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AN L-ARABINO-D-GALACTAN AND AN L-ARABINO-D-GALACTAN-CONTAINING PROTEOGLYCAN FROM RADISH *(Raphanus sativus)* SEEDS

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ABSTRACT

An L-arabino-D-galactan and an L-arabino-D-galactan-containing proteoglycan were isolated from hot phosphate-buffered saline extracts of radish seeds by ethanol fractionation, ion-exchange chromatography, and gel filtration, and found homogeneous by ultracentrifuge analysis and high-voltage electrophoresis. The proteoglycan consisted of 86% of a polysaccharide component containing L-arabinose and D-galactose as major sugar constituents, together with small proportions of D-xylose, D-glucose, and uronic acids, and 9% of a hydroxyproline-containing protein. Methylation analysis, periodate oxidation, and enzymic degradations indicated a backbone chain of (1→3)-linked β-D-galactosyl residues with side chains at O-6 of (1→6)-linked β-D-galactosyl residues and uronosyl groups. The α-L-arabinofuranosyl residues were located mainly in the outer regions as nonreducing groups, as well as O-2- or -5-linked inner chain residues, and O-2,5- or -3,5-linked branching residues. Reductive, alkaline degradation of the proteoglycan indicated that the polysaccharide chains were partly linked through *O*-glycosyl linkages to the threonine residues of the polypeptide chains. The proteoglycans from radish leaves and seeds appeared to share common antigenic determinant(s). The radish-seed arabinogalactan had a high content (81%) of L-arabinose and its basic structure seemed to be similar to that of the polysaccharide component of the proteoglycan.

INTRODUCTION

Arabinogalactans (AG) and arabinogalactan-containing proteoglycans (AGP) having a (1→3)- and (1→6)-linked D-galactan as a backbone have been isolated and characterized from various higher plants, but little is known about their roles in plant tissues, although several physiological functions have been proposed[1,2]. In a previous paper[3], we reported the isolation, from mature leaves of

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cruciferous plants, such as radish, rape and turnip, and properties of L-fucose-containing AGPs exhibiting blood-group H-like activity. Subsequent structural and serological studies of radish leaf AGPs provided evidence that a sugar sequence of α-L-fucopyranosyl-(1→2)-α-L-arabinofuranosyl-(1→ was responsible for the H-like activity inhibiting the hemagglutination of human O erythrocytes with eel anti-H agglutinin[4,5]. By monitoring the serological activity of the extracts of radish seeds and various tissues developed after germination, we found that the hot phosphate-buffered saline extracts of the resting seeds did not exhibit appreciable H-like activity, which suggested the presence of AG or AGP (or both) having different chemical compositions from those present in leaf tissues. We describe herein the isolation and characterization of AG and AGP of radish seeds, and compare their properties and structures with the AGPs of mature leaves.

EXPERIMENTAL

Materials. — Seeds of a cultivar, Aokubi, of radish, *Raphanus sativus* L. var. *hortensis* Backer were purchased from Tokita Seed and Plant Co., Ltd., Saitama, Japan. A partially purified β-D-galactanase (EC 3.2.1.90) was prepared from Driselase, a commercial cellulase product of basidiomycete, *Irpex lacteus* (Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan), as previously reported[4]. An α-L-arabinofuranosidase (EC 3.2.1.55) of *Rhodotorula flava*[6] and a purified[7] β-D-xylanase (EC 3.2.1.8) from *Streptomyces* sp. E-86 were generous gifts from Dr. N. Shibuya, National Food Research Institute, Ibaraki, Japan, and from Dr. I. Kusakabe, Institute of Applied Biochemistry, University of Tsukuba, Ibaraki, Japan, respectively. *Escherichia coli* β-D-galactosidase (grade VIII) (EC 3.2.1.23) was obtained from Sigma Chemical Co., Missouri, U.S.A. An L-fucose-specific lectin was purified from a fungus, *Aleuria aurantia*, fruit-bodies according to the procedure of Kochibe *et al.*[8].

Analytical methods. — The contents of total sugars, reducing sugar, uronic acid, and protein were measured colorimetrically[3,4]. Hydroxyproline was determined according to Kivirikko *et al.*[9]. Total nitrogen and amino acid composition were analyzed as previously reported[3]. Qualitative analyses of sugars and polysaccharides were carried out[3] by paper chromatography (p.c.) and high-voltage electrophoresis on a glass-fiber paper (Toyo-Roshi GA-100). G.l.c. of sugars as alditol acetate derivatives was performed with a Shimazu GC-6A gas chromatograph[3], and g.l.c.–m.s. with a Hitachi M-80 (70 eV) and data processor M-003, equipped with a Hewlett–Packard fused-silica capillary column (0.31 mm × 25 m) containing cross-linked methyl silicone programmed from 150 to 240° (2°/min). Trimethylsilyl[10] derivatives were separated by g.l.c. in a column (0.3 × 100 cm) of Silicone OV-17 programmed from 80 to 200° (5°/min).

Optical rotation was measured with a Jasco DIP-360 digital polarimeter, at 20°, in a 5-cm cell. Apparent molecular weights of the AGP and its degraded-products were estimated in a Sepharose CL-6B column (1 × 85 cm) equilibrated with

14.5mM phosphate buffer, pH 7.2, containing 0.13M NaCl and 0.02% NaN_3 (PBSN) by use of FITC-dextrans as molecular weight markers. Sedimentation analysis was performed in a Hitachi–Beckman Model E ultracentrifuge on a 0.5% solution of the polysaccharides in 0.9% NaCl at 40 000 for AG and 64 000 r.p.m. for AGP at 20°. The intrinsic viscosities of the AG and AGP were determined at 25° with a Cannon–Fenske viscometer for solutions in 1.0M NaCl of varying concentrations of the polysaccharides (AG, up to 10 mg/mL; AGP, up to 20 mg/mL). Standard soluble starch gave an $[\eta] = 0.060$ dL/g under these conditions.

Extraction and purification of AG and AGP. — Grinding of dry seeds (700 g) gave an oily powder that was defatted in boiling 80% ethanol for 30 min and cooled, and the aqueous ethanol decanted. The powder was soaked in ethanol overnight at room temperature, and the insoluble materials were collected and dried at 50°, and the seed coats removed by sieving in a draft of air. The resulting yellowish powder was further defatted by soaking in ethanol and then in acetone, for two days each, and air dried. The residual materials (278 g) were extracted with PBSN (2.5 L) in a boiling-water bath for 30 min with occasional shaking. The suspension was filtered to give a turbid extract. After extraction of the residues again, the combined filtrates were brought to 0.3 vol. of ethanol by dropwise addition of ethanol, and centrifuged to remove a precipitate (6.2 g). To the supernatant, ethanol was added (to 4 vol.) to give a precipitate that was kept at 4° overnight and collected by centrifugation. The precipitate (23.7 g) was suspended in water (350 mL), deproteinized by heating at 65° with 45% (w/v) phenol for 10 min under rigorous stirring[11], and cooled in an ice bath. Centrifugation of the mixture separated the water layer and a phenol layer that contained denatured proteins (2.8 g). Addition of ethanol (4 vol.) to the aqueous layer yielded a precipitate of crude polysaccharides, which was washed with ethanol and dried *in vacuo* (P_2O_5). A part (3 g) of the polysaccharide fraction (12.2 g) was dissolved in water, dialyzed against several changes of water for 2 days at 4°, and applied onto a column (3.5 × 32 cm) of DEAE-cellulose (HCO_3^-). Fractions (15 mL) were collected and monitored for sugar. Washing the column with water (1.5 L) afforded a fraction of neutral polysaccharide (27 mg). Elution with a linear gradient of $NaHCO_3$ (0–0.5M, 4 L) gave a minor peak at the beginning of the gradient and a major, broad peak with a steep profile at the leading edge, which was separated into Fraction I (114 mg; eluted with 0.05–0.15M $NaHCO_3$) and Fraction II (227 mg; 0.15–0.3M $NaHCO_3$). Finally, washing the column with 0.2M NaOH gave a brownish material (41 mg). The polysaccharides in Fractions I and II were separately subjected to gel filtration on a Sepharose 6B column (4 × 110 cm) equilibrated with PBSN. The polysaccharide in Fraction I, composed of AG, emerged mainly at the void volume, whereas the polysaccharides in Fraction II were resolved into two components, an AG and a low-M_r AGP. The fractions corresponding to AG or AGP were combined, dialyzed, and lyophilized (yields: AG, 140 mg; AGP, 50 mg). Further purification of the AG and AGP was carried out by rechromatography on a column (2 × 14 cm) of DEAE-cellulose (HCO_3^-). Upon elution with a linear gradient of $NaHCO_3$ (0–0.5M), the

AG emerged as a single peak with 0.1M $NaHCO_3$, and the AGP was eluted with 0.16M $NaHCO_3$ as a broad, asymmetric peak. The yields of AG and AGP were 68 and 30 mg, respectively. With repeated applications of the purification procedure to the polysaccharide fraction, 263 and 120 mg, respectively, of the purified AG and AGP were obtained from 700 g of radish seeds.

Methylation analysis. — Methylation of the polysaccharides (1–2 mg) was carried out by the Hakomori method[12], as described by Sandford and Conrad[13], except that polyalkoxide formation was allowed to proceed for 20 h in the case of AG and its modified products. Hydrolysis of methylated poly- and oligo-saccharides was performed as described previously[4]. The partially methylated monosaccharides were analyzed by g.l.c. and g.l.c.–m.s. after conversion to alditol acetates[3,4]. The g.l.c.–m.s. data were assigned by comparison with the mass-spectrometry data[14]. The molar response of each partially methylated sugar was corrected by the method of Sweet *et al.*[15].

Periodate oxidation. — AG (72 mg) and AGP (62 mg) were oxidized with 50mM $NaIO_4$ (50 mL) at 4° in the dark. Formic acid produced and IO_4^- consumed were determined by titration and the method of Fleury and Lange[16]. The oxidized products were reduced with M $NaBH_4$ to yield polyalcohols (65 mg from AG; 50 mg from AGP). Smith degradation[17] was performed with M trifluoroacetic acid (4 mL) for 48 h at 25°. The degraded products were fractionated with 75% methanol into insoluble polymers (25 mg from AG; 15 mg from AGP) and soluble products (37 mg from AG; 29 mg from AGP) containing 20 and 27% of the total sugars, respectively. Chromatography of the soluble fraction derived from AGP on a Dowex 1 (HCO_3^-) column separated acidic from neutral sugars, the latter being further resolved by preparative p.c. to give three neutral oligosaccharides (**1**, **2**, and **3**).

Carboxyl group reduction. — AGP (7 mg) was reduced according to the method of Taylor and Conrad[18] to give a carboxyl-reduced product (4.5 mg).

Enzymic degradations. — (*a*) *α-L-Arabinofuranosidase.* AG and AGP (8 mg each) were treated by *R. flava* α-L-arabinofuranosidase (1 unit) in 10mM citrate-phosphate buffer, pH 3.0 (2 mL) for 15 h at 37°. No significant release of sugar was observed upon addition of additional enzyme to the reaction mixtures. The reaction was terminated by heating the mixture in a boiling-water bath for 5 min. The mixture was de-ionized with Dowex 50W (H^+) resin. The enzyme-modified AG (1.7 mg) could be recovered from the digest by elution with 0.2M $NaHCO_3$ of a DEAE-cellulose column (1 × 5 cm; HCO_3^-) after washing the column with water to remove L-arabinose. Likewise, gel filtration on a Sephadex G-15 column (2.5 × 30 cm) separated the enzyme-modified AGP (4.4 mg) from L-arabinose.

(*b*) *β-D-Xylanase.* Removal of D-xylosyl residues from the native AG and AGP was attempted by incubating the samples (2 mg each) at 37° in a reaction mixture (1 mL) containing *Streptomyces* β-D-xylanase (6 μg) in 20mM phosphate buffer, pH 6.0.

(*c*) *β-D-Galactanase.* AGP (22 mg) was incubated with *I. lacteus* β-D-galactanase (40 μg) in 25mM acetate buffer, pH 4.6 (4 mL) at 37°. The liberation of

reducing sugar (apparent conversion to D-galactose, 12%) reached a plateau after 20 h, and the digest was fractionated on a Sephadex G-75 column (2 × 44 cm). In contrast to AGP, no measurable release of reducing sugar was observed from AG after prolonged incubation with β-D-galactanase.

(*d*) β-D-*Galactosidase*. Action of *E. coli* β-D-galactosidase on oligosaccharides was examined by incubating the samples (0.2 mg each) in a mixture containing the enzyme (10 μg) in 25mM phosphate buffer, pH 7.2 (0.2 mL) for 5 h at 37°.

Cleavage of alkali-labile sugar–protein linkage. — The AGP (5 mg) was incubated with 0.5M NaOH containing 0.5M $NaBH_4$ (1 mL) for 24 h at 25°. A portion (0.1 mL) of the mixture was made neutral with 6M acetic acid, evaporated with repeated additions of methanol, and analyzed by gel filtration on a Sephadex G-100 column (2 × 50 cm) equilibrated with PBSN. The molecular size of the alkali-treated sample was compared with that of the native AGP, based on the elution profiles of sugar and protein. Another portion of the sample was reduced[19] with $PdCl_2$–HCl and alkaline $NaBH_4$, de-ionized by passage through a Sephadex G-15 column, and analyzed for amino acid composition.

Serological tests. — The blood-group H-like activity of the AG and AGP was assayed by inhibition of hemagglutination of human O erythrocytes with eel anti-H agglutinin (titer 1:8) and expressed as the minimum concentration causing complete inhibition of hemagglutination[3]. The Ouchterlony double-diffusion method[20] was employed to examine the precipitation reactions of the polysaccharides with eel anti-H precipitin, rabbit anti-radish-leaf AGP sera[5], and *A. aurantia* L-fucose-specific lectin. β-Glucosyl Yariv antigen, synthesized by the method of Yariv *et al.*[21], was also tested for the precipitation reaction with the AGP in gel.

RESULTS AND DISCUSSION

Properties of AG and AGP. — Two acidic polysaccharides, AG and AGP, were separated and purified from hot PBSN extracts of radish seeds by ethanol fractionation, chromatography on a DEAE-cellulose column, and gel filtration on a Sepharose 6B column. On ultracentrifuge analysis, the purified AG sedimented as a single symmetric peak showing apparent homogeneity, whereas the purified AGP gave a broad and asymmetric peak indicative of relatively diverse molecular sizes. Similarly, on high-voltage electrophoresis on glass-fiber paper, AG migrated as a single component, whereas AGP migrated as a single but broad peak indicating diversity of charge and chemical composition. Further, AGP was eluted as a single component of diverse molecular size from a Sepharose CL-6B column. Table I summarizes the physical, chemical, and serological properties of AG and AGP. AG is a high-M_r polysaccharide consisting of mainly L-arabinose and D-galactose together with a small proportion of D-xylose and uronic acid(s). It gave a highly viscous solution when dissolved in water. In contrast, AGP has an apparent M_r of 52 000 with an intrinsic viscosity comparable with that of similar AGPs, such as radish leaf AGP (R-II)[3], rape leaf AGP[3], *Gladiolus* style AGP[22], and wheat

TABLE I

PHYSICAL PROPERTIES, CHEMICAL COMPOSITIONS, AND HEMAGGLUTINATION-INHIBITING ACTIVITIES OF AG AND AGP

Properties	*AG*	*AGP*
Mol. wt.	K_{av} 0.14[a]	52 000
$s_{20,w}$ (S)	14.8	5.6
$[\alpha]_D^{20}$ (c 0.5, water) (degrees)	−109	−39.0
$[\eta]$ (dL/g)	1.70	0.129
Kjeldahl N (%)	<0.1	1.3
Total sugar content (%)	97.9	86.1
Sugar composition (mol/mol, %)		
D-Xylose	2.3	3.2
L-Arabinose	81.0	44.8
D-Galactose	13.6	41.6
D-Glucose	0	0.2
Uronic acids	3.1	10.2
Hemagglutination inhibiting activity (mg/mL)	>1	1

[a]Partition coefficient determined on a Sepharose CL-2B column (3 × 88 cm) and PBSN as eluent, which was calibrated by heat-killed bacterial cells and D-glucose.

endosperm AGP[23]. The monosaccharide composition of AGP is similar to that of AG, but the ratio of L-arabinose to D-galactose (1.1:1) of AGP is clearly different from that of AG (6.0:1). The uronic acid constituents of AGP were identified as D-glucuronic acid and 4-*O*-methyl-D-glucuronic acid (molar ratio 1.4:1) by p.c. and g.l.c. analysis of the products of hydrolysis of carboxyl-reduced AGP. The carboxyl group of the uronic acids in the AG could not be reduced under the same condition as those used for AGP. The serological test for blood-group H-like activity revealed that the hot water-soluble polysaccharides of radish seeds were devoid of the activity inhibiting hemagglutination of human O erythrocytes with eel anti-H

TABLE II

AMINO ACID COMPOSITION OF AGP

Amino acid	*Mol/100 mol*	*Amino acid*	*Mol/100 mol*
Lysine	5.0	Glycine	9.0
Histidine	2.4	Alanine	17.5
Arginine	[a]	1/2 Cystine	2.9
Hydroxyproline	7.6	Valine	13.4
Aspartic acid	6.1	Methionine	1.6
Threonine	8.2	Isoleucine	1.3
Serine	7.5	Leucine	7.7
Glutamic acid	4.8	Tyrosine	[a]
Proline	3.4	Phenylalanine	1.6

[a]Not detected.

agglutinin. The activity (1.25 mg/mL) of the crude polysaccharide fraction remained unchanged in the purified AG and AGP (~1 mg/mL). This is distinctly in contrast with the potent activity of radish leaf AGPs (5–10 μg/mL), which contain appreciable proportions of L-fucose responsible for the serological activity[3]. Thus, the content of D-xylose, instead of L-fucose, is a characteristic feature of radish seed AG and AGP. The amino acid composition of seed AGP indicates a high content of hydroxyproline, serine, threonine, glycine, alanine, and valine (see Table II). The content of hydroxyproline was estimated to be 11 μg/mg by colorimetry (8.8 μg/mg by amino acid analysis). A small proportion of glucosamine (1.9 μg/mg) was also detected. The protein content of AGP was 8.6%, based on amino acid analysis. The protein moiety of radish seed AGP is generally similar in amino acid composition to those of the AGPs from leaf tissues of radish and rape[3].

Glycoside linkages. — The polysaccharide components of radish seed AG and AGP were fully methylated, hydrolyzed, and analyzed for methylated monosaccharides by g.l.c. and g.l.c.–m.s. (see Table III). Data from column A showed that the AG has a highly branched structure. A majority of the L-arabinosyl residues are present as nonreducing end groups and O-2,5-linked branching residues in the furanose form, and some of the sugar residues are located as interchain furanosyl residues substituted at O-2 and O-5. A preponderance of D-galactopyranosyl residues are O-3,6-linked branching residues, together with small proportions of O-3- and O-6-linked inner residues.

As shown in column D, the methylation analysis of the AGP suggested that the same linkages as those present in AG were preponderant for the L-arabinofuranosyl and D-galactopyranosyl residues, together with O-3,5-linked branching L-arabinofuranosyl residues and nonreducing D-galactosyl groups. A small proportion of D-xylose units in AG and AGP appears to be located as nonreducing end groups or O-4- (or -2)-linked residues, or both. The appearance of nonreducing D-glucopyranosyl groups in AGP after carboxyl group reduction (Table III, column E) indicates that the pyranosyluronic units are located as nonreducing end groups.

Periodate oxidation of AG and AGP was complete after 5 days, in which time 0.38 and 0.75 mol of periodate was consumed with the concomitant production of 0.06 and 0.17 mol of formic acid per sugar residue, respectively. Smith degradation of AG and AGP, followed by 75% methanol fractionation, yielded two products, a methanol-insoluble polymer and soluble oligosaccharides. The polymer originating from AG had $[\alpha]_D^{20}$ −10.7° (*c* 0.4, water), was excluded from a Sephadex G-100 column, and consisted of L-arabinose, D-galactose, and uronic acid(s) in the molar proportion of 62:29:9. Methylation analysis of this polymer (Table III, column B) indicated a marked decrease in the proportion of O-2,5-linked branching L-arabinofuranosyl residues with a concomitant appearance of nonreducing D-galactopyranosyl groups and an increase in the proportion of O-2-linked L-arabinofuranosyl, and O-3- and O-6-linked D-galactopyranosyl residues. The polymer resulting from AGP had $[\alpha]_D^{20}$ +10.8° (*c* 0.4, water), still retained the

TABLE III

METHYLATION ANALYSES OF NATIVE, AND MODIFIED, OR DEGRADED AG AND AGP

Sugar component[a]	*Mode of linkage*	T[b]	*Proportions (mol/100 mol)*						
			AG[c]			*AGP*[d]			
			A	*B*	*C*	*D*	*E*	*F*	*G*
2,3,5-Me_3-L-Ara	L-Ara*f*-(1→	0.65	31	25	1	23	22	3	1
2,3,4-Me_3-D-Xyl	D-Xyl*p*-(1→	0.78	1	[e]	2	[f]	3		1
3,5-Me_2-L-Ara	→2)-L-Ara*f*-(1→	0.94	1	18	[f]	1	1	1	
2,3,4,6-Me_4-D-Glc	D-Glc*p*-(1→	1.00					5		
2,3,4,6-Me_4-D-Gal	D-Gal*p*-(1→	1.16		5	7	3	3	35	15
2,3-Me_2-L-Ara	→5)-L-Ara*f*-(1→	1.22	10	10	2	18	14	1	7
2,3- or 3,4-Me_2-D-Xyl	→2 or →4)-D-Xyl*p*-(1→	1.38			1	1	1		1
2,4,6-Me_3-D-Gal	→3)-D-Gal*p*-(1→	1.76	2	16	5	13	7	26	7
2-Me-L-Ara	→3,5)-L-Ara*f*-(1→	1.92				3	3		
3-Me-L-Ara	→2,5)-L-Ara*f*-(1→	2.04	41	9		6	6		
2,3,4-Me_2-D-Gal	→6)-D-Gal*p*-(1→	2.55	1	4	2	8	12	10	16
2,4-Me_2-D-Gal	→3,6)-D-Gal*p*-(1→	4.38	13	13	6	24	23	24	13
Free L-arabinose[g]					74				39

[a]As per-*O*-acetylalditol. [b]Retention time on a Silar-10C column, relative to that of 2,3,4,6-Me_4-D-Glc. [c]A, Native; B, polymer obtained by Smith degradation; C, product of digestion with α-L-arabinosidase. [d]D, Native; E, carboxyl-reduced; F, polymer obtained by Smith degradation; G, Product of digestion with α-L-arabinosidase. [e]Not detectable. [f]Trace. [g]Reducing sugar obtained by digestion with an α-L-arabinosidase.

TABLE IV

CHARACTERIZATION OF OLIGOSACCHARIDES OBTAINED BY SMITH DEGRADATION OF AGP

Properties	*Oligosaccharide*		
	1	**2**	**3**
Yield (%)[a]	6.2	4.5	2.4
R_{Glc}[b]	0.82	0.24	0.06
Sugar composition (molar ratio)[c]			
D-Galactose	1	2	3
Glycerol	1.1	0.9	0.9
Methylated sugar (molar ratio)[d]			
2,3,4,6-Me_4-D-Gal	1	1	1
2,3,4-Me_3-D-Gal	0	0.8	1.7

[a]Percentage of 75% methanol-soluble fraction. [b]In 6:4:3 butanol–pyridine–water. [c]G.l.c. analysis as per-*O*-(trimethylsilyl)ated derivatives after complete digestion with β-D-galactosidase. [d]As per-*O*-acetylalditol.

protein component, and gave a single component with a decreased M_r of 37 000 on a Sephadex G-100 column. The decrease of the M_r of native AGP by Smith degradation resulted from the oxidation of a large part of the L-arabinofuranosyl residues, leaving a product that consisted of L-arabinose and D-galactose in the molar proportion of 6:94. Colorimetric determination of uronic acid showed that the majority (~90%) of the uronosyl groups were oxidized by the Smith degradation of AGP. Methylation analysis of this polymer (Table III, column F) indicated that the L-arabinofuranosyl residues (other than a small proportion of O-2-linked) had disappeared, which resulted in the concomitant appearance of nonreducing D-galactopyranosyl groups and an increase of O-3-linked D-galactopyranosyl residues. The D-xylosyl residues, present as a minor sugar constituent in AG and AGP, were completely oxidized by periodate.

Analysis by p.c. of the methanol-soluble fraction indicated that the Smith degradation of either AG or AGP produced a large proportion of glycerol, together with a small quantity of L-arabinose. Among the degraded products of AGP, several oligosaccharides were detected, from which three neutral sugars were isolated and their structures analyzed. The oligosaccharides were characterized by R_{Glc} values, digestion with *E. coli* β-D-galactosidase, and analysis of the composition and type of glycoside linkages (Table IV). They were shown to be β-D-Gal*p*-(1→1)-D-glycerol (**1**), β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→1)-D-glycerol (**2**), and β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→1)-D-glycerol (**3**). These results suggests the presence, in seed AGP, of consecutive (1→6)-linked β-D-galactopyranosyl residues, substituted at O-3 with periodate-oxidizable sugar residues, as observed[4] for the Smith degradation products of leaf AGPs.

Action of enzymes. — Release of 74 and 39% of L-arabinose (identified as the sole sugar by p.c.) from AG and AGP by the action of α-L-arabinofuranosidase

indicated that this sugar was present in α-L-furanose form. Upon analysis of the sugar composition, it was found that both the enzyme-modified AG and AGP were converted into polysaccharides consisting of D-xylose, L-arabinose, D-galactose, and D-glucose in the molar proportions of 13:14:73:0 and 6:6:86:2, respectively, in addition to uronic acids. Methylation analysis (Table III, column C) revealed that the enzyme effectively removed the α-L-arabinofuranosyl residues from AG, irrespective of their type of linkage. As a result, an increase of the nonreducing D-galactopyranosyl groups and a decrease of the O-3,6-branched D-galactopyranosyl residues implied that the L-arabinofuranosyl residues, in native AG, were largely located in the outer chains of the L-arabinan region, linked through O-3 or O-6 (or both) to the terminal or interchain D-galactopyranosyl residues. Similarly, methylation analysis of the enzyme-modified AGP (Table III, column G) suggested that the L-arabinofuranosyl residues were present as outer chains linked through O-3 to the terminal or the interchain (1→6)-linked β-D-galactopyranosyl residues, which probably constitute the side chains of the D-galactan component of AGP.

Based on the aforementioned results of structural analysis, it is probable that the core structure of the polysaccharide portion of AGP is a galactan composed of a backbone of (1→3)-linked β-D-galactopyranosyl residues, to which (1→6)-linked side chains of β-D-galactosyl residues are attached through O-6. The results also suggest that AG has essentially the same basic structure as that of the polysaccharide component of AGP, although there are marked differences in sugar compositions and physical properties.

Several enzymic and chemical attempts were made to locate the D-xylosyl residues in AG and AGP. The sugar composition of AG and AGP remained unchanged after treatment with *Streptomyces* β-D-xylanase, by the action of which larchwood β-D-(1→4)-xylan (Sigma) is readily hydrolyzed (conversion to D-xylose, 40%). Thus, no release of D-xylo-oligosaccharides and D-xylose-containing fragments was observed, thus eliminating the presence of the structures that contain consecutive or branched (1→4)-linked β-D-xylosyl residues. The observation that an aqueous solution of these polysaccharides did not form a blue iodine complex[24] in the presence of calcium chloride excluded the possibility of contamination by a xyloglucan, the isolation of which had been reported[25] with seeds of rape, *Brassica campestris*.

The native AG appeared to be resistant to *I. lacteus* β-D-galactanase, presumably owing to a highly branched structure of α-L-arabinofuranosyl residues, which might make the galactan region of the molecule inaccesible to attack by the enzyme. On the other hand, the enzyme degraded native AGP to give products that were fractionated on a Sephadex G-75 column into three fractions, (Fig. 1; A, B, and C) consisting of D-xylose, L-arabinose, D-galactose, and D-glucose in the molar proportions of 1:50:48:1, 7:57:35:1, and 0:42:57:1, respectively. Detection of D-xylose in Fractions A and B led to the assumption that D-xylosyl residues are not distributed randomly in AGP but may be located as clusters or chains linked to the arabinogalactan framework. Methylation analysis of fractions A and B revealed

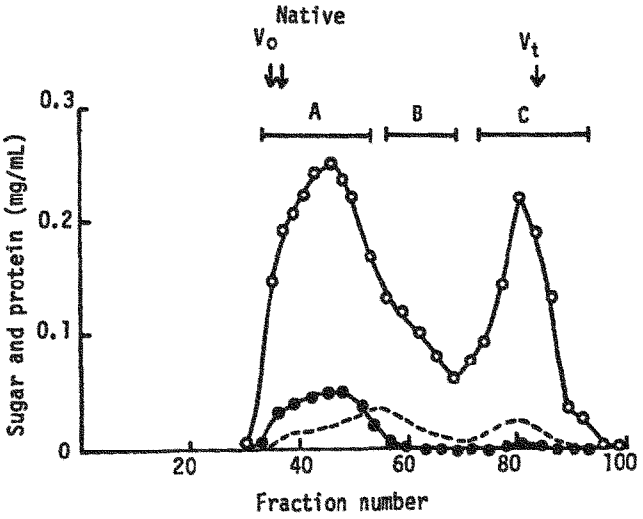

Fig. 1. Fractionation on a Sephadex G-75 column of the digest of AGP by β-D-galactanase: (○——○) Total sugar, (------) uronic acid, and (●——●) protein. Fractions of 1.65 mL each were collected. Arrows indicate the elution peaks of Blue Dextran (V_0), D-glucose (V_t), and the native AGP.

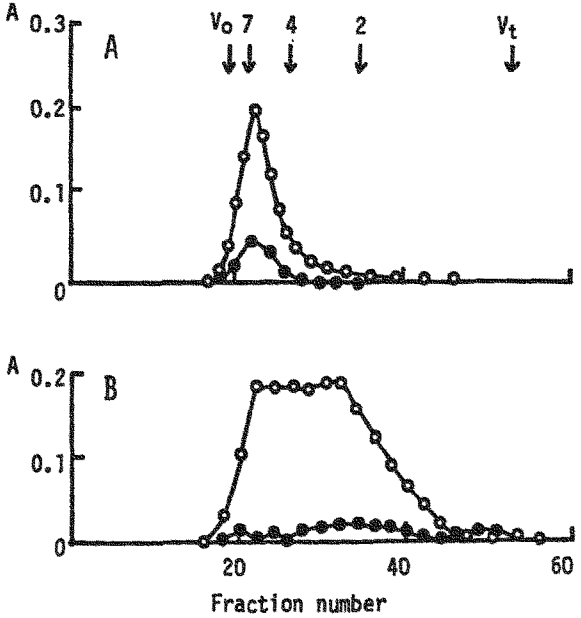

Fig. 2. Profiles of elution from a Sephadex G-100 column of the native AGP (A) and the product of reductive alkaline treatment (B). Arrows indicate mol. wts. ($\times 10^4$) estimated by markers. See legend to Fig. 1 for symbols.

TABLE V

DIFFERENCES IN CONTENT OF AMINO ACIDS IN AGP BEFORE AND AFTER REDUCTIVE ALKALI TREATMENT

Amino acid[a]	*Before*	*After*
Hydroxyproline	73	70
Serine	52	56
Threonine	45	30
Alanine	141	147
2-Aminobutanoic acid	0	9

[a]The residues of amino acids are given in μmol/g of the AGP.

that the original proportions of glycoside linkages in native AGP was nearly retained in these fractions, even after β-D-galactanase digestion, except for a slight but appreciable decrease of O-3- and O-6-linked D-galactosyl residues (data not shown). Fraction C gave, on p.c., D-galactose as the major product, with small amounts of L-arabinose and oligosaccharides. These were shown, by methylation analysis and partial degradation with *E. coli* β-D-galactosidase, to be composed mainly of (1→6)-linked β-D-galactosyl residues.

Carbohydrate–protein linkage. — The profiles of elution from a Sephadex G-100 column (Fig. 2) revealed that alkaline borohydride treatment resulted in the conversion of AGP to polydisperse forms having a reduced molecular size as a result of the cleavage, by β-elimination reaction, of the carbohydrate–protein linkage. The amino acid analysis of the products (Table V) showed a reduction of the content of threonine with a concomitant increase of the content of 2-aminobutanoic acid derived from 2-amino-2-butenoic acid, which was produced by the reductive alkali cleavage of the threonine-*O*-glycosyl linkage of AGP. The proportions of serine and other amino acids remained essentially unaltered. It can be inferred from these results that the polysaccharide component of seed AGP is *O*-glycosyl linked to a threonyl residue in the polypeptide chain, although other alkali-resistant linkages, such as *O*-galactosyl–hydroxyproline[23] and *O*-arabinosyl–hydroxyproline[26,27] cannot be ruled out. The *O*-glycosyl linkage between carbohydrate and protein is apparently a structural feature of AGPs present in the tissues of cruciferous plants, as this type of linkage was observed[4,28] in radish and rape leaf AGPs, in which the seryl instead of the threonyl residue was shown to be involved.

Precipitation reactions. — Double-diffusion on agar plate was carried out to examine the precipitation reactions of AG and AGP with rabbit anti-radish-leaf AGP antisera, eel anti-H precipitin, *A. aurantia* L-fucose-specific lectin, and Yariv antigen. AG gave negative results with all the reagents tested, a result that reflect its structure rich in L-arabinofuranosyl residues in the peripheral regions. In contrast, AGP failed to react with the L-fucose-specific lectins, but formed clear precipitin lines with the anti-radish-leaf AGP sera and β-D-glucosyl Yariv antigen. The ability of AGP to react with the antisera provided a serological evidence to

support the presence of common antigenic groups in leaf and seed AGPs, probably the side chains consisting of (1→6)-linked β-D-galactopyranosyl residues, which were sufficiently exposed to bind to the antibody[5]. Further, as with leaf AGPs and their enzymically and chemically modified products, the antigenic structure of seed AGP that bind to the antisera was not modified after α-L-arabinofuranosidase digestion and carboxyl reduction; Smith degradation of the AGP, however, afforded an unreactive product that had lost a large part of the side chains. Regardless of the structures of polysaccharide components, the precipitation reaction with the Yariv antigen occurred with both native AGP and its derivative still carrying the protein portion. This suggests that the binding sites of the β-D-glucosyl dye was localized specifically in the polypeptide portion, which is consistent with the result obtained with *Acacia senegal* AGP[29]. Failure of AGP to bind to eel anti-H precipitin and *A. aurantia* lectin provided an additional indication that seed AGP is devoid of L-fucose, a serological marker[5] of leaf AGPs.

In conclusion, a high-M_r AG and a low-M_r AGP, both of which contain D-xylose and uronic acids as minor sugar constituents, have been isolated from radish seeds and characterized to be L-arabino-3,6-D-galactan of Aspinall's type II (ref. 30) having branched α-L-arabinofuranosyl residues attached to the side chains. Apart from their content of D-xylose, radish seed AG and AGP appear to be related to *Brassica napus* high-M_r (refs. 31, 32) and *B. campestris* AG (refs. 33, 34), respectively, in the physical properties, sugar compositions, and glycoside linkages.

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REFERENCES

1 A. E. Clarke, R. L. Anderson, and B. A. Stone, *Phytochemistry*, 18 (1979) 521–540.
2 G. B. Fincher, B. A. Stone, and A. E. Clarke, *Annu. Rev. Plant Physiol.*, 34 (1983) 47–70.
3 K. Nakamura, Y. Tsumuraya, Y. Hashimoto, and S. Yamamoto, *Agric. Biol. Chem.*, 48 (1984) 753–760.
4 Y. Tsumuraya, Y. Hashimoto, S. Yamamoto, and N. Shibuya, *Carbohydr. Res.*, 134 (1984) 215–228.
5 Y. Tsumuraya, K. Nakamura, Y. Hashimoto, and S. Yamamoto, *Agric. Biol. Chem.*, 48 (1984) 2915–2917.
6 E. Uesaka, M. Sato, M. Raiju, and A. Kaji, *J. Bacteriol.*, 133 (1978) 1073–1077.
7 I. Kusakabe, T. Yasui, and T. Kobayashi, *Nippon Nogei Kagaku Kaishi*, 51 (1977) 439–448.
8 N. Kochibe and K. Frukawa, *Biochemistry*, 19 (1980) 2841–2846.
9 K. I. Kivirikko, O. Laitinen, and D. J. Prockop, *Anal. Biochem.*, 19 (1967) 249–255.
10 C. C. Sweeley, R. Bentley, M. Makita, and W. W. Wells, *J. Am. Chem. Soc.*, 85 (1963) 2497–2507.

11 O. WESTPHAL AND K. JANN, *Methods Carbohydr. Chem.*, 5 (1965) 83–91.
12 S. HAKOMORI, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
13 P. A. SANDFORD AND H. E. CONRAD, *Biochemistry*, 5 (1966) 1508–1517.
14 P.-E. JANSSON, L. KENNE, H. LIEDGREN, B. LINDBERG, AND J. LÖNNGREN, *Chem. Commun., Univ. Stockholm*, (1976) 1.
15 D. P. SWEET, R. H. SHAPIRO, AND P. ALBERSHEIM, *Carbohydr. Res.*, 40 (1975) 217–225.
16 P. FLEURY AND J. LANGE, *J. Pharm. Chim.*, 17 (1933) 107–113, 196–208.
17 I. J. GOLDSTEIN, G. W. HAY, B. A. LEWIS, AND F. SMITH, *Methods Carbohydr. Chem.*, 5 (1965) 361–370.
18 R. L. TAYLOR AND H. E. CONRAD, *Biochemistry*, 11 (1972) 1383–1388.
19 F. DOWNS AND W. PIGMAN, *Methods Carbohydr. Chem.*, 7 (1976) 200–204.
20 J. S. GARVEY, N. E. CREMER, AND D. H. SUSSDORF, *Methods Immunol.*, (1977) 313–327.
21 J. YARIV, M. M. RAPPORT, AND L. GRAF, *Biochem. J.*, 85 (1962) 383–388.
22 P. A. GLEESON AND A. E. CLARKE, *Biochem. J.*, 181 (1979) 607–621.
23 G. B. FINCHER, W. H. SAWYER, AND B. A. STONE, *Biochem. J.*, 139 (1974) 535–545.
24 B. D. E. GAILLARD, N. S. THOMPSON, AND A. L. MORAK, *Carbohydr. Res.*, 11 (1969) 509–519.
25 I. R. SIDDIQUI AND P. J. WOOD, *Carbohydr. Res.*, 17 (1971) 97–108.
26 T. YAMAGISHI, K. MATSUDA, AND T. WATANABE, *Carbohydr. Res.*, 50 (1976) 63–74.
27 G.-J. VAN HOLST AND F. M. KLIS, *Plant Physiol.*, 68 (1981) 979–980.
28 K. OGURA, Y. TSUMURAYA, Y. HASHIMOTO, AND S. YAMAMOTO, *Agric. Biol. Chem.*, 49 (1985) 2851–2857.
29 Y. AKIYAMA, S. EDA, AND K. KATÔ, *Agric. Biol. Chem.*, 48 (1984) 235–237.
30 G. O. ASPINALL, in F. A. LOEWUS (Ed.), *Biogenesis of Plant Cell Wall Polysaccharides*, Academic Press, New York, 1973, pp. 95–115.
31 O. LARM, O. THEANDER, AND P. ÅMAN, *Acta Chem. Scand., Ser. B*, 29 (1975) 1011–1014.
32 O. LARM, O. THEANDER, AND P. ÅMAN, *Acta Chem. Scand., Ser. B*, 30 (1976) 627–630.
33 I. R. SIDDIQUI AND P. J. WOOD, *Carbohydr. Res.*, 24 (1972) 1–9.
34 S. C. CHURMS, A. M. STEPHEN, AND I. R. SIDDIQUI, *Carbohydr. Res.*, 94 (1981) 119–122.