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Structures of aldouronic acids liberated from kenaf xylan by endoxylanases from *Streptomyces* sp.

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ABSTRACT

Streptomyces sp. SK519 was cultivated in the medium containing 0.5% birch wood xylan to produce extracellular xylanase. The xylanase was isolated from the culture supernatant by ammonium sulfate precipitation and partially purified by absorbing on the insoluble xylan. Alkali-extracted kenaf glucuronoylan of which main chain primarily consisting of (1-4)-linked β-D-xylopyranose (Xyl*p*), some of which carry α-(1-2)-linked 4-*O*-methylglucopyranosyl acid (MeGlcA*p*) and glucopyranosyluronic acid (GlcA*p*) as side chain was digested with the xylanase preparation. At the final stage of digestion, main neutral oligosaccharides were xylotriose (Xyl_3) and xylobiose (Xyl_2) followed by xylotetraose (Xyl_4). Xylose was only formed in a trace amount. As acidic oligosaccharides liberated, following seven aldopentao- and aldotetraouronic acids were isolated in a pure state, respectively, by preparative anion-exchange chromatography. Their structures were determined by analysis of constitutional sugar residues and ^{1}H and ^{13}C NMR spectroscopy.

Xyl-β-1,4-[MeGlcA-α-1,2-]Xyl-β-1,4-Xyl-β-1,4-Xyl;
[MeGlcA-α-1,2-]Xyl-β-1,4-Xyl-β-1,4-Xyl-β-1,4-Xyl;
Xyl-β-1,4-[GlcA-α-1,2-]Xyl-β-1,4-Xyl-β-1,4-Xyl
[GlcA-α-1,2-]Xyl-β-1,4-Xyl-β-1,4-Xyl-β-1,4-Xyl
[MeGlcA-α-1,2-]Xyl-β-1,4-Xyl-β-1,4-D-Xyl
[GlcA-α-1,2-]Xyl-β-1,4-Xyl-β-1,4-Xyl
Xyl-β-1,4-Xyl-β-1,3-Rha-α-1,2-GalA-α-1,4-Xyl

1. Introduction

In a previous paper (Komiyama, Kato, Aimi, Ogihara, & Shimizu, 2008), we reported the chemical structure of xylans isolated from the bast and core of kenaf (*Hibiscus cannabinus*). The main chain of these xylans consisted of (1-4)-linked β-D-xylopyranosyl (Xyl*p*) residues, some of which carried α-(1-2)-linked 4-*O*-methyl-D-glucopyranosyluronic acid (4-*O*-Me-GlcA*p*) and glucopyranosyluronic acid (GlcA*p*) residues as single side chains. Furthermore, 4-*O*-(α-GalA*p*)-D-Xyl*p* was isolated from the partial hydrolysates of these xylans. This aldobiouronic acid has been revealed to be an integral part of wood xylans and to be located at the reducing end of wood xylans (Andersson, Samuelson, Ishihara, & Shimizu, 1983; Johansson & Samuelson, 1977; Shimizu & Samuelson, 1973; Shimizu, Ishihara, & Ishihara, 1976).

In the glycoside hydrolase classification, β-(1-4)-xylanases (E.C.3.2.1.8) are mainly found in families 10 and 11 (Collins, Gerday, & Feller, 2005). The endo-xylanases in the latter are only active on xylooligosaccharides and xylans. Me-GlcA*p* side chain represents serious hindrance to their activity, resulting in the production of aldopentaouronic, *O*-β-D-Xyl*p*-(1-4)-[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 as acidic sugar. On the other hand, family 10 xylanase can attack the xylosidic linkage on the non-reducing end of a substituted residue giving aldotetraouronic acid *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.

This paper reports the structure of aldouronic acids liberated from the xylan from kenaf core by the partially purified endo-acting xylanases from *Streptomyces* sp. SK519. The structures of such oligosaccharides can give detailed information on the structure of the polysaccharides as well as about the substrate specificity of the applied enzymes.

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2. Experimental

2.1. General methods

Monosaccharides were analyzed by 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 A × 1 column (TOSOH Co.) with 0.5% borate–1.0% ethanolamine–HCl buffer (pH 7.9). Relative percentage amounts were calculated electronically.

Thin layer chromatography for separation of xylooligosaccharides was carried out using Silica Gel 60 (Merck, 10 × 4 cm) as adsorbent and solvent system, 2-PrOH:BuOH:H_2O (12:3:3). The reducing sugars were detected with 5% ethanolic phosphomolybdic acid.

Size exclusion chromatography for neutral xylooligosaccharides was performed on KS 802 [4.6 × 250 mm × 2 (in series), Shodex] at 80 °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.).

FT-IR spectra were recorded on JASCO Model FT/IR-410 spectrometer using a KBr disc containing 1% finely ground sample.

2.2. NMR spectroscopy

NMR spectra of oligosaccharides were recorded at 40 °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 reported relative to internal TSP. Standard pulse sequences were utilized to obtain COSY, NOESY, HMQC and HMBC spectra.

2.3. Substrates

Kenaf xylan was extracted from the extractive-free stem core of kenaf (Tainung 2, *H. cannabinus Linn.*) with 10% KOH as described in a previous paper (Komiyama et al., 2008). Grained birch xylan and *p*NP-β-D-xylopyranoside (*p*NP-β-Xyl) were purchased from Sigma.

2.4. Identification of the strain

The strain SK519, which was isolated from soil, was obtained from Life Science Research Center, Nihon University. The genotype of strain SK519 was investigated by comparing the nucleotide sequence of its 16S rDNA to the sequence database Basic Local Alignment Search Tool for Nucleotide (BLASTN; http://www.ncbi.nlm.nih.gov/blast/). Chromosomal DNA from SK519 was isolated using a High Pure Polymerase Chain Reaction (PCR) Template Preparation Kit (Roche). The 16S rDNA was amplified from the isolated DNA by PCR using a MicroSeq 16S rDNA Full Gene PCR kit (Applied Biosystems), and the resulting PCR product was purified using Quantum Prep PCR Kleen Spin Columns (Bio-Rad). DNA sequence analysis was performed by the dideoxynucleotide method (Sanger, Nicklen, & Coulson, 1977). The nucleotide sequence of the PCR product was determined using an automated DNA sequencer (ABI PRISM 3100 Genetic Analyzer; Applied Biosystems).

2.5. Production and partial purification of xylanase

Cultivation of strain SK519 was carried out at 28 °C for 60 h in the medium (pH 7.0) containing 0.5% birch xylan, 1.0% polypeptone, 0.1% yeast extract, 0.15% K_2HPO_4, 0.05% $MgSO_4{\cdot}7H_2O$, 0.01% KCl, and 0.0015% $FeSO_4$ at 28 °C for three days with shaking (110 strokes/min). The supernatant separated from the culture by centrifugation (4500*g*, 25 min, 4 °C) was added with ammonium sulfate to make 20–80% saturation. Resulted precipitate was collected by centrifugation (4500*g*, 25 min, 4 °C) and dissolved in 10 mM sodium phosphate buffer (pH 6.0), and then the enzyme solution was dialyzed against the same buffer. Birchwood xylan was added to the dialyzed solution (amount of xylan added; 0.15 g/ml) and stirred gently at 0 °C for 1 h. The xylan was collected by centrifugation (4500*g*, 30 min, 4 °C), suspended to 10 mM sodium phosphate buffer (pH 6.0), and incubated at 37 °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).

Purity of the enzyme preparation was confirmed by SDS–PAGE. Proteins in the polyacrylamide-gel were visualized by silver staining. Protein concentrations of the enzyme solutions were determined by the Lowry's method using bovine serum albumin (Sigma) as a standard.

2.6. Assay of enzyme activity

Xylanase activity was assayed by measuring the reducing sugars released from birch wood xylan using Somogyi–Nelson method. The enzyme solution (50 μl) was incubated with 200 μl of 10 mM sodium phosphate buffer (pH 7.0) containing 1.25% birch xylan at 37 °C. After incubation, the enzymatic reaction was terminated by heating at 100 °C for 10 min on a hot dry bath. One unit of xylanase activity was defined as the amount of enzyme required to produce 1 mM of reducing sugar equivalent to xylose per min under the assay condition.

β-Xylosidase activity was assayed using pNP-β-Xyl as a substrate, and was monitored by measuring the amount of *p*-nitrophenol released from the substrate by the enzymatic reaction. The enzyme solution (20 μl) was incubated with 80 μl of 1.25 mM *p*NP-β-Xyl in 50 mM sodium phosphate buffer (pH 7.0) at 37 °C. After incubation, the enzymatic reaction was stopped by adding 0.1 ml of 0.3 M Na_2CO_3. The amount of *p*-nitrophenol liberated in the reaction mixture was determined by measuring the absorbance at 405 nm.

2.7. Hydrolysis of kenaf xylan by partially purified xylanase

Xylan (5 g) from stem core of kenaf was suspended in 100 ml of water and heated at 100 °C for 15 min in a water bath giving translucent solutions. This solution was diluted with 100 ml of 0.1 M acetate buffer, pH 5.5, and then incubated with the partially purified xylanase solutions containing 13.8 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 at different intervals by size exclusion chromatography as described above.

After incubation for 120 h, the hydrolysis products were separated into neutral sugars and acidic sugars in the usual way (Shimizu et al., 1976; Komiyama et al., 2008).

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 (AcO^-, 15 × 930 mm) by elution with A, 0.08 M sodium acetate (pH 5.9) giving 10 fractions. Fractions 5–10 were obtained in trace amounts, so, they were neglected in this work. Fractions 1–4 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. They were further rechromatographed on a preparative column of Aminex A-27 (AcO^-, 10 × 830 mm) by elution with C, 0.25 M acetic acid and D, 0.5 M acetic acid.

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

Fraction 1 representing aldopentaouronic acid gave one fraction Fr. 1:S1 having *Dv* 2.5 on rechromatography in eluent C together with a few minor fractions which were neglected in this study. Fr. 1:S1 gave 2-*O*-(4-*O*-Me-α-D-GlcAp)-D-Xyl*p*, 4-*O*-Me-GlcA and Xyl on hydrolysis with 2 M TFA at 120 °C for 2 h. Its ^{13}C NMR spectrum, however, indicated that it was a mixture of possible isomers. The isomers were separated into two fractions, Fr. 1:S1a and 1:S1b on rechromatography in eluent E (0.03 M acetic acid) with *Dv* 21.0 and 22.4, respectively. The ratio of Fr. 1:S1a and 1:S1b was 83:17. Fr. 1:S1a and Fr. 1:S1b were 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) and as *O*-(4-*O*-Me-α-D-GlcA*p*)-(1-2)-*O*-β-D-Xyl*p*-(1-4)-*O*-β-D-Xyl*p*-(1-4)-*O*-β-D-(1-4)-Xyl*p*-(1-4)-D-Xyl ([MeGlcA-α-1,2-]Xyl-β-1,4-Xyl-β-1,4-Xyl-β-1,4-Xyl), respectively, on basis of ^{13}C and ^{1}H NMR spectra. The spectrum of Fr. 1:S1a was identical with that reported by Nacos et al. (2006).

Fraction 2 gave two peaks Fr. 2:S1 and Fr. 2:S2 on rechromatography in eluent D with *Dv* 3.4 and 4.0, respectively. Fr. 2:S1 gave 2-*O*-(α-D-GlcA*p*)-D-Xyl*p*, GlcA and Xyl on hydrolysis with 2 M TFA at 120 °C for 2 h. Its ^{13}C NMR spectrum, however, indicated that it was a mixture of possible isomers in the same manner as Fr. 1:S1. The isomers were also separated into two fractions, Fr. 2:S1a and 2:S1b on rechromatography in eluent E with *Dv* 27.8 and 29.8, respectively. The ratio of Fr. 2:S1a and 2:S1b was 74:26. Fr. 2:S1a and Fr. 2:S1b were identified as *O*-β-D-Xyl*p*-(1-4)-[*O*-α-D-GlcA*p*-(1-2)]-*O*-β-D-Xyl*p*-(1-4)-*O*-β-D-Xyl*p*-D-(1-4)-Xyl(Xyl-β-1,4-[GlcA-α-1,2-]Xyl-β-1,4-Xyl-1,4-Xyl) and as *O*-α-D-GlcA*p*-(1-2)-*O*-β-D-Xyl*p*-(1-4)-*O*-β-D-Xyl*p*-(1-4)-*O*-β-D-Xyl*p*-(1-4)-D-Xyl*p* ([GlcA-α-1,2-]Xyl-β-1,4-Xyl-β-1,4-Xyl-β-1,4-Xyl) on basis of ^{13}C and ^{1}H NMR spectra.

Fr. 2:S2 gave Rha and Xyl in 1:1.7 on hydrolysis with 2 M TFA at 120 °C for 1 h. Its *Dv*'s in eluents B and C were 1.3 and 4.0, respectively, which were identical with those of the authentic sample *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(Shimizu et al., 1976). This structure was confirmed by its ^{1}H and ^{13}C NMR spectra.

Fraction 3 representing aldotetraouronic acid gave one peak Fr. 3:S1 on rechromatography in eluent D with *Dv* 2.2 with a few minor peaks which were neglected in this study. Its *Dv* and ^{13}C NMR spectrum coincided with that of the authentic sample *O*-(4-*O*-Me-α-GlcA*p*)-(1-2)-*O*-β-D-Xyl*p*-(1-4)-*O*-β-D-Xyl*p*-(1-4)-D-Xyl which was isolated from the acid hydrolysate of kenaf xylan in a previous paper (Komiyama et al., 2008; Nacos et al., 2006).

Fraction 4 gave one peak Fr. 4:S1 having *Dv* 2.9 on rechromatography in eluent D with a few minor peak which were neglected in this study. Its *Dv* and ^{13}C NMR spectrum coincided with that of the authentic sample *O*-(α-GlcA*p*)-(1-2)-*O*-β-D-Xyl*p*-(1-4)-*O*-β-D-Xyl*p*-(1-4)-D-Xyl which was isolated from the acid hydrolysate of kenaf xylan in a previous paper (Komiyama et al., 2008).

3. Results and discussion

Strain SK519 was identified from the nucleotide sequence encoding its 16S rDNA. A search for similarity with 16S rDNA in the BLASTN database confirmed that the isolate was most closely related to many strains of genus *Streptomyces*. From these results, we named this bacterium *Streptomyces* sp. SK519. This strain showed high xylan degrading ability, and secreted the xylanase and β-xylosidase. Xylanase was isolated from supernatant of SK519 culture by ammonium sulfate precipitation and partially purified by absorbing on the insoluble xylan. Two main proteins of different molecular weights (by SDS–PAGE) existed in the enzyme solution obtained (Fig. 1). Through the process of the enzyme purification, xylosidase activity was recovered with 56.6% from culture fluid of strain SK519 and β-xylosidase activity was removed completely.

Xylan was extracted from the extractive-free stem core meal of kenaf with 10% KOH as described in a previous paper (Komiyama et al., 2008). The main chain of these xylans consists of β-(1-4)-linked D-Xyl*p* residues, some of which carry α-(1-2)-linked 4-*O*-Me-D-GlcA*p* and GlcA*p* as side chain. Furthermore, it was confirmed that GalA*p* residue is an integral part of the xylans.

Two percent suspension of the xylan (5 g) was incubated with the partially purified xylanases from *Streptomyces* sp. SK519. Amount of reducing sugar formed were determined by the method of Somogyi–Nelson and it increased with progress of hydrolysis (Fig. 2). After three days incubation, the amount of reducing unit was about 30%.

The reaction product of xylan by the xylanase preparation was analyzed by SEC. Fig. 3 shows the changes of ratio of each xylooligosaccharide produced in process of incubation time. At the start of the reaction, the xylooligosaccharides from xylobiose to xyloheptaose, and even a small amount of xylooctaose appeared. With progress of hydrolysis, the reaction profile was getting less and less complex as the oligosaccharides were converted into smaller ones. Xylobiose steadily increased while xylotriose leveled off. After

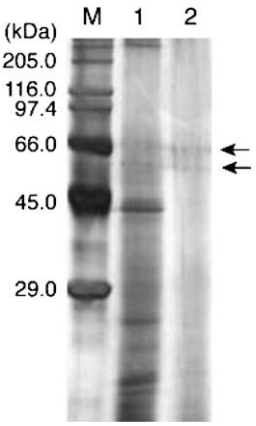

Fig. 1. SDS–PAGE of partially purified xylanases. Lane M contains molecular size standards (Sigma). Lane 1 contains the proteins in culture supernatant. Lane 2 contains the partially purified enzyme. The arrows indicate two main proteins.

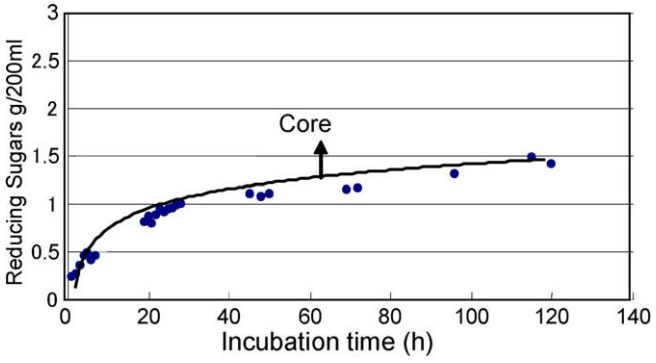

Fig. 2. Time–course of reducing sugar production from kenaf xylan by partially purified xylanase preparation (*Streptomyces* sp. SK519).

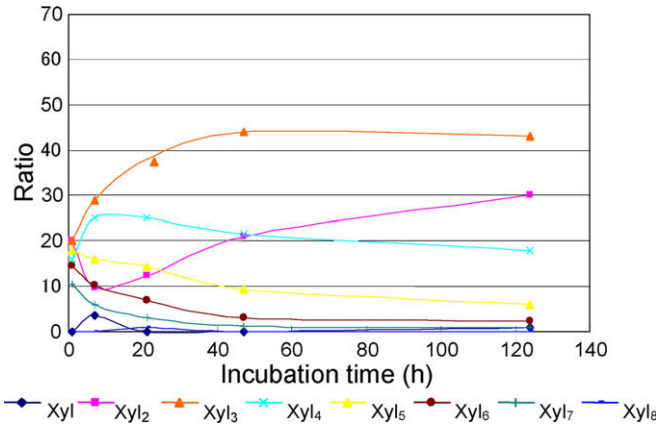

Fig. 3. Time–course of oligosaccharide production from kenaf xylan by partially purified xylanase preparation (*Streptomyces* sp. SK519).

exhaustive hydrolysis (five days) xylobiose, xylotriose and xylotetraose were main products. D-Xylose was only detected in a trace amount.

The final product of enzymatic hydrolysis was resolved into a neutral (67.6%) and an acidic portion (20.4%) with an anion exchange resin in the usual way (Shimizu et al., 1976). The neutral sugars consisted of mainly xylotriose, xylobiose and xylotetraose as shown in Fig. 3. D-Xylose was negligible indicating that the xylanase preparation was free from 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 these four fractions were severely overlapped, they were further separated from each other using eluent B, and each group was rechromatographed and purified in eluents C and D according to its strength of acid (Havlicek & Samuelson, 1973).

Finally, seven acidic sugars were isolated in a pure state. Their yields and *Dv* values are summarized in Table 1.

They were identified by *Dv* values (Shimizu & Samuelson, 1973; Shimizu et al., 1976), hydrolysis products and ^{1}H and ^{13}C NMR spectroscopy. The anomeric configurations of the glycosyl residues were determined by ^{1}H and ^{13}C NMR spectroscopy. Assignments of signals were carried out using 2D NMR spectroscopy and based on published data for some related compounds (Azuma & Koshijima, 1983; Cavagna, Deger, & Puls, 1984; Gast, Attala, & Mckelvey, 1990; Nacos et al., 2006; Simas et al., 2004).

When Fractions 1 and 2 representing aldopentaouronic acid were rechromatographed in eluent C, the former gave one peak Fr. 1:S1, whereas the latter gave two peaks Fr. 2:S1 and Fr. 2:S2. The analysis of constitutional sugars of Fr. 1:S1 and Fr. 2:S1 revealed that the former consisted of Xyl and 4-*O*-Me-GlcA, whereas the latter consisted of Xyl and GlcA. The ^{13}C NMR spectra of Fr. 1:S1 and Fr. 2:S1, however, showed that these fractions were composed mainly of aldopentaouronic acids with 4-*O*-Me-GlcA*p* or GlcA*p* attached to internal Xylp residues and of a minor aldpentaouronic acids with 4-*O*-Me-GlcA*p* or GlcA*p* attached to the terminal non-reducing Xylp residues. These isomers were separated on anion exchange chromatography using eluent E, giving two fractions, respectively, Fr. 1:S1a and 1:S1b, and Fr. 2:S1a and Fr. 2:S1b.

These acids were identified on the basis of ^{1}H and ^{13}C NMR spectra as follows:

Fr. 1:S1a: Xyl-β-1,4-[MeGlcA-α-1,2-]Xyl-β-1,4-Xyl-β-1,4-Xyl;
Fr. 1:S1b: [MeGlcA-α-1,2-]Xyl-β-1,4-Xyl-β-1,4-Xyl-β-1,4-Xyl;
Fr. 2:S1a: Xyl-β-1,4-[GlcA-α-1,2-]Xyl-β-1,4-Xyl-β-1,4-Xyl
Fr. 2:S1b: [GlcA-α-1,2-]Xyl-β-1,4-Xyl-β-1,4-Xyl-β-1,4-Xyl

The ^{13}C NMR spectrum of Fr. 1:S1a was identical with that reported by Nacos et al. (2006). The other ^{13}C spectra of these acids are shown in Fig. 4, and their carbon shifts are summarized in Table 2.

Fr. 2:S2 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-GalAp-(1-4)-D-Xyl by comparing its *Dv*'s with those of the authentic sample (Shimizu et al., 1976). The ^{13}C NMR spectrum and the assignment of signals are shown in Fig. 5 and Table 3. This structure was fully characterized by electrospray-ionization mass spectrometry and by ^{1}H and ^{13}C NMR spectroscopy after labeling its reducing end with 2-aminobenzeamide in the presence of sodium cyanoborohydride in the previous study (Ishii et al., 2008). The structure of this oligosaccharide was confirmed by means of ^{1}H NMR of its 2-aminobenzamide derivative (Peña et al., 2007), and MALDI-MS/MS of its perdeuteromethylated derivative (Brown et al., 2007).

Fractions 3 and 4 representing aldotetraouronic acids were rechromatographed in eluent D giving mainly one peak, Fr. 3:S1 and Fr. 4:S1, and identified as [MeGlcA-α-1,2-]Xyl-β-1,4-Xyl-β-1,4-Xyl and [GlcA-α-1,2-] Xyl-β-1,4-Xyl-β-1,4-Xyl respectively. Their *Dv* and ^{13}C NMR spectra were identical with those of the authentic samples from the acid hydrolysate of kenaf xylan reported in a previous paper (Komiyama et al., 2008). These acids were isolated, before, from the hydrolysate of rice straw arabinoglucuronoxylan by *Streptomyces* xylanase (Yoshida et al., 1990).

It is well known that individual microorganisms produce several endo-β-1,4-xylanases (Collins et al., 2005). At least three xylanases have been purified from *Streptomyces* (Bieley, Kluepfel, Morosoli, & Sharek, 1993; Marui, Nakanishi, & Yasui, 1985; Morosoli, Bertrand, Mondou, Shareck, & Kluepfel, 1986; Sreenath & Joseph, 1982). These enzymes are normally classified into family 10 and 11. Family 11 xylanase liberates the aldopentaouronic acid carrying 4-*O*-Me-GlcA*p* at the second Xyl*p* residue from non-reducing terminal as the smallest acidic sugar from 4-*O*-methyl-glucurono- xylan, whereas family 10 xylanase liberates the aldotetraouronic acid carrying the substituent at non-reducing

Table 1
Yield and *Dv* of aldouronic acids released from kenaf xylan by *Strepotomyces* xylanases

Fr, No.	Alduronic acids	Yield mg	NaOAc		AcOH		
			0.08 M	0.02 M	0.25 M	0.5 M	0.03 M
Fr. 1:S1	Xyl-β-1,4-[MeGlcA-α-1,2-]Xyl-β-1,4-Xyl-β-1,4-Xy	53.6	0.1	0.8	2.5		21.0
Fr. 1:S2	[MeGlcA-α-1,2-]Xyl-β-1,4-Xyl-β-1,4-Xyl-β-1,4-Xyl	11.0	0.1	0.8	2.5		22.4
Fr. 2:S1a	Xyl-β-1,4-[GlcA-α-1,2-]Xyl-β-1,4-Xyl-β-1,4-Xyl	12.3	0.4	1.3	3.4		27.8
Fr. 2:S1b	[GlcA-α-1,2-]Xyl-β-1,4-Xyl-β-1,4-Xyl-β-1,4-Xy	4.3	0.4	1.3	3.4		29.8
Fr. 2:S2	Xyl-β-1,4 -Xyl-β-1,3-Rha-α-1,2-GalA-α-1,4-Xyl	14.0	0.4	1.3	4.0		
Fr. 3:S1	[MeGlcA-α-1,2-] Xyl-β-1,4-Xyl-β-1,4-D-Xyl	52.9	0.6	2.4	5.4	2.6	
Fr. 4:S1	[GlcA-α-1,2-]Xyl-β-1,4-Xyl-β-1,4-Xyl	20.0	0.9	3.7		2.9	

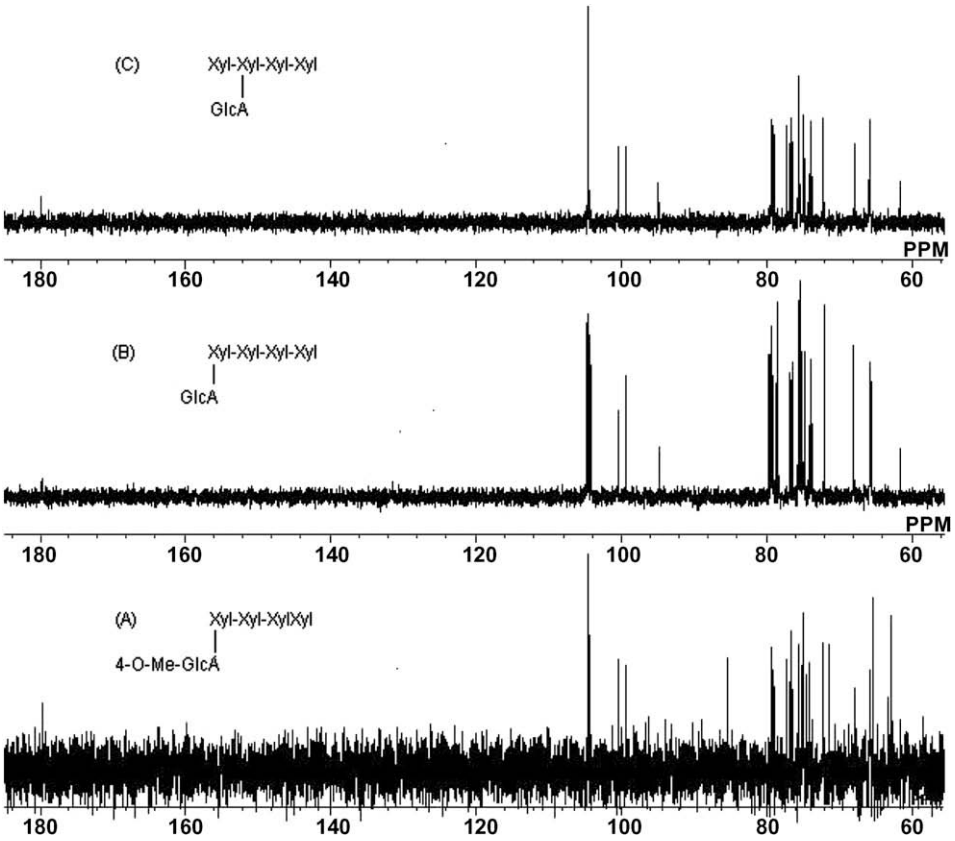

Fig. 4. ^{13}C NMR spectra of aldouronic acids. (A) **Fr. 1:S1b**: [MeGlcA-α-1,2-]Xyl-β-1,4-Xyl-β-1,4-Xyl-β-1,4-Xyl; (B) **Fr. 2:S1b**: [GlcA-α-1,2-]Xyl-β-1,4-Xyl-β-1,4-Xyl-β-1,4-Xyl; (C) **Fr. 2:S1a**: Xyl-β-1,4-[GlcA-α-1,2-]Xyl-β-1,4-Xyl-β-1,4-Xyl.

Table 2
Assignments of signals in the ^{13}C NMR spectra of aldouronic acids in the hydrolysate of xylan by *Strepotomyces* xylanases

		X^4-(G)-X^3-X^2-X^1		X^4-(GOMe)-X^3-X^2-X^1		(G)-X^4-X^3-X^2X^1		(GOMe)-X^4-X^3-X^2-X^1	
		β	α	β	α	β	α	β	α
X^1	1	100.51	94.85	100.34	94.86	100.43	94.88	100.33	94.89
	2	76.81	74.19	76.82	74.20	76.85	74.24	76.85	74.23
	3	76.73	73.76	76.74	73.77	76.77	73.81	76.76	73.79
	4	79.20	79.36	79.20	79.38	79.20	79.41a	79.19	79.41
	5	65.80	61.64	65.81	61.65	65.76b	61.70	65.73a	61.69
X^2	1	104.50		104.51		104.53		104.48b	
	2	75.49b		75.49		75.55c		75.55b	
	3	76.52		76.52		76.53d		76.53c	
	4	78.77		78.79		78.92		78.89	
	5	65.70a		65.70		65.85b		65.84a	
X^3	1	104.21		104.23		104.53		104.53b	
	2	79.36		79.24		75.55c		75.51b	
	3	75.21		75.20		76.57d		76.56c	
	4	79.64		79.66		79.41a		79.29	
	5	65.65a		65.70		65.85b		65.84a	
X^4	1	104.83		104.83		104.53		104.53b	
	2	75.66		75.67		79.38a		79.29	
	3	78.44		78.45		77.30		77.21	
	4	72.03		72.04		72.27		72.25	
	5	68.04		68.05		67.81		67.79	
GlcU	1	99.33		99.34		99.37		99.37	
	2	73.94		74.07		73.98		74.11	
	3	75.47b		75.07		75.53c		75.03	
	4	74.83		85.31		74.89		85.34	
	5	74.93		74.98		74.93		74.93	
	6	179.78		179.65		179.89		179.64	
	OMe	–		62.73		–		62.79	

[a,b,c] Assignments bearing the same alphabets may be interchanged.

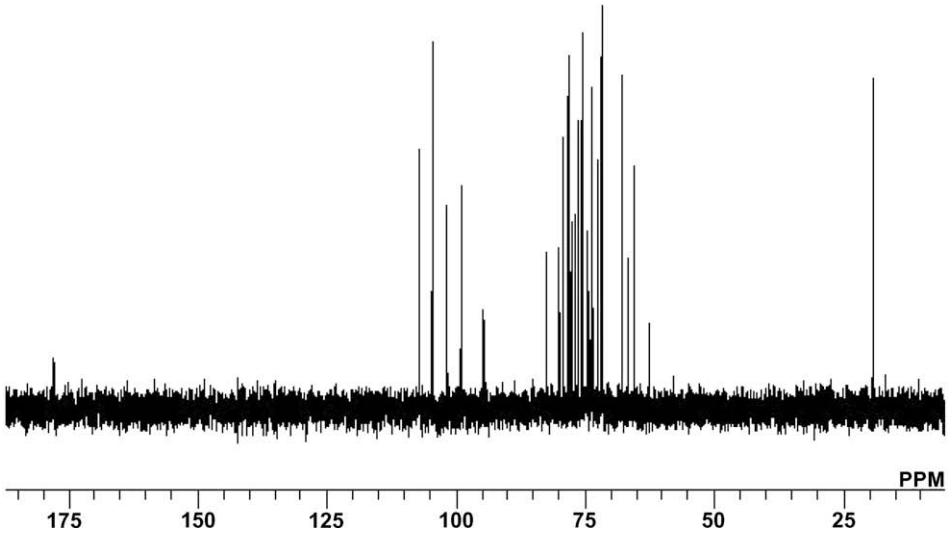

Fig. 5. ^{13}C NMR spectrum of *O*-β-D-Xyl*p*-(1-4)-*O*-β-D-Xyl*p*-(1-3)-*O*-α-L-Rha*p*-(1-2)-*O*-α-D-GalA*p*-(1-4)-D-Xyl (Fr. 2:S2).

Table 3
Assignments of signals in the ^{13}C NMR spectrum of *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 (Fr. 2:S2)

		X″-X′-R-G-X	
		β	α
X	1	99.14	94.74
	2	76.95	74.30
	3	77.65	74.53
	4	80.11	79.96
	5	66.83	62.69
X′	1	107.12	
	2	75.94	
	3	76.37	
	4	79.29	
	5	65.62	
X″	1	104.60	
	2	75.54	
	3	78.37	
	4	71.95	
	5	67.97	
R	1	104.70	
	2	72.52	
	3	82.68	
	4	73.73	
	5	71.86	
	6	19.43	
G	1	101.87	
	2	77.84	
	3	71.79	
	4	73.70	
	5	74.74	
	6	177.89	

terminal (Biley, Vršanska, Tenkanen, & Kluepfel, 1997). The presence of Fr. 1:S1a, 2:S1a, 3:S1 and 4:S1 indicated that the xylanase preparation used was a mixture of family 10 andz 11 xylanases.

On the other hand, the presence of Fr. 1:S1b and 2:S1b which are the aldopentaouronic acids carrying 4-*O*-Me-GlcA*p* or GlcA*p* residue at terminal non-reducing Xylp residues suggested that the xylanase preparation contained another xylanase differing in the mode of action from the above mentioned two xylanases.

Timell (1962) isolated a series of acidic oligosaccharides from aldotetra- to aldooctaouronic acid consisting of Xyl*p* and 4-*O*-Me-GlcA*p* residues from the hydrolysate of white birch 4-*O*-methylglucuronoxylan by a commercial "Pectinase" preparation. These aldouronic acids carried the substituent at the terminal non-reducing xylose residue. It can be considered that the xylanase requires the substituent on the xylan backbone cleaving the first 1,4-β-xylosidic linkage on the non-reducing terminal side of the substituted residue (Collins et al., 2005). Aldopentaouronic acid carrying 4-*O*-Me-GlcA*p* at non-reducing Xyl*p* residues were also isolated from the hydrolysate of birch 4-*O*-methyl-glucuronoxylan by the intracellular xylanase of brown rotting fungus *Tyromyces palustris* (Shimizu et al., 1976).

As for Fr. 2:S2, this acidic sugar was isolated from birch (*Betula platyphylla,* and *B. veruucosa*) and spruce (*Picea abies*) and further studies revealed this acid is located at the reducing end of xylan backbone (Andersson et al., 1983; Johansson & Samuelson, 1977; Shimizu et al., 1976). Recently, Peña et al. (2007) showed by ^{1}H NMR spectroscopy that the reducing end of *Arabidopsis* xylan contains this glycosyl sequence. It has been shown that this glycosyl sequence contributes markedly to the retardation of the alkaline peeling during early stage and during alkali treatments under milder conditions (Johansson & Samuelson, 1977). Dekker (1985) has suggested before that this sequence is linked to the rhamonogalacturonan. To our surprise, recently, various researchers (Brown et al., 2007; Peña et al., 2007; Lee 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).

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References

Andersson, S.-I., Samuelson, O., Ishihara, M., & Shimizu, K. (1983). Structure of reducing end-groups in spruce xylan. *Carbohydrate Research, 111*, 283–288.

Azuma, J., & Koshijima, T. (1983). ^{13}C NMR spectroscopy of carbohydrates. ^{13}C NMR spectroscopy of xylan and related carbohydrate. Technical Note 17, Wood Research Institute, Kyoto University, pp. 137–146.

Biely, P., Kluepfel, D., Morosoli, R., & Shareck, F. (1993). Mode of action of three endo-β-1,4-xylanases of *Streptomyces lividans*. *Biochimica et Biophysica Acta, 1162*, 246–254.

Biley, P., Vršanská, M., Tenkanen, M., & Kluepfel, D. (1997). Endo-β-1,4-xylanase famillyes: Differences in catalytic properties. *Journal of Biotechnology, 57*, 151–166.

Brown, D. M., Goubet, F., Wong, V. W., Gooacre, R., Stephens, E., Dupree, P., et al. (2007). Comparison of five xylan synthesis mutants reveals new insight into the mechanisms of xylan synthesis. *The Plant Journal, 52*, 1154–1168.

Cavagna, M. F., Deger, H., & Puls, J. (1984). 2D NMR analysis of the structure of an aldotriouronic acid obtained from birch wood. *Carbohydrate Research, 129*, 1–8.

Collins, T., Gerday, C., & Feller, G. (2005). Xylanases, xylanase families and extremophilic xylanases. *FEMS Microbiology Reviews, 29*, 3–23.

Dekker, R. F. H. (1985). Biodegradation of the hemicellulose. In T. Higuchi (Ed.), *Biosynthesis and biodegradation of wood components (pp. 505-533)*. Academic Press.

Gast, J. C., Attala, R. H., & Mckelvey, R. D. (1990). The ^{131}C NMR spectra of the xylo- and cello-oligosaccharides. *Carbohydrate Research, 84*, 137–146.

Havlicek, Y., & Samuelson, O. (1973). Partition chromatography of cyclitols on ion-exchange resins. *Journal of Chromatography, 85*, 93–100.

Ishii, T., Konishi, T., Ono, H., Ohnishi-Kameyama, M., Togashi, H., & Shimizu, K. (2008). Assignment of the 1H and 13C NMR spectra of 2-aminobenzamide-labeled xylooligosaccharides and enzyme assay for xylan. *Carbohydrate Polymers, 74*, 579–589.

Johansson, M., & Samuelson, O. (1977). Reducing end groups in birch xylan and their alkaline degradation. *Wood Science Technology, 11*, 251–263.

Komiyama, H., Kato, A., Aimi, H., Ogihara, J., & Shimizu, K. (2008). Chemical structure of Kenaf xylan. *Carbohydrate Polymers, 72*, 638–645.

Lee, C., Zhong, R., Richardson, E. A., Himmelsbach, D. S., McPhail, B. T., & Ye, Z-H. (2007). The PARVUS gene is expressed in cells undergoing secondary wall thickening and is essential for glucuronoxylan biosynthesis. *Plant Cell Physiology, 48*(12), 1659–1672.

Marui, M., Nakanishi, K., & Yasui, T. (1985). Purification and properties of three types of xylanases induced by methyl β-xyloside from *Streptomyces* sp.. *Agricultural and Biological Chemistry, 49*(12), 3399–3407.

Morosoli, R., Bertrand, J-L, Mondou, F., Shareck, F., & Kluepfel, D. (1986). Purification and properties of a xylanase from *Streptomyces lividans*. *Biochemical Journal, 239*, 587–592.

Nacos, M. K., Katapodis, P., Pappas, C., Daferera, D., Tarantilis, P. A., Christakopoulos, P., et al. (2006). Kenaf xylan–A source of biologically active acidic oligosaccharides. *Carbohydrate Polymers, 66*, 126–134.

Nakamura, A., Hatanaka, C., & Nagamatsu, Y. (2000). Ultraviolet spectrometric determination of natural monosaccharides by HPLC with ethanolamine. *Bioscience Biotechnology and Biochemistry, 64*(1), 178–180.

Peña, M. J., Zhong, R., Zhou, G-K., Richardson, E. A., O'Neill, M. A., Darvill, A. G., et al. (2007). Arabidopsis irregular xylem8 and irregular xylem9: Implicatons for the complexity of glucuronoxylan biosynthesis. *The Plant Cell, 19*, 549–563.

Samuelson, O. (1963). *Ion exchange separation in analytical chemistry*. New York: Almqvist and Wiksell, Stockform, Wiley.

Sanger, F., Nicklen, S., & Coulson, AR. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences, 74*, 5463–5467.

Shimizu, K., & Samuelson, O. (1973). Uronic acids in birch xylan. *Svensk Papperstidning., 76*, 150–155.

Shimizu, K., Ishihara, M., & Ishihara, T. (1976). Hemicellulases of brown rotting fungus, *Tyromyces palustris* 2. The oligosaccharides from the hydrolysate of a hardwood xylan by the intracellular xylanases. *Mokuzai Gakkaishi, 22*, 618–625.

Simas, F. F., Gorin, P. A. J., Guerrini, M., Naggi, A., Sassaki, G. L., Delgobo, C. L., et al. (2004). Structure of a heteroxylan of gum exuate of the palm Scheelea phalerata (uricuri). *Phytochemistry, 65*, 2347–2355.

Sreenath, H. K., & Joseph, R. (1982). Purification and properties of extracellular xylan hydrolases of *Streptomyces exfoliates*. *Folia Microbiologica, 27*, 107–115.

Timell, T. E. (1962). Enzymatic hydrolysis of a 4-O-methylglucuronoxylan from the wood of white birch (1962). *Svensk Papperstidning, 65*(1), 435–447.

York, W. S., & O'Neill (2008). Biochemical control of xylan biosynthesis-which end is up? *Current Opinion in Plant Biology, 11*, 258–265.

Yoshida, S., Kusdakabe, I., Matsuo, N., Shimizu, K., Yasui, T., & Murakami, K. (1990). Structure of rice-straw arabinoglucuronoxylan and specificity of Streptomyces xylanase toward the xylan. *Agricultural and Biological Chemistry, 54*(2), 449–457.