galactosyl and glucosyl units attached at *O*-2 of 4-*O*-Me-D-GlcA*p*. About 30% of 4-*O*-Me-D-GlcA*p* units are branched at *O*-2.

In this paper, we report on structural analysis of enzymatically derived oligosaccharides from *E. globulus* xylan. Based on the structure of oligosaccharides, the structural feature of xylan and the specificity of xylanases are 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% borate-1.0% ethanolamine–HCl buffer (pH 7.9). Relative percentage amounts were calculated electronically.

Size exclusion chromatography (SEC) for neutral xylooligosaccharides was performed on KS 802 (4.6 × 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.).

2.2. NMR spectroscopy

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. 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.

2.3. Preparation of xylan

A 10-year-old *E. globulus* tree grown in Western Australia was supplied by Toshihiro Ona, Associate Professor of Kyushu University, Faculty of Agriculture. The wood sample was milled with a cutting mill (Fritsch Japan Co., Ltd.) and the fraction 60–80 mesh was collected by sieving.

The mill (20 g) was exhaustively extracted with methanol in an extracting equipment and then delignified by chlorite method. Xylan was extracted from the air-dried holocellulose with 10% KOH (200 ml) for 2 h at 25 °C under nitrogen with occasional application of ultrasound (28 kHz). The extract was neutralized with acetic acid and dialyzed against distilled water. The dialyzed solution was condensed and freeze-dried.

2.4. Enzymatic hydrolysis of xylan

The source of enzyme was the commercial cellulase preparation "Meicelase" from *Trichoderma viride* which was kindly supplied by Meiji Seika Co., Ltd. Xylanase activity was assayed by measuring the reducing sugars released from the xylan using Somogyi–Nelson method. The enzyme (5 mg) was incubated 1/20 M sodium acetate buffer (pH 5.5) containing 1.0% 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.

The xylan (5 g) was suspended in 100 ml of distilled water and heated at 100 °C for 10 min in a water bath giving translucent solution. This solution was diluted with 100 ml of 0.1 M acetate buffer (pH 5.5), and then incubated with the commercial cellulase preparation (30 mg, xylanase activity: 9.5 U) at 40 °C. Aliquots were taken at different time intervals and the incubation was stopped by heating (100 °C, 10 min). The degree of degradation was determined by estimating the release of reducing end-groups according to Somogyi–Nelson. The change in molecular mass distribution of the generated mixture was determined by SEC as described above.

After incubation for 120 h, the sodium ion was removed with a cation exchange resin (Dowex 50 W × 8, H^+ form), and the hydrolysis product was treated with 1/10 N NaOH at pH 8 for 4 h. The resulting solution was applied to the column of anion exchange resin (Dowex 1 × 8, AcO^- form). The neutral sugars were washed out with water until the test with anthrone on the effluent was negative (yield 2.7 g). The acidic sugars were then eluted with 5 M acetic acid (yield 0.7 g).

2.5. 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 (AcO^-, 15 × 930 mm) by elution with A, 0.08 M sodium acetate (pH 5.9) giving 4 fractions. Fractions 1 and 2 were overlapped with the vicinal fractions, so they were separated from each other by elution with B, 0.02 M sodium acetate (pH 5.9) on the same column. Fractions 1 and 2 were rechromatographed on a preparative column of Aminex A-27 (AcO^-, 10 × 830 mm) by elution with C, 0.25 M acetic acid giving two fractions, respectively (1-S1, 1-S2, 2-S1 and 2-S2). Fractions 3 and 4 were rechromatographed by elution with D, 0.5 M acetic acid on the same column as above giving three and four fractions, respectively (3-S1, 3-S2, 3-S3, 4-S1, 4-S2, 4-S3 and 4-S4).

Each acidic sugar was identified by volume distribution coefficients (*Dvs*) calculated in the usual way (Samuelson, 1963), acid hydrolysis, subsequent identification of the hydrolysis products and ^{13}C NMR and ^1H NMR spectroscopy.

Fraction 1 representing aldopentaouronic acid gave two fractions Fr. 1-S1 and Fr. 1-S2 having *Dvs* 2.63 and 4.02 with eluent C, respectively. Fr. 1-S1 gave only Xyl as neutral sugar on hydrolysis with 2 M TFA at 120 °C for 2 h. Fr. 1-S1 was 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*-β-D-Xyl*p*-(1 → 4)-D-Xyl (Xyl-β-1,4-[MeGlcA-α-1,2-]Xyl-β-1,4-Xyl-β-1,4-Xyl) on basis of ^{13}C and ^1H NMR spectra. The ^{13}C NMR spectrum of Fr. 1-S1 was identical with those reported in the previous paper (Komiyama et al., 2009) and by Nacos et al (2006).

Fr. 1-S2 was a new aldouronic acid giving Xyl and Gal in the ratio of 2.5:1.0 as neutral sugars on hydrolysis with 2 M TFA at 120 °C for 1 h. This acid was identified as *O*-β-D-Gal*p*-(1 → 2)-*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 (Gal-β-1,2-MeGlcA-α-1,2-Xyl-β-1,4-Xyl-β-1,4-Xyl) on the basis of 1D and 2D NMR spectra.

Fraction 2 gave two fractions Fr. 2-S1 and Fr. 2-S2 having *Dvs* 4.09 and 5.09 with eluent C, respectively. Fr. 2-S1 gave Rha and Xyl in the ratio of 1:2.0 as neutral sugars on hydrolysis with 2 M TFA at 120 °C for 1 h. It was identified as *O*-β-D-Xyl*p*-(1 → 4)-*O*-β-D-Xyl*p*-(1 → 3)-*O*-α-L-Rha*p*-(1 → 2)-*O*-α-D-GalA*p*-(1 → 4)-D-Xyl (Xyl-β-1,4-Xyl-β-1,3-Rha-α-1,2-α-GalA-β-1,4-Xyl) on the basis of its *Dvs* (Shimizu et al., 1976) and NMR spectra (Ishii et al, 2008; Komiyama et al., 2009).

Fr. 2-S2 was the main product and its *Dvs* and ^{13}C NMR spectrum 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-α-1,2-]Xyl-β-1,4-Xyl-β-1,4-Xyl) (Komiyama, Kato, Aimi, Ogihara, & Shimizu, 2008).

Fraction 3 gave three fractions Frs. 3-S1, 3-S2 and 3-S3 on rechromatography in eluent D with *Dvs* 2.51, 2.70 and 3.19, respec-

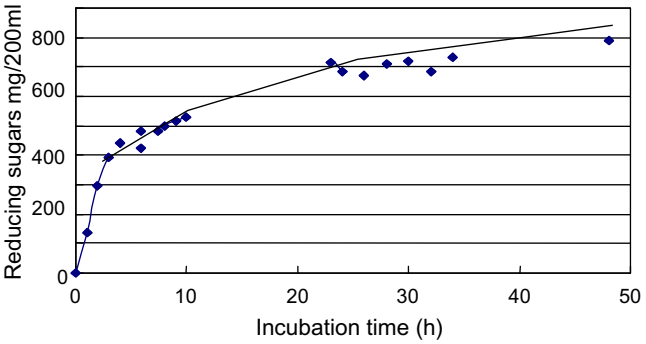

Fig. 1. Time-course of reducing sugar production from *Eucalyputus globulus* xylan by xylanase from *Trichoderma viride*.

tively. Fr. 3-S1 was the same acids as Fr. 2S-2, from the overlapping Fraction 2. Fr. 3-S2 and 3-S3 gave Rha and Xyl in the ratio of 1:1.9 and 1:2.0, respectively, as neutral sugars on hydrolysis with 2 M TFA at 120 °C for 1 h. Their *Dv*s were identical with those of authentic samples (Shimizu et al., 1976) and they were identified as *O*-β-D-Xyl*p*-(1 → 4)-*O*-β-D-Xyl*p*-(1 → 3)-*O*-α-L-Rha*p*-(1 → 2)-D-GalA (Fr. 3-S2: Xyl-β-1,4-Xyl-β-1,3-Rha-α-1,2-GalA) and *O*-β-D-Xyl*p*-(1 → 3)-*O*-α-L-Rha*p*-(1 → 2)-*O*-α-D-GalA*p*-(1 → 4)-D-Xyl (Fr. 3-S3: Xyl-β-1,3-Rha-α-1,2-α-GalA-β-1,4-Xyl), respectively.

Fraction 4 representing aldotriouronic acid gave four fractions. Fr. 4-S3 having *Dv* 4.26 in eluent D was the main acid and others, Frs. 4-S1 and 4-S2 were the same acids as Frs. 3-S2 and 3-S3, respectively, from the overlapping Fraction 3. Fr. 4-S3 was identified as *O*-(4-*O*-Me-α-D-GlcA*p*)-(1 → 2)-*O*-β-D-Xyl*p*-(1 → 4)-D-Xyl ([MeGlcA-α-1,2-]Xyl-β-1,4-Xyl) by comparison of its *Dv*s with those of the authentic sample (Shimizu et al., 1976). Its 13C NMR spectrum was identical with that reported in a previous paper (Cavagna, Degar, & Puls, 1984; Komiyama et al., 2008). Fr. 4-S4 was obtained in a trace amount and neglected in this paper.

3. Results and discussion

Extractive-free wood meal of *E. globulus* (60–80 mesh) which contained alcohol-benzene extractives 1.3% and Klason-lignin 20.8% was delignified by the chlorite method. Xylan was extracted in a yield of 26.9% from the air-dried holocellulose with 10% KOH for 2 h at 25 °C under nitrogen with application of ultrasound.

This xylan gave Xyl 88.4%, Gal 7.2%, Glc 3.2%, Rha 0.8% and Man 0.4% on hydrolysis with 2 M TFA at 100 °C for 2 h. This carbohydrate composition was very close to those found for the xylans from *E. globulus* by Shatalov et al. (1999) and Evtuguin et al. (2003). The molar ratio of Rha:Glc:Gal:Xyl was 1.0:3.4:8.2:100.2.

Two percent suspension of the xylan (5 g) was incubated at 40 °C with a commercial cellulase preparation "Meicelase" (30 mg, xylanase activity *U*: 9.5) from *T. viride*. Amount of reducing sugar formed were determined by the method of Somogyi–Nelson method and it increased with progress of hydrolysis as shown in Fig. 1. After two days incubation, the amount of reducing unit (as xylose) released was about 16%.

The reaction products from xylan by the enzyme preparation were analyzed by SEC. Fig. 2 shows the changes of ratio of each xylooligosaccharide produced in process of incubation time. At the start of the reaction, the xylooligosaccharides from xylobiose (Xyl_2) to xyloheptaose (Xyl_7) appeared. With progress of hydrolysis, the reaction profile was getting less and less complex. Xyl_2 steadily increased while Xyl_3 and Xyl_4 decreased. As the oligosaccharides were converted into smaller ones, Xyl increased little by little.

The final product of enzymatic hydrolysis was resolved into a neutral (54.9%) and an acidic (14.4%) portion with an anion exchange resin in the usual way (Shimizu et al., 1976). The neutral sugars consisted of mainly Xyl_2, Xyl_3 and Xyl (Fig. 2). An appreciable amount of Xyl was found to be present indicating that the enzyme preparation contained β-xylosidase.

The acidic sugars were first separated into main four groups (Fractions 1–4) according to their molecular size by means of anion exchange chromatography using eluent A as shown in Fig. 3. As Fractions 1 and 2 were severely overlapped, they were separated from each other using eluent B. Each group was rechromatographed and purified in eluents C and D. When acetic acid is used as eluent, the strength of the eluted acid has a predominant influence upon *Dv*. (Havlicek & Samuelson, 1973). Fig. 4 shows, as an example, the rechromatogram of Fr. 3 eluted with eluent D, giving three fractions Frs. 3-S1, 3-S2 and 3-S3.

Finally, seven acidic sugars were isolated in a pure state. They were identified by *Dv* values (Shimizu & Samuelson, 1973; Shimizu

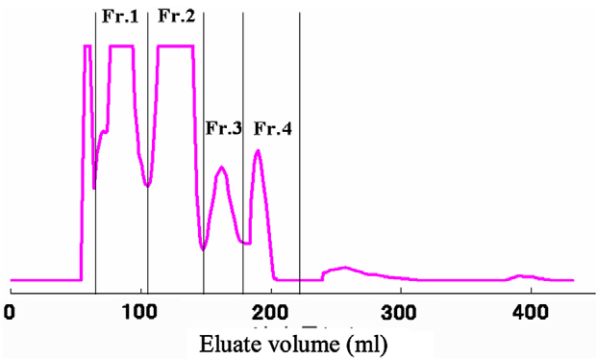

Fig. 3. Fractionation of the uronic acids formed from *E. globules* xylan by xylanase from *T. viride*; column (15 × 930 mm) of Diaion (AcO^-) resin eluted with 0.08 M NaOAc at 1.5 ml/min.

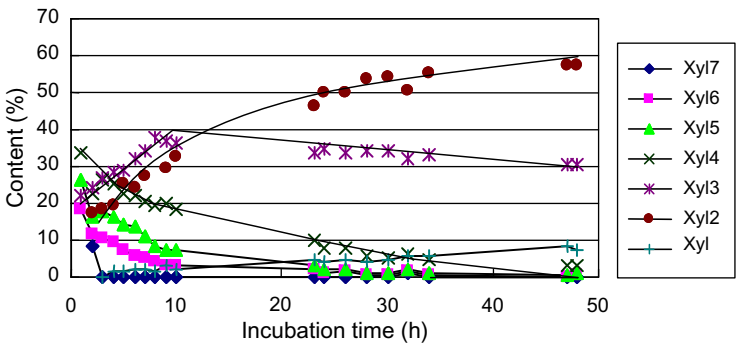

Fig. 2. Time-course of oligosaccharide production from *Eucalyputus globules* xylan by xylanase from *Trichoderma viride*.

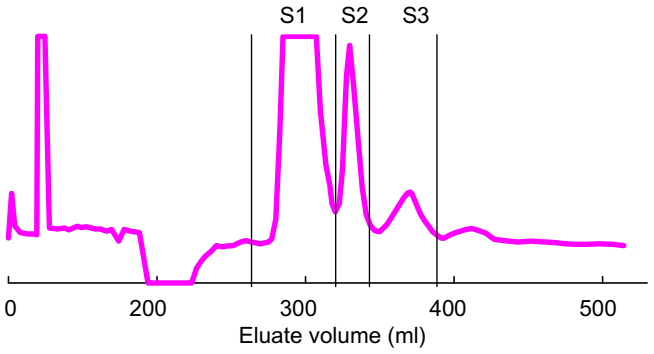

Fig. 4. Rechromatography of Fr. 3 in Fig. 3 on a column (10 × 840 mm) of Aminex 27 (AcO^-) resin with 0.5 M AcOH at 0.7 ml/min.

et al., 1976), hydrolysis products and ^1H and ^{13}C NMR spectroscopy. The anomeric configurations of the glycosyl residues were determined by ^1H and ^{13}C NMR spectroscopy. Assignments of signals were carried out using 2D NMR spectroscopy and based on published data for some related compounds (Agrawal, 1992; Azuma & Koshijima, 1983; Cavagna et al., 1984; Gast, Attala, & Mckelvey, 1990; Nacos et al., 2006; Shashkov et al., 2003; Simas et al., 2004). Their yields and *Dv* values were summarized in Table 1.

These acidic oligosaccharides were classified into three groups.

The first group, Frs. 1-S1, 2-S2 and 4-S3, was aldouronic acids from aldotrio- to aldopentao-uronic acids composed of D-Xyl and 4-*O*-Me-D-GlcA. They were identified as Xyl-β-1,4-[MeGlcA-α-1,2-]Xyl-β-1,4-Xyl-β-1,4-Xyl (Fr. 1-S1), [MeGlcA-α-1,2-]Xyl-β-1,4-Xyl-β-1,4-Xyl (Fr. 2-S2) and MeGlcA-α-1,2-Xyl-β-1,4-Xyl (Fr. 4-S3) on the basis of their *Dv* values and ^{13}C NMR spectra (Komiyama et al., 2009; Nacos et al., 2006).

It is well known that *Trichoderma* spp. produce multiple xylanases with high xylanolytic activity (Wong & Saddler, 1992). *Trichoderma* xylanases have been found to be active on xylans from different sources, usually producing xylooligomers, Xyl_2, and Xyl. Xyl is not usually the major product and it is typically produced after an accumulation of xylooligomers. In particular, five specific xylanases (XYL-I, XYL-II, XYL-III, XYLIV, and EGI) have been identified in *Trichoderma reesei* (Biely, Vršanská, & Claeyssens, 1991; Saloheimo, Siika-aho, Tenkanen, & Penttila, 2003; Tenkanen, Puls, & Poutanen, 1992; Törrönen et al., 1992; Xu, Takakuwa, Nogawa, Okada, & Morikawa, 1998). The former two belong to glycosyl hydrolase Family 11 and the third belongs to Family 10 (Xu et al. 1998). The fourth belongs to Family 5. The last is known to be a non-specific endoglucanase which can hydrolyze both cellulose and xylan. This enzyme catalyzes transglycosylation depending on the substrate concentration. It can be considered that the glucuronoxylan was synergetically hydrolyzed with these enzymes. Family 11 xylanases liberates the aldopentaouronic acid carrying 4-*O*-Me-GlcA*p* at the second Xyl*p* residue from nonreducing terminal as the smallest acidic sugar from 4-*O*-methyl-glucuronoxylan, whereas Family 10 xylanase liberates the aldotetraouronic acid carrying the substituent at nonreducing terminal (Biely, Vršanská, Tenkanen, & Kluepfel, 1997; Collins, Gerday, & Feller 2005). The presence of Fr. 1-S1 and 2-S2 indicated that the xylanases in "Meicelase" from *T. viride* contained both of Family 10 and 11 xylanases. The aldotetraouronic acid Fr. 2-S2 may be also possibly generated from Fr. 1-S1 by β-xylosidase (Biely et al., 1997).

In addition to Fr. 1-S1 and 2-S2, Fr. 4-S3, [MeGlcA-α-1,2-]Xyl-β-1,4-Xyl, was isolated in an appreciable amount. This aldouronic acid was not produced from kenaf xylan by partially purified xylanase from *Streptomyces* sp. SK515 (Komiyama et al., 2009), but this was produced when birch xylan was hydrolyzed by intracellular xylanase from a brown rotting fungus *Tyromyces palustris* (Shimizu et al., 1976). Recently, Saloheimo et al. (2003) isolated a novel xylanase (XYL-IV) from *T. reesei* which is homologous to the xylanases that are classified as belonging to Family 5 of glycosyl hydrolases. This xylanase generally cleaves closer to a substituted Xyl unit than either XYL-I or XYL-II and hydrolyzes [MeGlcA-α-1,2-]Xyl-β-1,4-Xyl-β-1,4-Xyl giving [MeGlcA-α-1,2-]Xyl-β-1,4-Xyl and Xyl. The liberation of Fr. 4-S3 indicates the presence of this xylanase in "Meicelase". Another unique xylanase, "appendage-dependent xylanase" (John, Rice, & Preston, 2006; Nishitani & Nevins, 1991; Vršanská, Kolenová, Puchart, & Biely, 2007) which is classified as belonging to Family 5 is known to generate the aldotriouronic acid (FR. 4-S3) from hardwood xylans together with β-xylosidase. But this enzyme has never been reported to be present in the enzymes secreted by *T. viride*.

The second group, Frs. 2-S1, 3-S2 and 3-S3, included aldouronic acids consisting of L-Rha, D-Xyl and 4-*O*-Me-D-GlcA. Fr. 2-S1 was identified as Xyl-β-1,4-Xyl-β-1,3-Rha-α-1,2-α-GalA-β-1,4-Xyl based on its *Dv* and ^{13}C NMR spectrum (Komiyama et al., 2009). Frs. 3-S2 and 3-S3 were identified as Xyl-β-1,4-Xyl-β-1,3-Rha-α-1,2-D-GalA and Xyl-β-1,3-Rha-α-1,2-α-GalA-β-1,4-Xyl, respectively, by comparison of their *Dv* values with those of the authentic samples (Shimizu et al., 1976).

Nowadays, it is well known that dicot and gymnosperm glucuronoxylans have a unique sequence of glycosyl residue Xyl-β-1,4-Xyl-β-1,3-Rha-α-1,2-α-GalA-β-1,4-Xyl at their reducing ends (Shimizu et al,,1976; Andersson et al.,1983; Johansson & Samuelson, 1977; Evtuguin et al., 2003; Peña et al., 2007). In the previous paper, we reported that kenaf xylan also had this glycosyl sequence (Komiyama et al., 2008). The presence of this terminal glycosyl sequence was confirmed in the acetylated heteroxylan from *E. globulus* by NMR spectra (Evtuguin et al., 2003). And it has been demonstrated that this glycosyl sequence is required for normal

Table 1
Yield and *Dv* of aldouronic acids released from *Eucalypus globulus* xylan.

Fraction	Aldouronic acids	Yield	NaOAC		AcOH	
No.		mg	0.08 M	0.02 M	0.25 M	0.5 M
Fr. 1-S1	Xyl-β-1,4-[MeGlcA-α-1,2-]Xyl-β-1,4-Xyl-β-1,4-Xyl	28.9		0.82	2.63	
Fr. 1-S2	Gal-β-1,2-MeGlcA-α-1,2-Xyl-β-1,4-Xyl-β-1,4-Xyl	4.6		0.92	4.02	
Fr. 2-S1	Xyl-β-1,4-Xyl-β-1,3-Rha-α-1,2-α-GalA-β-1,4-Xyl	1.6		1.31	4.09	
Fr. 2-S2	[MeGlcA-α-1,2-]Xyl-β-1,4-Xyl-β-1,4-Xyl	41.4		2.17	5.09	2.51
Fr. 3-S1	Same as Fr. 2-S2					
Fr. 3-S2	Xyl-β-1,4-Xyl-β-1,3-Rha-α-1,2-GalA	6.1		3.05		2.70
Fr. 3-S3	Same as Fr. 4-S2					
Fr. 4-S1	Same as Fr. 3-S2					
Fr. 4-S2	Xyl-β-1,3-Rha-α-1,2-α-GalA-β-1,4-Xyl	6.8		3.29		3.19
Fr. 4-S3	[MeGlcA-α-1,2-]Xyl-β-1,4-Xyl	15.4	0.76			4.26
Fr. 4-S4	Trace amount					

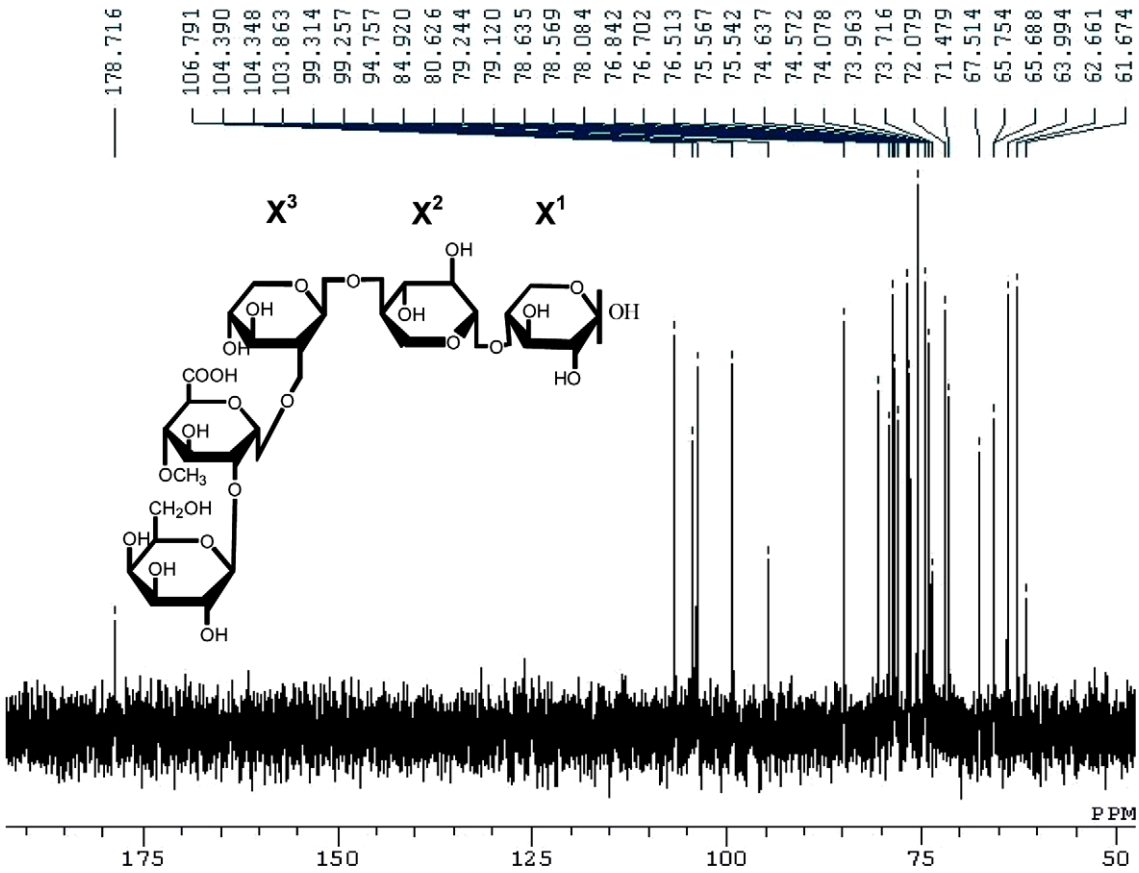

Fig. 5. ^{13}C NMR spectrum of Gal-β-1,2-MeGlcA-α-1,2-Xyl-β-1,4-Xyl.

xylan synthesis (Brown et al., 2007; Lee et al., 2007; Peña et al., 2007; York & O'Neil, 2008). In this study, it was proved by isolation of Fr. 2-S1, 3-S2 and 3-S3 that this glycosyl sequence was also an integral part of *Eucalyptus* xylan. The three acidic acids were previously isolated from the hydrolysate of birch xylan by *T. palustris* xylanase (Shimizu et al., 1976).

It is well known that a blockage of OH-2 in 4-*O*-substituted reducing sugars by glycosidation or etherification retards the endwise alkaline degradation (Johansson & Samuelson, 1977). The reason why xylan is more stable towards alkaline degradation is explained by the retarding effect of 2-*O*-substituted GalA and the presence of 4-*O*-Me-D-GlcA substituent along the xylan chain. But an appreciable proportion of the reducing xylose end groups present in the xylan in wood is lost during alkaline extraction forming new reducing GalA end groups. Fr. 3-S2 can be considered to be liberated from this glycosyl sequence at the reducing end.

The last group, Fr. 1-S1, was a novel aldouronic acid composed of D-Xyl and D-Gal and 4-*O*-Me-D-GlcA. This acid was identified as Gal-β-1,2-MeGlcA-α-1,2-Xyl-β-1,4-Xyl-β-1,4-Xyl on the basis of 1D and 2D NMR spectra. The ^{13}C NMR spectrum is shown in Fig. 5 and the assignments of chemical shifts in the ^{13}C and ^{1}H NMR spectra are shown in Tables 2 and 3, respectively.

The HMBC spectrum showed the cross-peaks indicating interglycosidic coupling between H-1 of D-Gal residue and C-2 of 4-*O*-Me-D-GlcA residue, between H-2 of 4-*O*-Me-D-GlcA residue and C-1 of D-Gal residue, between H-1 of 4-*O*-Me-D-GlcA residue and C-2 of non-reducing terminal D-Xyl, and between H-2 of non-reducing terminal D-Xyl and C-1 of 4-*O*-Me-D-GlcA residue. The presence of a single doublet at 5.46 ppm suggests 4-*O*-Me-D-GlcA residue is linked to neither to the reducing end nor to the adjacent xylosyl unit (refer to Puchart & Biely, 2008). Shatalov et al. (1999) and Evtuguin et al. (2003) found this structural unit in the xylan of

Table 2
Assignments of chemical shifts (in ppm) in the ^{13}C NMR spectrum of Gal-β-1,2-MeGlcA-α-1,2-Xyl-β-1,4-Xyl-β-1,4-Xyl.

	X^1		X^2		X^3	4-*O*-Me-GlcA	Gal
	α	β	α	β			
1	92.83	97.33	102.48	102.52	101.98	97.59	104.82
2	72.12	74.72	73.62	76.90	78.05	71.95	
3	71.80	74.77	74.57	74.97	72.86	73.49	
4	77.37	77.22	76.60	70.25	82.42	69.56	
5	59.70	63.82	63.76	65.68	70.82	76.23	
6						174.90	62.15
OMe						60.93	

Table 3
Assignments of chemical shifts (in ppm) in the ^{1}H NMR spectrum of Gal-β-1,2-MeGlcA-α-1,2-Xyl-β-1,4-Xyl-β-1,4-Xyl.

	X^1		X^2	X^3	4-*O*-Me-GlcA	Gal
	α	β				
1	5.18 (3.7)	4.59 (7.9)	4.48 (7.3)	4.65 (7.3)	5.46 (3.7)	4.72 (7.6)
2	3.56	3.27	3.28	3.46	3.92	3.59
3	3.76	3.55	3.56	3.52	4.02	3.67
4	3.76	3.79	3.79	3.65	3.36	3.92
5	3.51, 3.83	3.37, 4.06	3.52, 4.16	3.29, 3.99	4.63	3.68
6						3.82
OMe					3.52	

The values in parentheses are coupling constants *J* in Hz.

E. globulus, but they supposed that the D-Gal was present as the α-pyranoside form. In our experiment, the anomeric configuration of D-Gal unit was determined as β, because the coupling constant $J(1,2)$ of anomeric proton was 7.6 Hz. According to Agrawal (1992), β-anomeric protons appear as doublets between 4.4 and 4.8 ppm with $J(1,2)$ in the range 6–8 Hz in monosaccharides with gluco and galacto stereochemistry (Jimeno, Martin-Lomas, & Alemany, 1985; Shashkov et al., 2003).

The yield of this aldouronic acid was lower than estimated on the basis of the results reported by Shatalov et al. (1999) and Evtuguin et al. (2003). For quantitative study, more purified xylanase free from galactosidase, glucosidase and so on is needed.

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