Characterization of an Acidic Arabinoxylan from Cell Walls of Immature Barley Plants[†]

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An acidic arabinoxylan occurring in the hemicellulose I fraction (4% potassium hydroxide extract) of cell walls of immature barley plants was isolated and characterized by methylation and fragmentation analyses. The results indicated that the acidic arabinoxylan had a linear backbone chain of β -1,4-D-xylose residues, about 50% of which were substituted at the 2 and/or 3 position, mainly with arabinofuranose and glucuronic acid residues.

To characterize polysaccharides in the cell walls of immature barley plants, we separated them into six fractions using successive extraction with ammonium oxalate-oxalic acid, 4% potassium hydroxide, and 24% potassium hydroxide. We reported earlier the chemical structure of the β -D-glucan obtained from the hemicellulose I fraction, a polysaccharide fraction extracted with 4% potassium hydroxide, and that of the xyloglucan obtained from the hemicellulose II fraction, a polysaccharide fraction extracted with 24% potassium hydroxide.^{1,2)} Furthermore, we reported that the hemicellulose I fraction obtained from barley cell walls contained β -D-glucan and arabinoxylan in the approximate ratio of 30~40:60~70.¹⁾ This paper describes the chemical structure of arabinoxylan, another predominant polysaccharide of barley cell walls.

MATERIALS AND METHODS

Materials. The polysaccharides in cell walls of immature barley plants were separated into six fractions; PS, HC-IA,

HC-IB, HC-IIA, HC-IIB, and CL, and then HC-IB was fractionated into six subfractions (HC-IB-1 to -6) by graded ammonium sulfate precipitation as described in our previous paper.¹⁾ This study used fraction HC-IB-6, which consisted of arabinose, xylose, glucose, and galactose in the molar ratio of 36.2:54.1:3.7:6.3. The endo- β -1,4-xylanase from *Streptomyces* sp. (128 units/mg) was the same as reported previously.³⁾ 4-O- β -D-Xylopyranosyl-D-xylose was prepared from the enzymic hydrolyzate of rice straw β -1,4-xylan (our laboratory collection). Bio-Gel P-2 (200~400 mesh) was purchased from Bio-Rad, and Sepharose CL-6B, DEAE-Cellulose, Blue dextran, and Dextran T-500, T-250, T-110, T-70, and T-40 were from Pharmacia Fine Chemicals.

General methods. Carbohydrate solutions were concentrated under reduced pressure at 40~45°C. Ascending paper chromatography was done on Toyo No. 50 filter paper using butanol-pyridine-water (6:4:3, v/v) (method A) or by the descending method using ethyl acetate-water-acetic acid-formic acid (18:4:3:1, v/v) (method B). Sugars on the chromatogram were detected with alkaline silver nitrate.⁴⁾ Optical rotation was measured with a Nippon Bunko Model DIP-SL polarimeter. Gas-liquid chromatography (GLC) was done with a Yanagimoto Model G-80 gas chromatograph with a flame ionization detector at a nitrogen gas flow of 15 ml per min, on a glass column (0.4×200 cm) of 1.5% QF-1 on Chromosorb W (AW-DMCS) [column (a)] or a glass column (0.4×200 cm) of 3% OV-210 on Supelcoport

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[column (b)]. Total carbohydrate was measured by the phenol-sulfuric acid method.⁵⁾

Analysis of neutral sugars in poly- or oligo-saccharide fraction. Polysaccharide (1~2 mg) was hydrolyzed with 1 M sulfuric acid for 5~6 hr at 100°C, and oligosaccharide (0.2~1 mg) was hydrolyzed with 0.5 M sulfuric acid for 3~4 hr at 100°C. Each hydrolyzate was neutralized with barium carbonate, treated with Amberlite IR-120 resins (H⁺ form) to remove barium ions and then concentrated. Sugars were analyzed by GLC on column (a) at 140°C after converting them to alditol trifluoroacetates.⁶⁾

Chromatography of fraction HC-IB-6 on DEAE-Cellulose. HC-IB-6 (500 mg) was dissolved in 50 ml of distilled water and centrifuged. The supernatant was put on a column (1.5 × 25 cm) of DEAE-Cellulose (acetate form), and eluted stepwise with water (75 ml, fraction HC-IB-6-i), 50 mM potassium acetate (66 ml, HC-IB-6-ii), 0.1 M potassium acetate (144 ml, HC-IB-6-iii), 0.5 M potassium acetate (105 ml, HC-IB-6-iv), 0.1 M sodium hydroxide (72 ml, HC-IB-6-v), 0.2 M sodium hydroxide (66 ml, HC-IB-6-vi), and 0.5 M sodium hydroxide (69 ml, HC-IB-6-vii). These seven fractions were separately freeze-dried, following neutralization, dialysis against deionized water, and concentration. Yields of fractions HC-IB-6-i to -vii were 21, 42, 151, 217, 14, 10, and 10 mg, respectively. Fraction HC-IB-6-iv was further studied as an acidic arabinoxylan (A-AX) from cell walls of immature barley plants.

High voltage paper electrophoresis of A-AX. A-AX (5 mg in 0.1 ml of water) was put on a line 20 cm from the anodal end of a 5 × 60 cm strip of Toyo GA-100 glass filter paper soaked in 0.1 M sodium tetraborate (pH 9.3). Electrophoresis was done at 1350 v for 70 min in a high voltage paper electrophoresis apparatus HPE-V (Toyo Kagaku). After electrophoresis, the filter paper was cut into strips (5 × 1 cm). Each strip was soaked in 2 ml of deionized water, heated in a boiling-water bath for 5 min, and filtered on Toyo No. 2 filter paper. The carbohydrate in the filtrate was measured by the phenol-sulfuric acid method.

Gel filtration of A-AX on Sepharose CL-6B. A-AX (2 mg) in 0.1 M sodium hydroxide (0.3 ml) was put on a column (1.5 × 72 cm) of Sepharose CL-6B pre-equilibrated with 0.1 M sodium hydroxide, followed by filtration through the column with 0.1 M sodium hydroxide. Fractions of 1 ml each were collected and assayed for carbohydrate. The column had been calibrated using Blue dextran, Dextran T-500, T-250, T-110, T-70, and T-40, and glucose.

Sugar analysis of A-AX. A-AX (15 mg) was hydrolyzed with 2 ml of 0.5 M sulfuric acid for 14 hr at 100°C. The hydrolyzate was neutralized with barium carbonate and

filtered. The filtrate was passed through a column (0.4 × 6 cm) of Dowex-1 (acetate form), yielding the neutral sugar fraction. Acidic sugars were eluted from the column with 6 M acetic acid. Yields of the neutral and acidic fractions were 11.4 and 1.4 mg, respectively. Neutral sugars were analyzed by GLC as described under "Analysis of neutral sugars in poly- or oligo-saccharide fractions." Acidic sugars were analyzed by paper chromatography (method B).

Methylation analysis of A-AX. A-AX (10 mg) dissolved in dimethyl sulfoxide (3 ml) was methylated using methylsulfinyl carbanion (0.5 ml) and methyl iodide (0.5 ml) by the method of Hakomori,⁷⁾ and then dialyzed against tap water. The non-dialyzable materials were concentrated to dryness. The methylated A-AX was methylated twice more. The methylated A-AX was hydrolyzed with 90% HCOOH-0.25 M sulfuric acid and converted into the corresponding alditol acetates.⁸⁾ The partially methylated alditol acetates were analyzed by GLC on column (b) at 172°C.

Partial acid hydrolysis of A-AX and resolution of the hydrolyzate. A-AX (100 mg) in 15 ml of 50 mM trifluoroacetic acid was refluxed in a boiling-water bath for 90 min. The resulting solution was mixed with 60 ml of ethanol; the mixture was kept at -20°C for 24 hr and centrifuged. The supernatant was concentrated to dryness. The dried material was dissolved in 1 ml of water and put on a column (1.5 × 100 cm) of Bio-Gel P-2 operated at 50°C, then filtered through the column with water. Fractions of 1.3 ml each were collected and assayed for carbohydrate. Tubes 38~50 (fraction F), 69~71 (E), 72~76 (D), 77~81 (C), 82~87 (B), and 89~95 (A) were separately combined and concentrated, each concentrate being rechromatographed on the same column. After freeze-drying, fractions A to F were obtained in a yield of 21.1, 5.0, 3.0, 2.1, 0.5, and 35.0 mg, respectively.

RESULTS

Isolation of an acidic arabinoxylan (A-AX)

The polysaccharide fraction HC-IB-6, obtained from cell walls of immature barley plants, contained arabinose, xylose, glucose, and galactose in a molar ratio of 36.2:54.1:3.7:6.3 as neutral sugar components as described previously.¹⁾ When fraction HC-IB-6 was chromatographed on DEAE-Cellulose (acetate form), it gave seven carbohydrate fractions, HC-IB-6-i to -vii (Fig. 1). Since the yield of fraction HC-IB-6-iv eluted with 0.5 M potassium acetate was much higher than those of any other fractions and neutral

FIG. 1. Chromatography on DEAE-Cellulose of a Polysaccharide Fraction HC-IB-6 Obtained from Cell Walls of Immature Barley Plants.

HC-IB-6 (500 mg in 50 ml of water) was put on a column (1.5 × 25 cm) of DEAE-Cellulose (acetate form). The column was eluted stepwise with water, potassium acetate, and sodium hydroxide. Tubes 1~25, 26~58, 59~97, 98~135, 136~158, 159~178, and 179~200 were separately combined to give fractions HC-IB-6-i to -vii.

TABLE I. YIELDS AND NEUTRAL SUGAR COMPOSITION OF SUBFRACTIONS OBTAINED FROM FRACTION HC-IB-6 BY DEAE-CELLULOSE CHROMATOGRAPHY

Fraction ^a	Yield ^b (mg)	Neutral sugar composition (mol%)			
		Ara	Xyl	Glc	Gal
HC-IB-6-i	21	31.4	59.2	1.7	7.7
-ii	42	31.9	60.2	trace	7.8
-iii	151	33.8	60.9	0.7	4.6
-iv	217	30.8	61.7	1.3	6.2
-v	14	19.1	53.2	9.0	18.6
-vi	10		N.D. ^c		
-vii	10		N.D. ^c		

^a See Fig. 1.

^b From HC-IB-6 of 500 mg dry weight.

^c Not determined.

FIG. 2. High Voltage Paper Electrophoresis of A-AX.

Electrophoresis was done on Toyo GA-100 glass filter paper at 1350 V for 70 min with 0.1 M sodium tetraborate (pH 9.3).

sugar composition of fraction HC-IB-6-iv was not very different from those of any other fractions (Table I), fraction HC-IB-6-iv was designated as acidic arabinoxylan (A-AX) and studied further. A-AX was homogeneous by high voltage paper electrophoresis (Fig. 2), and also by gel filtration on a calibrated Sepharose CL-6B column, and its average molecular weight was about 5.0×10^5 . It had $[\alpha]_D -66^\circ$ ($c=1.1$, in water).

Structural characterization of A-AX

1. Analysis of acid hydrolyzate of A-AX. Acid hydrolysis of A-AX (15 mg) and then chromatography on Dowex-1 (acetate form) of the hydrolyzate yielded neutral (11.4 mg) and acidic (1.4 mg) sugar fractions. The former was shown by GLC on column (a) to be composed of xylose, arabinose, galactose, and glucose in a molar ratio of 62:31:6:1. The latter was shown by paper chromatography (method B) to be composed of a sugar having R_{Xyl} 0.30, glucuronic acid, and galacturonic acid (Fig. 3). The sugar having R_{Xyl} 0.30 (about 0.5 mg), which was obtained from strips (6~8.5 cm from origin) by elution with water, followed by freeze-drying of the eluate, was methylated by the method of Hakomori. The uronic acid residue in the sugar, esterified in the methylation reaction, were reduced with lithium aluminium hydride⁹⁾ and the primary hydroxyl group formed from the uronic acid

FIG. 3. Paper Chromatogram of Acidic Sugars Obtained from Acid-hydrolyzate of A-AX.

A mixture of acidic sugars (1.4 mg) obtained from an acid hydrolyzate of A-AX was chromatographed on Toyo No. 50 filter paper (method B). After development, the filter paper was cut into strips. Each strip was soaked in 1 ml of water, heated in a boiling-water bath for 5 min and filtered on Toyo No. 2 filter paper. The carbohydrates in the filtrates were measured by the phenol-sulfuric acid method. Upper part shows a guide strip treated with alkaline silver nitrate (1, authentic sugars; GlcA, glucuronic acid; GalA, galacturonic acid; X, xylose; 2, sample).

residue was methylated by the method of Hakomori. The fully methylated neutral sugar thus prepared was hydrolyzed and converted into the corresponding alditol acetates. Analysis of the partially methylated alditol acetates by GLC on column (b) found non-reducing terminal glucose (55%) and 2- or 4-linked xylose (45%) in the methylated sugar. Accordingly, the original acidic sugar having R_{Xyl} 0.30 was considered to be glucuronosyl-(1→2)-xylose. The molar ratio of glucuronosyl-xylose, glucuronic acid, and galacturonic acid was calculated to be 60:40: trace from paper chromatography (Fig. 3).

From these results, the molar ratio of xylose, arabinose, galactose, glucose, glucuronic acid, and galacturonic acid in A-AX is 59:28:5:1:8: trace.

2. Methylation analysis. Methylation analysis showed that 2,3,5-tri-*O*-methyl-arabinose (25.5%), 2,3,4-tri-*O*-methyl-xylose (2.8%), 2,3-di-*O*-methyl-xylose (33.3%), and 2- and/or 3-mono-*O*-methyl-xylose (28.7%) were prominent structural units in the methylated A-AX (Fig. 4). The result suggests that A-AX is comprised mostly of a backbone containing

FIG. 4. Gas Chromatogram of the Partially Methylated Alditol Acetates Obtained from the Acid Hydrolyzate of the Methylated A-AX.

The sample was analyzed on column (b) at 172°C at a nitrogen flow rate of 15 ml/min. Peaks 1, 2, 3, and 4 are 2,3,5-Me₃-Ara (2,3,5-tri-*O*-methyl-1,4-di-*O*-acetyl-arabinitol), 2,3,4-Me₃-Xyl, 2,3- and/or 3,4-Me₂-Xyl and 2- and/or 3-Me₁-Xyl, respectively.

FIG. 5. Chromatography on Bio-Gel P-2 of Partial Acid-hydrolyzate of A-AX.

Partial acid-hydrolyzate of A-AX was put on a column (1.5 × 100 cm) of Bio-Gel P-2 operated at 50°C, followed by filtration through the column with water. The arrows in the figure indicate the elution positions of Blue dextran (V_0) and xylose (X) used for calibration.

TABLE II. YIELDS, R_f VALUES, PRODUCTS OF COMPLETE ACID-HYDROLYSIS, AND RESULTS OF METHYLATION ANALYSIS OF OLIGOSACCHARIDES B TO E OBTAINED FROM A-AX BY PARTIAL ACID-HYDROLYSIS

Oligosaccharide	Yield ^a (mg)	R_f^b	Product ^c of complete acid- hydrolysis	Methylation analysis (mol%) ^d	
				2,3- and/or 3,4-Me ₂ -Xyl	2,3,4-Me ₃ -Xyl
OS-B	5.0	0.50	Xylose	48.8	51.2
OS-C	3.0	0.30	Xylose	65.6	34.4
OS-D	2.1	0.15	Xylose	71.4	28.6
OS-E	0.5	0.06	Xylose	81.0	19.0

^a From A-AX of 100 mg dry weight.

^b Mobility of each oligosaccharide on paper chromatography (method A). The R_f values of authentic xylose and 4-*O*- β -D-xylopyranosyl-D-xylose were 0.71 and 0.50, respectively.

^c Measured by GLC of the alditol trifluoroacetates.

^d 2,3-Me₂-Xyl = 2,3-di-*O*-methyl-1,4-di-*O*-acetyl-xylitol and so on.

some (1 $\rightarrow$ 4)-linked xylopyranosyl residues and of single arabinofuranosyl side chains.

3. *Analysis of partial acid-hydrolyzate of A-AX.* Partial acid-hydrolyzate of A-AX (100 mg) followed by fractionation of the hydrolyzate by gel filtration on Bio-Gel P-2 afforded six fractions, A to F (Fig. 5).

After separation of fraction A (monosaccharide fraction) by preparative paper chromatography (method A), arabinose, xylose, and galactose were obtained in yields of 17.1, 4.0, and 1.0 mg, respectively.

As fractions B to E, purified by rechromatography on the same Bio-Gel P-2 column, appeared to be homogeneous on paper chromatography, oligosaccharides in fractions B to E were designated as OS-B (oligosaccharide B), OS-C, OS-D, and OS-E, respectively. Their sugar compositions and results of methylation analysis are summarized in Table II with their yields and R_f values. Each oligosaccharide gave only xylose after complete acid hydrolysis. The result of methylation analysis was that the molar ratio of 2,3,4-tri-*O*-methyl-xylose and 2,3- and/or 3,4-di-*O*-methyl-xylose are approximately 1:1, 1:2, 1:3, and 1:4 for OS-B, OS-C, OS-D, and OS-E, respectively. OS-B was indistinguishable from authentic 4-*O*- β -xylopyranosyl-D-xylose on paper chromatography (method A). The log $R_f/(1 - R_f)$ of xylose, OS-B, -C, -D, and -E, when plotted against the degrees of polymerization, gave a linear regression suggesting that these sugars

FIG. 6. Chromatography on Bio-Gel P-2 of Fraction F Hydrolyzed with Endo- β -1,4-xylanase.

Fraction F (11 mg in 5 ml of 40 mM Na-acetate buffer, pH 5.5) was incubated with 0.1 mg of *Streptomyces* sp. endo- β -1,4-xylanase for 24 hr at 40°C under a covering of a few drops of toluene. After incubation, the reaction mixture was deionized with Amberlite IR-120 resins (H^+ form) and concentrated to a small volume. The concentrate was put on a column (1.5 $\times$ 150 cm) of Bio-Gel P-2 operated at 50°C, then filtered through the column with water. Fractions of 1 ml each were collected and assayed for total carbohydrate (A_{480} , —●—) and uronic acid by the carbazole-sulfuric acid method⁽¹⁰⁾ (A_{530} , —○—). Tubes 55~73, 115~125, and 127~133 were separately combined, concentrated to give fractions F-1 (yield: 4.8 mg), F-2 (1.8 mg), and F-3 (0.7 mg). Paper chromatography (method A) showed that fraction F-2 was xylobiose and F-3 was xylose.

belong to a homologous series.

Fraction F had $[\alpha]_D -10^\circ$ ($c = 1.2$, in water) and gave, on acid hydrolysis, xylose, glucuro-

nic acid, and glucuronosyl-xylose (paper chromatography, method B). Analysis by GLC on column (b) of the partially methylated alditol acetates from the hydrolyzate of the methylated fraction F, which was prepared by methylation, reduction with lithium aluminium hydride, and remethylation, found non-reducing terminal glucose (22%), non-reducing terminal xylose (11%), 2- and/or 4-linked xylose (54%), and 2,4- and/or 3,4-linked xylose (12%) in methylated fraction F. Upon hydrolysis with *Streptomyces* sp. endo- β -1,4-xylanase, fraction F yielded xylose (9%), xylobiose (25%), uronic acid-containing oligosaccharides (66%) (Fig. 6). These results suggest that the glucuronic acids in fraction F are non-reducing terminal residues.

DISCUSSION

An acidic arabinoxylan obtained from cell walls of immature barley plants has a linear backbone chain of β -1,4-D-xylose residues, about 50% of which were substituted at the 2 and/or 3 position, mainly with arabinofuranose and glucuronic acid residues. The chemical structure of the acidic arabinoxylan is similar to those of arabinoxylans that have been characterized from a number of monocot tissues.¹¹⁻¹⁸ McNeil *et al.*¹² reported that barley aleurone arabinoxylan contain no urosyl residues. It is not certain whether the structural difference between A-AX and barley aleurone arabinoxylan is derived from the difference of barley tissues used. A-AX contained a minor amount of non-reducing ter-

minal galactose residue. This must occur as the non-reducing terminal groups of side chains in A-AX, although the details of the structure were not studied.

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