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Note

# Preparation of (1 → 4)-β-D-xylooligosaccharides from an acid hydrolysate of cotton-seed xylan: suitability of cotton-seed xylan as a starting material for the preparation of (1 → 4)-β-D-xylooligosaccharides

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## Abstract

Cotton-seed residual cake, which is a byproduct of the process of oil extraction from the seed, was delignified with sodium hypochlorite (1% available chlorine). Xylan was then prepared from the delignified wet material by alkali extraction with 15% sodium hydroxide. The cotton-seed xylan contained 64.7% xylose and 9.4% uronic acid. The xylan was hydrolyzed with 0.125 M sulfuric acid at 90 °C for 15 min. The resultant hydrolysis products were separated by gel-permeation chromatography on BioGel P-4 and Toyopearl HW-40F columns connected in series, with water as an eluate. Xylose and xylooligosaccharides with a degree of polymerization ranging from DP 2 to 15 were separated under such conditions, and each xylooligosaccharide-containing peak fraction afforded a single band on fluorophore-assisted carbohydrate electrophoresis. These results suggest that cotton-seed xylan is suitable for the preparation of xylose and xylooligosaccharides. © 2002 Elsevier Science Ltd. All rights reserved.

*Keywords:* Xylan; Cotton-seed; Xylooligosaccharide

β-Xylanase (EC 3.2.1.8) is a key enzyme for the degradation of β-(1 → 4)-xylan, the major plant cell-wall polysaccharide of hemicellulose, and produces β-(1 → 4)-xylooligosaccharides of different lengths. Over the last decade xylanase has attracted considerable research interest, because the enzyme has many potential applications in food, animal feed, and in the paper and pulp industries.[1,2] Considering such industrial applications of the xylanase, it is essential to analyze the enzymatic properties. In order to gain better understanding of reaction mechanism of xylanase, (1 → 4)-β-D-xylooligosaccharides ($X_n$s) are especially important substrates that serve as model compounds for the optimization of hydrolysis processes. β-(1 → 4)-Xylooligosaccharides have been prepared from partial acid or enzymatic hydrolysates of xylans from land plants, such as birch wood,[3,4] beechwood,[5] corncob,[6,7] and oat spelts.[8] We have also prepared β-(1 → 4)-xylobiose[9] and β-(1 → 4)-xylooligosaccharides[10] from the hydrolysis with *Streptomyces* β-xylanase of commercial hardwood xylan, which is a byproduct in the process of mercerization of rayon or staple fiber manufacturing. However, the resulting xylan contained β-(1 → 2)- and β-(1 → 3)-D-xylopyranosyl linkages and afforded heteroxylooligosaccharides by the enzymatic hydrolysis.[11] Such heterooligosaccharides made it difficult to separate individual $X_n$s by chromatographic fractionation. In addition, the hardwood xylan is no longer available from the supplier. Therefore other xylans, instead of the hardwood xylan, were necessary for the preparation of $X_n$s, and then we found that cotton-seed cake contained a large amount of xylan (40–45% as pentosan). The cotton-seed xylan was a glucuronoxylan with a simple chemical structure[12] and was expected to be a suitable material for the preparation of $X_n$s. Consequently, this paper deals with a simple method for the preparation of a series of $X_n$s from cotton-seed xylan and the suitability of cotton-seed xylan compared with birchwood and oat-spelt xylans.

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Table 1
Sugar composition of three kinds of xylans

| Source of xylan | Sugar content (%) | Component sugars (%) [a] | | | |
|---|---|---|---|---|---|
| | | Xyl | Ara | Glc | Uronic acid |
| Cotton seed | 75.0 | 64.7 | – | – | 9.4 |
| Birchwood | 72.0 | 56.0 | – | – | 10.3 |
| Oat spelt | 73.1 | 52.8 | 6.7 | 7.3 | 1.8 |

[a] –, not detected.

Table 1 shows the composition of cotton-seed xylan compared with other xylans. The total sugar content of cotton-seed xylan was 75.0%, and the value was almost the same as those of birchwood and oat-spelt xylans. Cotton-seed xylan contained only xylose and uronic acid, and this xylan indicated the highest xylose content among the three kinds of xylans. By contrast, oat-spelt xylan contained, not only xylose, arabinose and uronic acid, but also glucose. John et al.[8] reported that commercial oat-spelt xylan (Roth, Karlsruhe, Germany) contained α-glucan (ca. 16%), and they used the xylan after treating the material with α-amylase and amyloglucosidase. Glucose may have arisen from such a glucan, although the oat-spelt xylan used in this study was purchased from Sigma Chemical Co. Cotton-seed xylan is, therefore, adequate for use as a starting material for the preparation of $X_n$s.

Enzyme and acid are the hydrolytic agents which give $X_n$s by fragmentation (hydrolysis) of xylan. Previously, we used *Streptomyces* β-xylanase for obtaining $X_n$s from hardwood xylan.[10] However, it was difficult to control the production of $X_n$s with the desired degree of polymerization (DP). Thus, the fragmentation of cotton-seed xylan into oligomers was accomplished by partial-acid hydrolysis. Fig. 1 shows the fluorophore-assisted carbohydrate electrophoresis (FACE) of degradation products from cotton-seed xylan by acid hydrolysis with sulfuric acid ranging in the final concentration from 0.125 to 2 M. In the case of acid hydrolysis, the distribution of DPs of the products were dependent on the concentration of sulfuric acid, and we used 0.125 M sulfuric acid for further study, as such reaction conditions afforded oligomers with DPs up to about 15. Since the mobility of acidic saccharides is higher than those of neutral saccharides,[13] several bands located between $X_n$ are probably heterooligosaccharides having uronic acid substituents.

Products from three kinds of xylans by acid hydrolysis with 0.125 M sulfuric acid were compared by FACE. The origin of xylan is the major factor that influences the resulting products by partial-acid hydrolysis, and the products from cotton-seed xylan were almost the same as those from birchwood xylan, although the products from oat-spelt were slightly different. Especially, oat-spelt xylan afforded a large amount of monomer. This is due to the fact that arabinofuranosyl substituents are particularly sensitive toward acid hydrolysis.

Next, $X_n$s in the acid hydrolysate of cotton-seed xylan were fractionated by gel-permeation chromatography on three kinds of combinations of two columns connected in series. Xylose and $X_n$s with DPs ranging from 2 to 15 were separated on BioGel P-4 and Toyopearl HW-40F columns with a good resolution, and chromatography on BioGel P-4 and P-2 columns also achieved the separation of $X_n$s up to a DP 15. However, the resolution of BioGel P-4 and P-2 columns was slightly lower than that of BioGel P-4 and Toyopearl HW-40 columns. Toyopearl HW-50 and HW-40 columns, in contrast, could separate only $X_n$s up to a DP 8. $X_n$s were separated by charcoal chromatography in our previous study.[10] The advantage of charcoal chromatography is the high loading capacity of the column compared with other separation methods. (Charcoal has been used industrially for the purification of sugars.) Nevertheless, it was difficult to separate $X_n$s with high DPs, and acidic oligosaccharides ($X_n$s with uronic acid substituents) were overlapped with $X_n$s on the chromatography. Timell[3] reported that acidic oligosaccharides were removed using Dowex 1-X4 anion-exchange resin in the acetate form before charcoal chromatography in order to avoid overlapping peaks. Thus, the methods that use a charcoal column are less

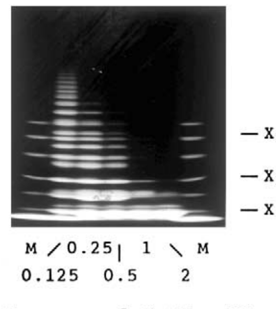

Fig. 1. Effect of concentration of sulfuric acid on the hydrolysis products of cotton-seed xylan. M, authentic xylohexaose to xylose from top to bottom.

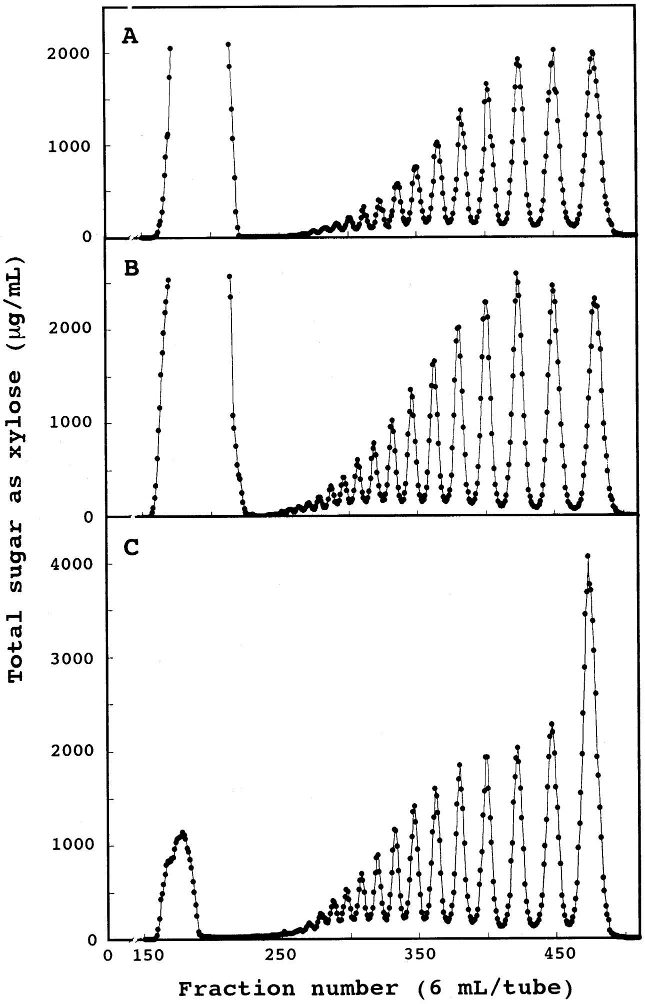

Fig. 2. Elution profiles of hydrolysis products of three kinds of xylans on BioGel P-4 and Toyopearl HW-40F columns connected in series. (A) Cotton-seed xylan; (B) birchwood xylan; (C) oat-spelt xylan.

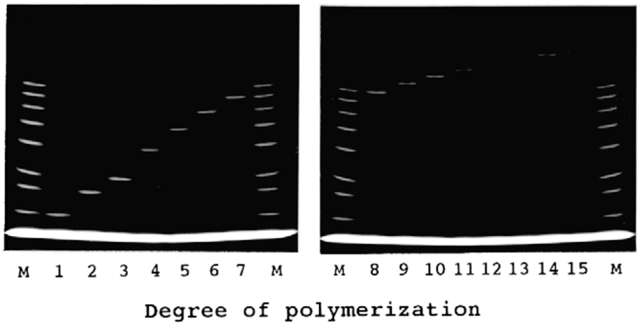

Fig. 3. FACE analysis of xylooligosaccharides isolated from an acid hydrolysate of cotton-seed xylan. M, authentic xylooctaose to xylose, from top to bottom.

satisfactory because of low separation efficiency and the fact that they are time consuming. Moreover, continuous practice is impossible, because the procedure is based on gradient elution, and the column must be regenerated. Therefore, and gel-permeation chromatography on polyacrylamide gel has been successfully introduced.[4,5,8] Havlicek and Samuelson[4] used a BioGel P-2 column for the fractionation of X$_n$s in the acid hydrolysate of birch xylan after treating with ion-exchange resin to remove acidic saccharides and separated X$_n$s with DP ranging from 2 to 18. Körner et al.[5] separated X$_n$s up to a DP 7 by using a BioGel P-4 column with 0.05 M Tris–HCl buffer, pH 7.8 as eluent. Under such chromatographic conditions, the series of X$_n$s were eluted according to size-exclusion principles, whereas acidic saccharides composed of xylose and uronic acid were separated by partition principles, which resulted in xyloheptaose being superimposed on glucuronosylxylose.[5] This method, therefore, has disadvantages for the separation of X$_n$s with high DP. Moreover, for sugars which are separated by this method, one needs to remove buffer salts. On the other hand, it is known that BioGel and Toyopearl gels are resistant to the permeation of acidic saccharides into pores of the gel particles, so that acidic saccharides are eluted near the void fraction with water as the eluent. So the above-named gels with water as eluent were used in this study for the separation of X$_n$s without removal of acidic saccharides before the chromatographic run. The use of distilled water as an eluent has another advantage. This is because separated sugars can be obtained in pure form by evaporation or lyophilization without removal of buffer salts. Moreover, gel-permeation chromatography is a suitable separation method that can be adapted for an autopreparative system using an autosampler (or injection pump), a refractive index detector, and a fraction collector, which is responsive to the detector signal. In each case, acidic saccharides were all practically eluted near the void fraction. Especially, a good resolution was achieved by using BioGel P-4 and Toyopearl HW-40F columns (A in Fig. 2).

X$_n$s in the individual peak fractions obtained by the chromatography on BioGel P-4 and Toyopearl HW-40F columns were analyzed by FACE (Fig. 3). Each X$_n$ gave a single band on FACE. Additionally, each X$_n$ afforded 2,3,4-tri-*O*-methyl-D-xylose and 2,3-di-*O*-methyl-D-xylose in the molar ratio corresponding to their DP as indicated by methylation analysis.[14] These were completely hydrolyzed by β-xylosidase from *Aspergillus niger* (Sigma) (data not shown).

Hydrolysis products from birchwood and oat-spelt xylans were also subjected to the chromatographic separation on BioGel P-4 and Toyopearl HW-40F columns (Fig. 2). The elution profile of hydrolysis products from birchwood (B in Fig. 2) was quite similar to that from cotton-seed (A in Fig. 2), whereas the products from oat-spelt xylan gave a slightly different pattern (C in Fig. 2). In the case of cotton-seed and birchwood xylans, each fraction, except for the void fraction, contained only $X_n$s. In the case of oat-spelt xylan, however, most of each fraction contained not only $X_n$s but also an additional two or three saccharides having different $R_f$ values on FACE. These oligosaccharides are probably arabinoxylooligosaccharides and maltooligosaccarides. These results suggest that glucuronoxylan, such as cotton-seed and birchwood xylans are suitable for the above preparative procedures, as those do not produce $X_n$s with arabinofuranosyl substituents. By comparing with birchwood xylan, cotton-seed xylan is easy to prepare because the lignin in cotton-seed cake is capable of removal with sodium hypochlorite without powdering.

In conclusion, cotton-seed xylan is suitable for use as a starting material for the production of xylose and $X_n$s because xylans with high xylose content and simple chemical structure are limited in nature. Moreover, chromatographic separation by using BioGel P-4 and Toyopearl HW-40F columns connected in series is a useful method as it enables the separation of $X_n$s up to a DP 15 with high resolution.

## 1. Experimental

*Xylans.*—Cotton-seed xylan was prepared by alkali extraction. Cotton-seed cake (1 kg, Okamura Oil Mill, Ltd., Osaka Japan), which is a byproduct from the process of oil extraction from cotton-seed, was soaked in sodium hypochlorite solution (2.5 L, 1% available chlorine) at rt for 24 h to remove lignin and colored materials. After washing with water, the solid material was dewatered by filtration through cotton cloth. The delignified wet material was soaked in 15% NaOH at rt for 24 h to extract the xylan. After filtration through cotton cloth, the filtrate was neutralized with 3 M $H_2SO_4$. The precipitate was collected by centrifugation at 8500*g* for 30 min and dialyzed against running water to remove salts. After 3 days, the precipitate was collected by centrifugation, washed with EtOH, and then dried. The yield of cotton-seed xylan was 214.6 g. Oat-spelt xylan and birchwood xylan were purchased from Sigma Chemical Co. (St. Louis, MO)

*Analysis of xylan.*—Xylan was hydrolyzed with $H_2SO_4$ according to the method described previously.[15] After acid hydrolysis, total sugar was measured by the orcinol–HCl method[16] with D-xylose as the standard, and component sugars were analyzed by gas–liquid chromatography after conversion into alditolacetate derivatives as described previously.[15] Uronic acid was measured by the *m*-hydroxydiphenyl method[17] with D-glucuronic acid as a standard.

*Partial-acid hydrolysis of xylan.*—Each xylan containing 2 g of polyxylose was added to 20 mL of 15% NaOH solution. The slurry was neutralized with HCl, dialyzed against running water to remove the salts, filled up to 50 mL, and used as a 4% xylan suspension. The suspension (50 mL) was heated at 90 °C, and 50 mL of hot $H_2SO_4$ (90 °C, 0.25–4 M) was added to the suspension. The reaction was done at 90 °C for 15 min, and then 50 mL of water was added to the reaction mixture to cool down. After cooling down, the reaction mixture was neutralized with barium carbonate, and the resultant precipitate was removed by centrifugation. The supernatant was filtered through an Advantec No. 5C filter paper (Toyo Roshi Kaisha, Ltd., Tokyo, Japan) and concentrated to a syrup by a rotary evaporator. The syrup was filtered through an Advantec No. 2 filter paper and filled up to 10 mL with water.

*Gel-permeation chromatography.*—The column system for gel-permeation chromatography consisted of two water-jacketed columns (XK50/100 column, Amersham Pharmacia Biotech, Buckinghamshire, UK) connected in line with a tube, and the gel beds of the first and the second columns were 865 × 50 and 815 × 50 mm i.d., respectively. Combinations of gel media used in this study were as follows; (A) BioGel P-4 (fine, Bio-Rad Laboratories, Hercules, CA) and Toyopearl HW-40F (Tosoh Corporation, Tokyo, Japan); (B) BioGel P-4 (fine) and BioGel P-2 (fine); (C) Toyopearl HW-50F and Toyopearl HW-40F. Samples containing about 700 mg in 10 mL were applied to the columns after filtration through a Millex HV filter membrane (0.45 μm, Millipore Corporation, Bedford, MA), and were eluted with degassed water at 50 °C and a flow rate of 1.5 mL/min. The eluate was fractionated into 6-mL portions. Sugars in each fraction was measured by the orcinol–HCl method[16] and by the *m*-hydroxydiphenyl method.[17] Sugar composition in each fraction was analyzed by FACE.[13]

*FACE analysis.*—Oligosaccharides were labeled with 8-aminonaphthalene-1,3,6-trisulfonic acid (Molecular Probes, OR, USA) and analyzed by polyacrylamide gel electrophoresis on a 30–40% acrylamide gel as described in our previous paper.[13]

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