

Purification and Characterization of Two Arabinoxylans from Wheat Bran

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ABSTRACT

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Water-soluble hemicellulose (WSH) was extracted and purified from wheat bran. The yield of WSH was 7.6% of the bran. WSH was mainly comprised of arabinoxylan (86.4%). Two major arabinoxylans of the WSH were fractionated by a diethylaminoethyl (DEAE)-Sephacryl CL-6B column. One of them (AX-1) passed through the DEAE-Sephacryl column, but the other (AX-2) was adsorbed on the column and was eluted with a linear gradient of 0.1–0.3M NaCl. These fractions were further purified by Sephacryl S-200. The carbohydrate yields of AX-1 and AX-2 were around 40 and 60% of the WSH, respectively. AX-1 was comprised principally of xylose (50.4%), arabinose (28.7%), and glucose (17.3%), whereas AX-2 contained some uronic acid (4.7%) and proteinaceous materials (4.8%) as well as xylose (42.4%) and arabinose (45.5%); little glucose (2.4%) was found in AX-2. Size-exclusion high-

performance liquid chromatography of AX-1 and AX-2 showed that they had almost the same peaks in the symmetric molecular mass distribution that corresponded to a molecular mass of around 300,000–350,000 Da of pullulan. Enzymatic hydrolysis of the polysaccharides contained in AX-1, catalyzed by endo-1,4- β -D-xylanase, reduced the molecular weight of the fragments to less than 10,000 Da. However, polysaccharides of AX-2 did not show any change in molecular weight distribution. Methylation analysis suggested that AX-2 had a more branched structure than AX-1, because AX-2 had a lower ratio (1/1.88) of 2,3-dimethylxylose to 2- or 3-methylxylose than did AX-1 (1/0.59). It also showed that AX-1 was constituted of 37% unsubstituted, 22% single-substituted, and 40% double-substituted xylopyranosyl residues, whereas the percentages for AX-2 were 21, 39, and 40%, respectively.

Wheat bran is a good source of polysaccharides such as hemicellulose and cellulose. In these cell-wall polysaccharides, the main component is the hemicellulose arabinoxylan, which mainly consists of two pentose sugars, arabinose and xylose (Adams 1955, Schmorak et al 1957). Wheat bran arabinoxylan has a structure characterized by a β -(1,4)-xylan backbone with branching α -L-arabinofuranosyl groups (Brillouet et al 1982, Brillouet and Joselean 1987). Besides structural studies, numerous studies have been made on the chemical, physical, and nutritive quality of arabinoxylan (D'Appolonia and MacArthur 1976, Southgate et al 1976, Cummings et al 1978, Nomani et al 1979, Bertrand et al 1981, Fincher and Stone 1986, Meuser and Suckow 1986). Some authors also reported that pentosans can influence the rheological behavior of doughs and the texture of bakery products (D'Appolonia 1971, Shogren et al 1987).

In order to fractionate arabinoxylan from wheat bran, some techniques were developed. A number of investigators separated wheat bran into classes of various polysaccharides according to their solubility in different solvents (Schweitzer and Wursch 1979, Ring and Selvendran 1980, Anderson and Clydesdale 1980, Brillouet et al 1982). However, most of these reports are concerned with small-scale preparation and a limited characterization of the arabinoxylan.

In this study, water-soluble hemicellulose (WSH) was prepared in quantity from wheat bran by a new preparation method. Finally, to clarify not only their nutritive effect but their effect on rheological behavior of doughs, arabinoxylans were purified from this WSH. They were characterized and their structure was investigated.

MATERIALS AND METHODS

Wheat Bran

Industrial wheat bran obtained from hard spring wheat was produced by Nisshin Flour Milling Co., Ltd. (Saitama, Japan). Its protein content (Kjeldahl method) was 14.5%, moisture 14.8%, and ash 4.35%, as determined by AACC approved methods (AACC 1983). The wheat bran was stored in the cold (-20°C) before use.

Preparation of WSH

WSH was prepared as summarized in Figure 1. To remove soluble proteins and starch, wheat bran (2 kg) was suspended in 20 L of warm water at 50°C , and the mixture was vigorously stirred by a large-scale agitator (model Super F, Nisshin Engineering, Tokyo) at a circumferential speed of 25 m/sec, for 3 min. After completion of the agitation, solid matters were separated from the solution by a centrifugal filter (model O-20, Tanabe Tekko, Tokyo). The residue was washed twice with water. The residues obtained (with a water content of about 50% by weight) were suspended in 10 L of 0.2M NaOH at 80°C , and the mixture

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was stirred using the same agitator as described above at a circumferential speed of 20 m/sec, for 1.5 hr. The suspension was continuously centrifuged at $6,000 \times g$. The supernatant (a crude extraction of WSH) was neutralized by adding 2M HCl. After addition of 10 L of water to the supernatant containing hemicellulose, the solution was dialyzed against water for 3 hr by the ultrafiltration system (model RUW-2, Nitto Denko) with a polysulfonic membrane (UF-3520, model P-18, total membrane area of 0.76 m^2 , i.d. of 11.5 mm, Nitto Denko, Osaka, Japan) having a molecular weight cutoff of 20,000 Da. The solution was concentrated by the same system to obtain 5 L of concentrate. The flow rate of the solution and the pressure against the membrane were 13 L/min and 8 kg/cm^2 , respectively. During dialysis, the rate of flux was maintained at $20 \text{ L/hr} \cdot \text{m}^2$ and the temperature of the solution was kept at 50°C by an electronic heater with a thermostat. The concentrated solution was deionized by an ion-exchange system with 500 ml of cation and anion exchange resins (models IR-120B and IRA-93, respectively, Orugano, Tokyo). The flow rate of solution was 125 ml/min. The solution obtained was immediately frozen at -40°C . The frozen solution was lyophilized, pulverized, ground, and sieved by an impact mill (Ultra centrifugal mill with a 0.5-mm filter, Retsch, Haan, Germany), to obtain WSH.

Purification of Arabinoxylans from WSH by Chromatography

A diethylaminoethyl (DEAE)-Sephacryl CL-6B column (Pharmacia, $100 \times 300 \text{ mm}$) was equilibrated to an effluent pH of 8.5 with 20 mM tris-HCl buffer. After being dialyzed against the same buffer overnight at 4°C , the WSH solution (15 ml of 1% WSH) was applied to the DEAE-Sephacryl column. Elution was done first with 150 ml of the equilibrating buffer and then with a linear gradient of 0–0.5N NaCl in the same buffer, using a flow rate of 0.6 ml/min. The eluent was collected in 5-ml fractions and was continuously monitored by light absorption at 280 nm. Also the total carbohydrates in the fractions were determined by measuring the absorbance at 480 nm according to the phenol-sulfuric acid method of Dubois et al (1956). After the arabinoxylan fractions were pooled and dialyzed against 100 mM tris-HCl buffer (pH 8.5) overnight at 4°C , the retentate was concentrated to about 20 ml, using an ultrafiltration system (Asahipak C5P, Asahi Kasei Kogyo Co., Tokyo) with a membrane having a molecular weight cutoff of 13,000.

The arabinoxylan concentrates from the DEAE-Sephacryl CL-6B column were loaded onto a Sephacryl S-200 column (Pharmacia, $30 \times 1,000 \text{ mm}$) that had been equilibrated with 100 mM tris-HCl buffer (pH 8.5). Using a flow rate of 0.4 ml/min, the eluent was collected in 5-ml fractions, and total carbohydrates in the fractions were determined by measuring the absorbance

at 480 nm according to the phenol-sulfuric acid method (Dubois et al 1956). Arabinoxylan fractions were pooled and dialyzed against distilled water overnight at 4°C ; the solution was frozen immediately and lyophilized. These purified arabinoxylans were used for the subsequent experiments.

Hydrolysis of Carbohydrates

To WSH or arabinoxylan isolates (10 mg), 5 ml of 2N trifluoroacetic acid (TFA) was added, and a steady stream of nitrogen was bubbled in for 60 sec. The ampoules were immediately sealed and placed in an oven at 105°C for 2 hr. Aliquots of the hydrolyzed solution were cooled and centrifuged at $3,000 \times g$ for 10 min. Two milliliters of supernatant was evaporated at 50°C to remove TFA on a rotary vacuum evaporator, and the dried sample was dissolved in 0.8 ml of distilled water.

Analysis of Sugar Composition

Aliquots of the hydrolyzed WSH or arabinoxylan isolates were filtered through $0.45\text{-}\mu\text{m}$ filters, and the filtrates ($20 \mu\text{l}$) were directly analyzed at 80°C by high-performance liquid chromatography (HPLC), using a chromatograph (model 655A-12, Hitachi, Tokyo) equipped with a solvent-delivery system controlled by a model L-5000 LC controller, a model 655A-40 automatic sample injector, and a refractometer (Shodex RISE-61, Showa Denko, Tokyo). The packed column ($3 \times 200 \text{ mm}$), with silica of $10\text{-}\mu\text{m}$ particle size, was used for separation of sugars. Data were recorded and quantitated with a Hitachi Chromato integrator D-2000. The solvent was superpurified water produced by Milli-Q (Millipore, Bedford, MA), eluted at 0.7 ml/min over 20 min, and degassed on line by a degasser (model 546B, Gaschrom Kogyo). Glucose, xylose, and arabinose concentrations in arabinoxylan isolates were estimated from a standard curve of the respective sugars. Arabinoxylan content was determined as the combined weight of arabinose and xylose.

Determination of Uronic Acids

Uronic acids were determined by the metaphenylphenol method using glucuronic acid as a standard (Blumenkranz and Asboe-Hansen 1973).

Determination of Phytic Acids

Phytic acids were determined according to the method of Tangendjaja et al (1980). The HPLC system used was described above. A reversed-phase C-18 column ($4 \times 300 \text{ mm}$) was obtained from Gaschrom Kogyo (Tokyo). Pure phytic acid was purchased from Wako (Tokyo).

Endo-1,4- β -D-Xylanase Treatment of Arabinoxylan

The endo-1,4- β -D-xylanase (EC 3.2.1.8.) used in this study was purified from cellulose Onozuka RS (Yakult Co. Ltd, Tokyo). The xylanase (64 units) obtained was used to hydrolyze WSH or arabinoxylan isolates (1 ml contained 30 mg of total carbohydrates) with 100 mM acetate buffer (pH 5.5). After incubation (40°C , 60 min), 0.5 ml of 2N NaOH was added to the reaction mixture and the solution was immediately heated (95°C , 10 min) to inactivate xylanase. After filtration ($0.45 \mu\text{m}$), the solution was fractionated by size-exclusion HPLC (SE-HPLC).

SE-HPLC

To investigate the molecular weight distribution of polysaccharide chains in arabinoxylan isolates and xylanase-treated arabinoxylan isolates, SE-HPLC was done using the HPLC system described above. The Waters Ultrahydrogel 1000 column ($7.8 \times 300 \text{ mm}$) used in these experiments has a claimed separation range of 10–1,000 kDa for polysaccharides. Molecular weights were estimated by comparing sample peak retention times to a standard curve composed of the logarithmic average molecular weight of the pullulan standards (Shodex pullulan standards P-82, Showa Denko). The molecular weight markers of pullulan used were: P-800 (8.53×10^5), P-400 (3.8×10^5), P-200 (1.22×10^5), P-100 (1×10^5), P-50 (4.8×10^4), P-20 (2.37×10^4), P-10 (1.22×10^4), and P-5 (0.58×10^4). Superpurified water was used as

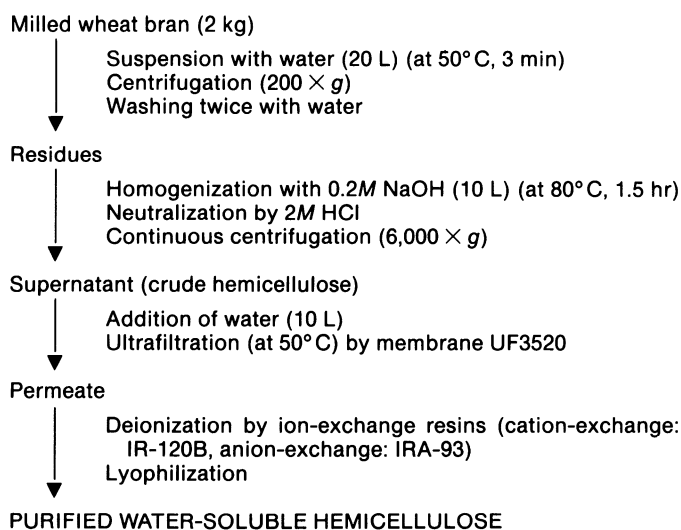


Fig. 1. Scheme for isolation and purification of water-soluble hemicellulose from wheat bran.

the eluent, with a flow rate 0.5 ml/min. After filtration (0.45 μ m), 100 μ l of 3% carbohydrate sample solution was applied. The eluent was collected in 0.5-ml fractions, and the total carbohydrates in these fractions were determined by measuring absorbance at 480 nm according to the phenol-sulfuric acid method (Dubois et al 1956).

Methylation Analysis of Arabinoxylan Isolates

Arabinoxylan isolates (5-mg samples) were subjected to micro-scale methylation by a modified method of Hakomori (1964), and the methylated product was extracted with chloroform. The extract was washed several times with water and concentrated to a syrup by evaporation. The methylated sample obtained was hydrolyzed in 1 ml of 90% formic acid solution at 100°C for 1 hr in a sealed test tube, and the hydrolysate was evaporated to obtain a dried sample. Furthermore, the hydrolysate sample was hydrolyzed in 1 ml of 1N TFA at 121°C for 1 hr in a sealed test tube and neutralized by the complete evaporation of TFA. After dissolution in 1 ml of water, the methylated sugars in the solution were hydrogenated with sodium borohydride and then acetylated with 2 ml of a mixture of equal portions of pyridine and acetic anhydride. The resulting alditol acetate was examined on a gas-liquid chromatograph fitted with a flame ionization detector, according to the method of Kusakabe et al (1977). The stainless steel column (3 \times 1,000 mm) was packed with 3% ethylene succinate cyanoethyl silicon copolymer medium on Gas Chrom Q (100–120 mesh) (purchased from Gaschro Kogyo, Tokyo), and the column temperature was maintained at 168°C. The carrier was nitrogen gas at a flow rate of 50 ml/min.

RESULTS AND DISCUSSION

Analysis of WSH

The yield of WSH was 7.6% of the original bran. WSH was mainly comprised of xylose and arabinose (86.4% as arabinoxylan)

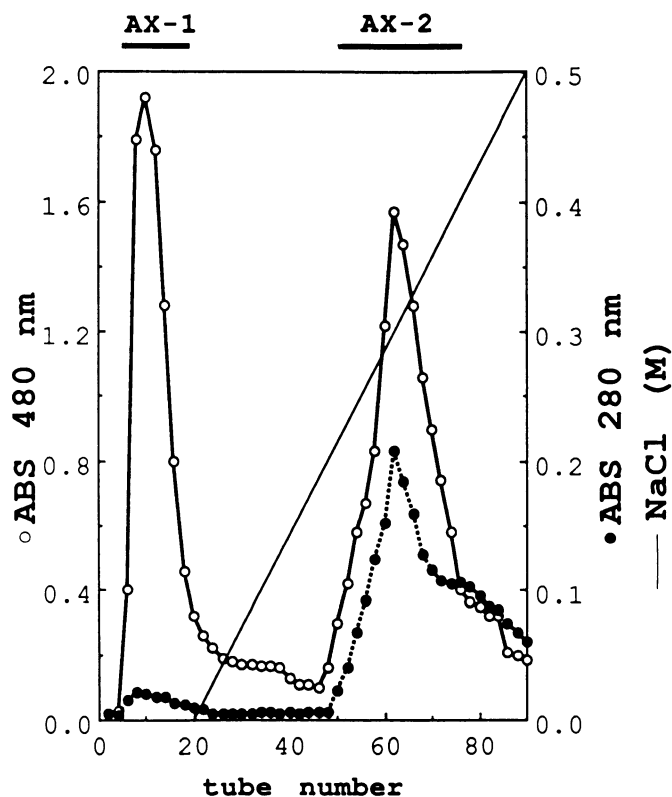


Fig. 2. Separation of arabinoxylan contents from soluble hemicellulose by diethylaminoethyl-Sepharose CL-6B column chromatography at a flow rate of 0.6 ml/min using 20 mM tris-HCl buffer (pH 8.5) with a linear gradient of 0–0.5M NaCl as the eluting buffer. Eluent was collected in 5-ml fractions. ○ = carbohydrates, ● = proteinaceous materials. Arabinoxylan fractions are denoted as AX-1 and AX-2.

with a molecular ratio of 1:0.92 (44.9:41.5%), and a little glucose (6.8%), uronic acid (glucuronic acid) (5.2%), crude protein (7.3%), and ash (0.27%). Phytic acid was found in the original bran (5.25%) but not in WSH. The total of these contents was a little more than 100% (105.97%), due to overestimation.

The high molecular ratio of arabinose to xylose in WSH suggested that mainly arabinoxylan with highly branched arabinose was extracted. Brillouet and Mercier (1981) reported that a highly branched arabinoxylan (hemicellulose B) was precipitated in 80% ethanol from a hemicellulose solution and that it was composed of 80.1% pentose (43% xylose and 37.1% arabinose), 9.4% glucose, and 4.3% uronic acid. The sugar composition of WSH was similar to these figures. However, the use of ethanol to fractionate hemicellulose was not satisfactory for obtaining pure arabinoxylan because of coaggregation with polysaccharides and other materials such as proteinaceous contents or minerals. Therefore, use of an ultrafiltration system and ion exchange resins might improve the purification.

Purification of Arabinoxylan from WSH by Chromatography

WSH was applied to the DEAE-Sepharose CL-6B column and separated into two major carbohydrate components: one of them (AX-1) passed through this column and the other (AX-2) was eluted with a linear gradient of 0.1–0.3M NaCl (Fig. 2). The total carbohydrates corresponding to AX-2 were equivalent to almost 60% of the soluble hemicellulose applied. While proteinaceous materials mainly coeluted with the carbohydrate material in AX-2, little proteinaceous material was detected in AX-1.

The two fractions were further purified by a Sephacryl S-200 column. The elution profiles (shown in Fig. 3) revealed that AX-1 had a little wider distribution in molecular weight than AX-2, but both had almost same elution time and elution patterns. These results demonstrated why the obtained WSH showed a single and symmetric peak (Fig. 4A). In addition, the elution profile of the proteinaceous portion contained in AX-2 showed a pattern almost the same as that of the carbohydrates. It is speculated from these results that the arabinoxylan chain in AX-2 might be linked to the peptide moiety by the same sugar amino acid linkage as was found between an arabinogalactan and a peptide moiety, i.e., a glycosidic link involving the hydroxy group of hydroxyproline (Strahm et al 1981). However, further work is required to clarify this hypothesis.

Analysis of Purified Arabinoxylan AX-1 and AX-2

As shown in Table I, some differences in composition were observed between AX-1 and AX-2. The main components of iso-

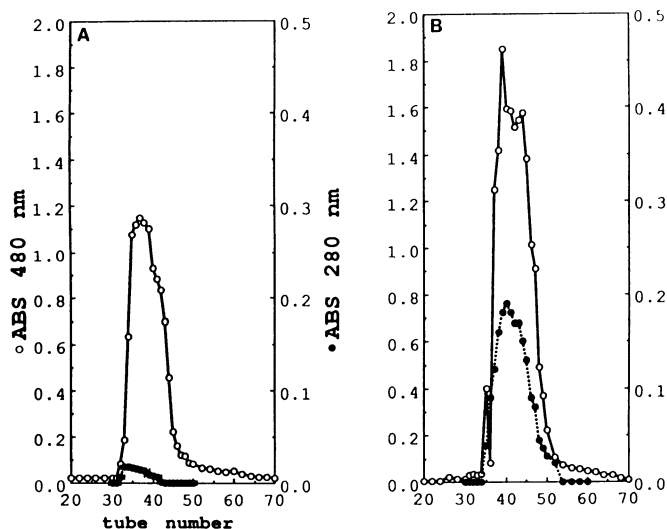


Fig. 3. Elution profile of arabinoxylan fractions AX-1 (A) and AX-2 (B) by Sephacryl S-200 column chromatography at a flow rate of 0.4 ml/min using 100 mM tris-HCl buffer (pH 8.5) as the eluent buffer. Eluent was collected in 5-ml fractions. ○ = carbohydrates, ● = proteinaceous materials.

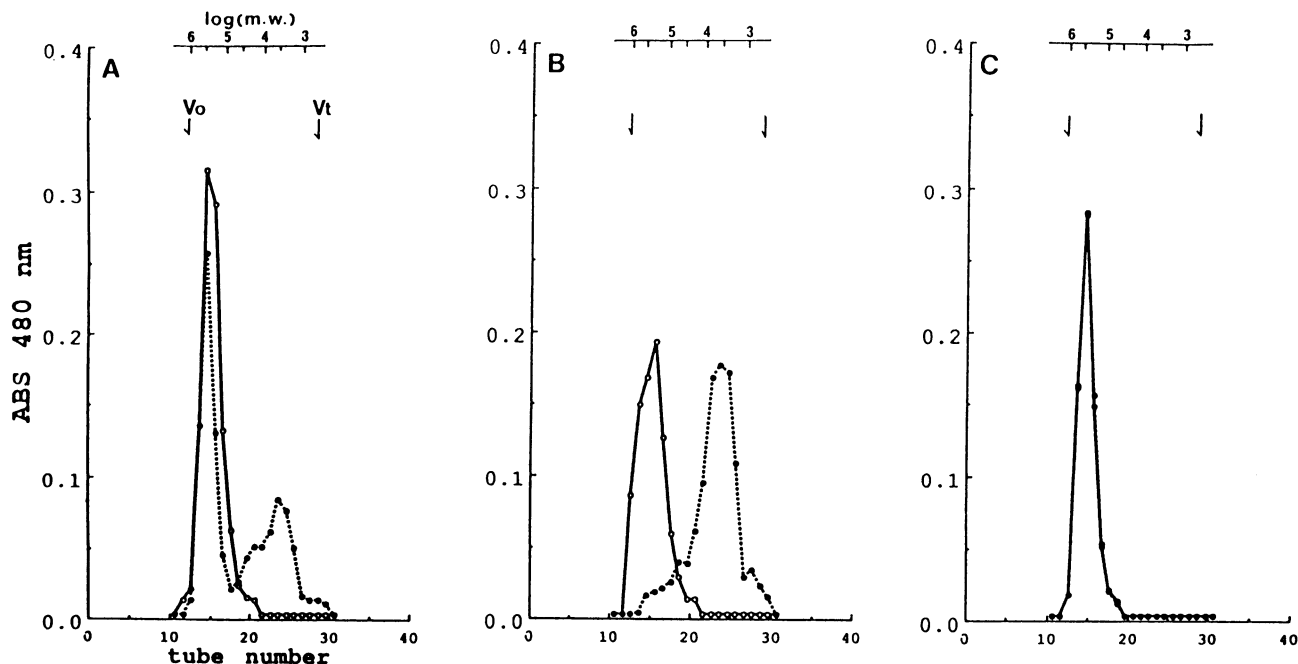


Fig. 4. Elution profile of arabinoxylans (line) and enzymatic hydrolysates of arabinoxylans (dotted line) catalyzed by endo-1,4,- β -D-xylanase, by size-exclusion high-performance liquid chromatography on an Ultrahydrogel 1000 column. V_0 and V_t show the void volume and total bed volume for this column, respectively. The logarithmic molecular weights (upper axis) were estimated from pullulan standards. A = water-soluble hemicellulose, B = fraction AX-1, C = fraction AX-2.

TABLE I
Comparison of Sugar and Protein in Arabinoxylans
(AX-1 and AX-2) Purified from Wheat Bran^a

Fraction	Sugar Composition				Crude Protein (%)
	Xylose (%)	Arabinose (%)	Glucose (%)	Uronic Acid (%)	
AX-1	50.4	28.7	17.3	0.6	0.8
Molar ratio	1	0.57	0.34	0.012	
AX-2	42.4	45.5	2.4	4.7	4.8
Molar ratio	1	1.07	0.057	0.11	

^a Values are the average of two replications. All duplicates were within 5% of the mean.

lated arabinoxylan AX-1 were xylose, arabinose, and glucose, whereas arabinoxylan AX-2 was mainly comprised of xylose and arabinose. It also contained some uronic acid (4.7%), proteinaceous materials (4.8%), and glucose (2.4%). These results suggested that AX-1 and AX-2 have different structures.

According to the report of Brillouet et al (1982), hemicellulose B extracted from wheat bran is constituted of at least two polysaccharides (arabinoxylan) that can be fractionated by ethanol precipitation. Compared to those results, AX-2 corresponds to a polysaccharide (arabinoxylan) precipitated in the range of 60–90% ethanol, which consisted mainly of xylose and arabinose with a molar ratio of 1:1.09–1.14 and also contained 5.4–5.7% uronic acid. Since some investigators (Metcalf et al 1968, D'Appolonia and MacArthur 1975, Lineback et al 1977) have suggested that pentosan from wheat flours consists of a xylan with highly branched arabinose, pentosan might have a structure similar to that of AX-2.

The gel filtration chromatography profiles of the original WSH and the two arabinoxylans purified on SE-HPLC are shown in Figure 4 (A, B, and C, respectively). The polysaccharides from the two arabinoxylans (AX-1 and AX-2) were eluted in a single and symmetric peak between the void volume (V_0) and the total bed volume (V_t). The molecular weight distribution of both purified arabinoxylans was estimated by SE-HPLC from a calibration plot of elution time vs. the molecular weight of pullulan. It showed

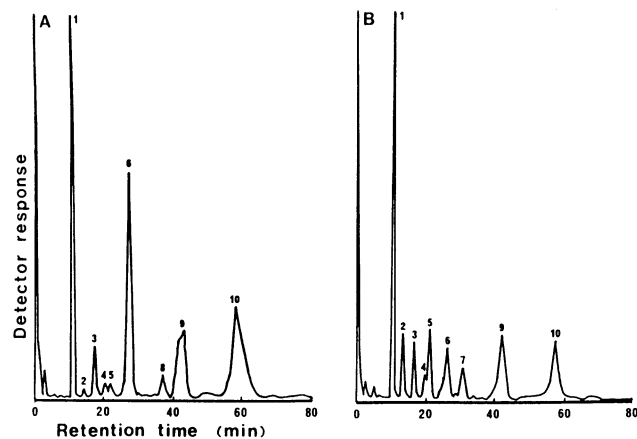


Fig. 5. Separation of methylated sugars from the purified arabinoxylans (A = fraction AX-1, B = fraction AX-2) by gas-liquid chromatography on stainless steel column packed with 3% ECNSS-M on Gas Chrom Q.

that AX-1 and AX-2 had almost the same peak with symmetric molecular weight distribution, which corresponded to a molecular weight of around 300–350 kDa of pullulan. AX-1 had a wider distribution of molecular weight than AX-2.

As shown in Figure 4A, 40% of the original WSH was decomposed by endo-1,4,- β -D-xylanase, but the rest of WSH resisted that enzymatic reaction. Enzymatic hydrolysis of AX-1 arabinoxylan, catalyzed by endo-1,4,- β -D-xylanase, caused the molecular weight distribution to change to a lower level that corresponded to a molecular weight lower than 10,000 Da (Fig. 4B). However, the molecular weight distribution of AX-2 did not change (Fig. 4C). These results suggest that xylanase did not work for AX-2 because of the unique structure of AX-2. According to Kusakabe et al (1983), xylanase is specific for the xylose residues that are devoid of branches of L-arabinofuranose residues or 2-O- β -D-xylopyranosyl-L-arabinose units in the arabinoxylan. This suggests that AX-1 is constituted from a main chain of 1,4-linked- β -D-xylopyranose residues having only a few L-arabinofuranosyl branches but that AX-2 is constituted from a main chain with many arabinofuranosyl branches.

TABLE II
Methylation Analysis of Arabinoxylans (AX-1 and AX-2)
Purified from Wheat Bran^a

Peak Number ^b	Methyl Sugar	Relative Molar Ratio	
		AX-1	AX-2
1	2,3,5-Trimethyl arabinose	24.5	27.1
2	2,3,4-Trimethyl xylose	0.6	5.5
3	3,5-Dimethyl arabinose	3.5	5.6
4	Not identified	NC ^c	NC
5	2,5-Dimethyl arabinose	1.0	9.3
6	2,3-Dimethyl xylose	25.0	8.1
7	Not identified	ND ^d	NC
8	Not identified	NC	ND
9	2- or 3-Methyl xylose	14.7	15.2
10	Xylose	27.0	15.6

^a Values are the average of two replications. All duplicates were within 5% of the mean.

^b Corresponding to the peaks shown in Figure 4.

^c NC = not calculated.

^d ND = not detected.

Methylation Analysis

As shown in Figure 5, gas-liquid chromatography on the methylated sugars from AX-1 and AX-2 revealed nine major derivatives. Although two minor peaks were not identified and 2- and 3-*O*-methylxylose could not be separated under these conditions, seven derivatives from the two arabinoxylans were identified and their amounts were compared (Table II). It was suggested that the structure of AX-2 would be more highly branched than AX-1, because AX-2 had a lower ratio of 2,3-dimethylxylose to 2- or 3-methylxylose (1:1.88), compared with the ratio for AX-1 (1:0.59). The presence of double-substituted xylose was detected, as reported by many researchers (Medcalf and Gilles 1968, Woolard et al 1976, Bacic and Stone 1981, Shibuya et al 1983, Shibuya and Iwasaki 1985, Brillouet and Joselean 1987). A higher proportion (5.5%) of 2,3,4-trimethylxylose in AX-2 than in AX-1 (0.6%) suggested that AX-2 contained more xylose in the terminal position, which was interpreted as AX-2 containing some short chains of 1,4-linked- β -D-xylopyranosyl residues. Thus, it was suggested that 37% of the 1,4-linked- β -D-xylopyranosyl residues in AX-1 are unsubstituted, 22% are singly branched at the 2- or 3-position, and 40% are doubly branched at the 2- or 3-position. On the other hand, AX-2 is constituted of 21% unsubstituted, 39% single-substituted, and 40% double-substituted xylopyranosyl residues. The values for AX-2 are close to other results reported for arabinoxylan from beeswing wheat bran (Brillouet and Joselean 1987).

Furthermore, arabinose in AX-1 mainly consisted of terminal residues such as 2,3,5-trimethylarabinose, but AX-2 contained some arabinose linked to the 2- or 3-position in addition to terminal arabinosyl residues. Some arabinosyl residues linked some short side chains at the *O*-2 or *O*-3 position. Although AX-2 had cation-exchange character, this characteristic might be due to the proteinaceous material or uronic acid linked to the arabinosyl residue. However, the present study did not ascertain the position where uronic acid was substituted. Further work using nuclear magnetic resonance is required to determine this.

CONCLUSION

WSH was prepared in quantity from wheat bran by a new preparation method using ultrafiltration and ion exchange resins. The WSH was composed of at least two arabinoxylans having different character and structure, although SE-HPLC showed that the two arabinoxylans had almost the same peaks and symmetric molecular weight distribution. These results suggested that the two arabinoxylans might have different nutritive effects and play different roles in breadmaking.

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