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STRUCTURE OF L-ARABINO-D-GALACTAN-CONTAINING GLYCOPROTEINS FROM RADISH LEAVES

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ABSTRACT

Two L-arabino-D-galactan-containing glycoproteins having a potent inhibitory activity against eel anti-H agglutinin were isolated from the hot saline extracts of mature radish leaves and characterized to have a similar monosaccharide composition that consists of L-arabinose, D-galactose, L-fucose, 4-*O*-methyl-D-glucuronic acid, and D-glucuronic acid residues. The chemical structure features of the carbohydrate components were investigated by carboxyl group reduction, methylation, periodate oxidation, partial acid hydrolysis, and digestion with exo- and endo-glycosidases, which indicated a backbone chain of (1→3)-linked β-D-galactosyl residues, to which side chains consisting of α-(1→6)-linked D-galactosyl residues were attached. The α-L-arabinofuranosyl residues were attached as single nonreducing groups and as O-2- or O-3-linked residues to O-3 of the β-D-galactosyl residues of the side chains. Single α-L-fucopyranosyl end groups were linked to O-2 of the L-arabinofuranosyl residues, and the 4-*O*-methyl-β-D-glucopyranosyluronic acid end groups were linked to D-galactosyl residues. The *O*-α-L-fucopyranosyl-(1→2)-α-L-arabinofuranosyl end-groups were shown to be responsible for the serological, H-like activity of the L-arabino-D-galactan glycoproteins. Reductive alkaline degradation of the glycoconjugates showed that a large proportion of the polysaccharide chains is conjugated with the polypeptide backbone through a 3-*O*-D-galactosylserine linkage.

INTRODUCTION

In their pioneering studies on the blood-group active substances in the plant

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kingdom, Springer *et al.*[1-3] have described complex polysaccharides having a potent H-activity in the aqueous extracts of twigs of the Japanese yews, *Taxus cuspidata* and *Sassafras albidum*. Subsequent chemical and immunological investigations demonstrated that, instead of the nonreducing, terminal L-fucosyl group of human and porcine H-active substances[4], 2-*O*-methyl-L-fucosyl or 3-*O*-methyl-D-galactosyl groups were the serologically active monosaccharides in the plant H-active polysaccharides that exhibit potent-inhibitory activity against hemagglutination of human O erythrocytes with heterologous anti-H agglutinins from eel sera. Recently, Yamamoto *et al.*[5-7] have presented serological data showing a widespread occurrence of blood-group A-, B-, and H-like substances in extracts of seeds, fruits, and leaves of various angiosperms. Several glycoconjugates having blood-group H-like activity were isolated from the leaves of cruciferous plants, such as radish, turnip, and rape, and characterized to be L-fucose-containing arabinogalactan glycoproteins[8] (AGPs). In the present paper, we report the chemical structure of the carbohydrate component of radish-leaf AGPs on the basis of chemical and enzymic analysis, and provide evidence for the involvement of an *O*-glycosyl linkage between carbohydrate and polypeptide chains.

EXPERIMENTAL

Materials. — Two AGPs (R-I and R-II) reacting with eel anti-H agglutinin were isolated from hot, buffered saline extracts of mature leaves of a radish, *Raphanus sativus* var. *hortensis* cv. Aokubi, as described previously[8]. α-L-Fucosidase from *Charonia lampas* was obtained from Seikagaku Kogyo Co., Ltd. (Chūo-ku, Tokyo), β-D-galactosidase (EC3.2.1.23) (grade VIII) of *Escherichia coli* from Sigma Chemical Co. (St. Louis, MO 63175, U.S.A.), Pronase from Kaken Chemical Co., Ltd. (Tokyo), and Driselase from Kyowa Hakko Kogyo Co., Ltd. (Tokyo). L-Araban was prepared from sugar-beet extracts[9]. α-L-Arabinofuranosidase (EC3.2.1.55) (70 units/mg of protein as assayed with sugar-beet L-araban as a substrate) was purified from the culture filtrate of *Rhodotorula flava* grown on L-araban-supplemented medium according to the method of Uesaka *et al.*[10]. A partially purified β-D-galactanase was obtained from Driselase (a commercial product of cellulase) by chromatography on a DEAE-cellulose column[11], which effectively eliminated contaminating β-D-galactosidase and α-L-fucosidase. After chromatography on a CM-cellulose column, the β-D-galactanase preparation was utilized for structural analysis of the radish AGPs.

Analytical methods. — Reducing sugars were quantitatively determined by the Nelson–Somogyi method[12]. The content of total sugar, uronic acid, nitrogen, and protein was estimated by methods described in the previous paper[8]. Sugars were qualitatively determined by paper chromatography (p.c.) on Whatman No. 1 and 3MM papers in (*A*) 6:4:3 1-butanol–pyridine–water and (*B*) upper layer of 4:1:5 1-butanol–acetic acid–water, and by paper electrophoresis as described previously[8]. For the detection of nonreducing sugars, the paper chromatograms were

air-dried and sprayed with 1% sodium metaperiodate in 50% aqueous acetone prior to revelation of the spots with alkaline silver nitrate. The conditions and equipment used for g.l.c., g.l.c.–m.s., ^{1}H-n.m.r. spectrometry, amino acid analysis, analytical gel-permeation chromatography, and de-ionization were described in a previous paper[8].

Hemagglutination inhibition was assayed by a serial, 2-fold dilution of H-active substances in 14.5mM phosphate buffer, pH 7.2, containing 0.13M NaCl (PBS) or 0.85% NaCl (saline), plus a constant amount of eel anti-H agglutinin. The H-activity of the samples was determined by the addition of human O erythrocytes in saline and expressed as the minimum concentration (μg/mL), or the reciprocal value (expressed as 2^n), of the lowest dilution causing a complete inhibition of hemagglutination.

Methylation analysis. — Dried samples (1–5 mg) of the AGPs or their derivatives were methylated in dimethyl sulfoxide (2 mL) by the method of Hakomori[13], the methylsulfinyl anion being prepared according to Conrad[14]. Each methylated polysaccharide was hydrolyzed with 8% H_2SO_4 for 4 h at 100° and the hydrolyzate made neutral[15] with $BaCO_3$. The permethylated oligosaccharides were extracted into chloroform and purified by passage through a Sephadex LH-20 column (1 × 30 cm). After elution with 2:1 chloroform–methanol, the oligosaccharides were hydrolyzed with 2M trifluoroacetic acid for 4 h at 100°, and the methylated monosaccharides were analyzed as alditol acetates by g.l.c. in a column (3 mm × 2 m) of 3% ECNSS-M at 180° or in a glass-capillary column (0.28 mm × 50 m) coated with G-SCOT and Silar-10C at 180°. The partially methylated alditol acetates were identified by g.l.c.–m.s. in a column of 3% ECNSS-M programmed from 150 to 180° (5°/min) or 3% OV-17 programmed from 200 to 250° (5°/min). The molar response of each methylated sugar was corrected according to Sweet *et al.*[16].

Periodate oxidation. — The oxidation of samples (100–200 mg) with 50mM sodium metaperiodate (100 mL) was carried out in the dark at 4°. Formic acid produced and periodate consumed were determined, at time intervals, by titration with 10mM NaOH and the procedure of Fleury and Lange[17], respectively. Liberation of formic acid reached a plateau after 96 h and the reaction was terminated by addition of 1,2-ethanediol (2 mL). After dialysis, Smith degradation[18] of the oxidized and $NaBH_4$-reduced products was performed[19] with M trifluoroacetic acid (10 mL) for 48 h at 25°. Examination of aliquots (0.7 mg) of the hydrolyzates on a Sephadex G-75 column (1 × 80 cm) showed no further decrease of M_r values after 48 h. The acid-resistant glycans were recovered by precipitation with methanol (to 75%) and repeatedly subjected to periodate oxidation, reduction, and mild acid hydrolysis. Chromatography on a column (2 × 10 cm) of Dowex 1 (HCO_2) anion-exchange resin separated the methanol-soluble products into neutral and acid fractions. The neutral fraction was chromatographed on a Bio-Gel P-2 column (1 × 150 cm), and the oligosaccharides were purified by preparative p.c. and characterized.

Reduction[20] of the carboxyl groups of AGPs. — The sample (10 mg) was dissolved in 0.14M 2-(*N*-morpholino)ethanesulfonic acid (5 mL, pH 4.75), and solid

3-(3-dimethylaminopropyl)-1-ethylcarbodiimide (70 mg) was added. The pH of the mixture was maintained at 4.75 with 0.1M HCl. After 1 h, additional carbodiimide was added, followed 1 h later by 2M $NaBH_4$ (12 mL), the pH being maintained at 7. The mixture was kept overnight and the excess of hydride decomposed with 6M acetic acid. The carboxyl-reduced products were dialyzed against water and lyophilized.

Enzymic degradation. — AGP R-II (40 mg) was treated with *R. flava* α-L-arabinofuranosidase (5 units) in 0.01M citrate–phosphate buffer, pH 3.0 (10 mL) for 20 h at 37°. Liberation of reducing sugars in aliquots (20 μL) was monitored colorimetrically at time intervals, and a small portion of the final product examined for sugar by p.c. Chromatographic separation of the digest on a Sephadex G-75 column (3 × 80 cm) gave a polysaccharide fraction that was lyophilized and characterized. The enzyme-modified AGP R-II (10 mg) was degraded with β-D-galactanase (170 μg) in 50mM acetate buffer (pH 4.0, 4 mL), for 24 h at 37° to give a mixture of various oligosaccharides that were resolved by gel-permeation chromatography on a Sephadex G-75 column (2 × 80 cm) and by preparative p.c., and examined for content of sugar and protein, and serological activity.

Samples (0.5 mg each) of poly- and oligo-saccharides were treated at 37° with *E. coli* β-D-galactosidase (10 μg) in 25mM phosphate buffer, (pH 7.2, 0.3 mL) for 5 h, or with *C. lampas* α-L-fucosidase (5 μg) in 25mM acetate buffer, pH 4, containing 0.15M NaCl (0.2 mL) for 20 h.

The AGPs (2 mg each) were digested with Pronase (0.5 mg) in 50mM Tris · HCl buffer (pH 7.8, 1 mL), in the presence of 10mM $CaCl_2$ for 24 h at 37°, and the resulting digests were chromatographed on a Sepharose CL-6B column.

Partial acid hydrolysis. — A sample (5 mg) of AGP R-II treated with α-L-arabinofuranosidase was hydrolyzed with 50mM trifluoroacetic acid (1 mL) at 100°, and aliquots (0.1 mL) of the hydrolyzate were removed, at time intervals over a period of 3 to 140 min, cooled, and precipitated with 80% ethanol. The precipitate was washed with ethanol and dried *in vacuo*, and the ethanol-soluble fraction was evaporated to dryness and analyzed by p.c. Both fractions were monitored for sugar content and serological activity.

Identification of aldobiouronic acid. — An aldobiouronic acid (13 mg) having R_{Glc} 0.31 (solvent *B*) and $[\alpha]_D^{20}$ +10° (*c* 0.3, water) was isolated from the hydrolyzate of the AGPs (300 mg) upon hydrolysis[8] with M H_2SO_4 for 4 h at 100°, and was shown to consist of D-galactose and 4-*O*-methyl-D-glucuronic acid by p.c. after hydrolysis with 2 M HCl for 8 h at 100°. Methylation and reduction[21] of the uronic acid carboxyl groups of the aldobiuronic acid gave a product that was remethylated and hydrolyzed, and the sugar constituents obtained were converted into the corresponding alditol acetates. Analysis by g.l.c. confirmed the presence of 2,3,4,6-tetra-*O*-methyl-D-glucose and 2,3,4-tri-*O*-methyl-D-galactose in the molar ratio of 1.0:0.9, indicating a 6-*O*-(4-*O*-methyl-D-glucopyranosyluronic acid)-D-galactose structure. The ^{1}H-n.m.r. spectrum of the disaccharide alditol showed a signal at δ 4.52 ($J_{1,2}$ 7.6 Hz) attributable to H-1 of the β-D-glucopyranosyluronic group. Based

on these results, the disaccharide was identified as 6-*O*-(4-*O*-methyl-β-D-glucopyranosyluronic acid)-D-galactose (lit.[22] $[\alpha]_D^{20}$ +3°).

Cleavage of alkali-labile carbohydrate-protein linkages. — A solution of AGP (10 mg) in 0.5M NaOH (1 mL) containing 0.5 M $NaBH_4$ was incubated for 24 h at 25°. The excess of hydride was decomposed with 6M acetic acid, and borate ions were removed as methyl borate. A portion of the product was chromatographed on a column of Sepharose CL-6B equilibrated with PBSN (PBS plus 0.02% NaN_3) in order to examine the effect of alkaline borohydride treatment on the sample. An elution profile of sugar, protein, and serological activity revealed that M_r of the AGP was reduced markedly by the β-elimination reaction[23]. The remainder of the sample was de-ionized by passage through a Bio-Gel P-2 column, hydrolyzed, and analyzed for amino acid composition. For the detection of the alditol derived from the reducing terminal sugar, the alkaline-reduced AGP (10 mg) was hydrolyzed with M H_2SO_4 for 4 h at 100°, the solution cooled, D-xylitol added as an internal standard, and the mixture made neutral ($BaCO_3$). After passage through a column of Dowex 50W (H^+) cation-exchange resin, the hydrolyzate was applied onto a column (1.2 × 1.5 cm) of Dowex 1-X2 (OH^-) anion-exchange resin. Elution with water separated the reducing sugars[24] from the alditol, which was analyzed by g.l.c. after acetylation.

RESULTS AND DISCUSSION

Properties of AGPs. — Two AGPs (R-I and R-II) have been purified to apparent homogeneity from extracts of mature radish leaves by ethanol precipitation, chromatography on DEAE-cellulose, and gel-permeation chromatography. The AGPs have apparent M_r values of 130 000 and 75 000 and are composed of 91 and 98% carbohydrate, and 10 and 3% protein, respectively[8]. Analysis by g.l.c. of the carboxyl-reduced AGPs indicated L-fucose, L-arabinose, D-galactose, 4-*O*-methyl-D-glucuronic acid, and D-glucuronic acid in the molar proportions of 3.8:27.8:58.0:8.6:1.8 for R-I, and 5.6:33.0:56.9:3.7:0.8 for R-II, respectively. The polypeptides of R-I and R-II are similar and rich in hydroxyproline, alanine, serine, and threonine, which correspond to two-thirds of the total amino acid content. A characteristic feature is the presence of L-fucose, which has been rarely found in glycoproteins isolated from various plants[25,26], and is apparently responsible for the serological activity[8].

Chemical structure of carbohydrate component.— The purified AGPs, R-I and R-II, and their carboxyl-reduced derivatives were methylated and the hydrolyzates analyzed by g.l.c.–m.s. (Table I). The carbohydrate components of R-I and R-II (columns A and D) contain the same linkages in a similar proportion. The preponderance of 2,4,6-tri-, 2,3,4-tri-, and 2,4-di-*O*-methyl-D-galactose suggests an inner structure of O-3- and O-6-linked D-galactopyranosyl residues with (1→3,6) branching. A small proportion of 2,3,4,6-tetra-*O*-methyl-D-galactose suggests some nonreducing terminal D-galactopyranosyl groups. A preponderance of 2,3,5-tri-*O*-

TABLE I

METHYLATION ANALYSIS OF NATIVE AND MODIFIED AGPs R-I AND R-II

Sugar component[a]	*Mode of linkage*	T[b]	T[c]	*Proportions (mol/100 mol)*								
				R-I[d]			*R-II*[d]					
				A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*
2,3,5-Me_3-L-Ara	L-Ara*f*-(1→	0.52	0.55	21	12	3	29	[e]	19	5		
2,3,4-Me_3-L-Fuc	L-Fuc*p*-(1→	0.65	0.67	6	4		5	6	6			
3,5-Me_2-L-Ara	→2)-L-Ara*f*-(1→	0.96	0.93	4	3		6	5	4			
2,3,4,6-Me_4-D-Glc	D-Glc*p*-(1→	1.00	1.00		10				6			
2,3,4,6-Me_4-D-Gal	D-Gal*p*-(1→	1.24	1.21	6	4	24	3	9	3	22	26	12
2,3-Me_2-L-Ara	→5)-L-Ara*f*-(1→	1.35	1.31	3	2		2		1			
2,4,6-Me_3-D-Glc	→3)-D-Glc*p*-(1→	1.93	1.88		2				1			
2,4,6-Me_3-D-Gal	→3)-D-Gal*p*-(1→	2.27	2.04	17	8	40	15	9	12	35	33	81
2,3,4-Me_3-D-Gal	→6)-D-Gal*p*-(1→	3.37	3.17	16	20	10	11	34	12	15	17	
2,4-Me_2-D-Gal	→6)- →3)-D-Gal*p*-(1→	6.26	5.67	27	35	23	29	16	36	23	21	7
Free L-arabinose[f]								21			3	

[a]Determined as *O*-acetylalditol derivatives. [b]Retention time relative to 2,3,4,6-Me_4-D-Glc on 3% ECNSS-M column. [c]As *b*, on Silar-10C column. [d]A, Native; B, carboxyl-reduced; C, polymer fractions of 1st Smith degradation, D, native; E, α-L-arabinosidase digest; F, carboxyl-reduced; G, polymer fraction of 1st Smith degradation; H, α-L-arabinosidase digest of G; and I, polymer fraction of 2nd Smith degradation. [e]Not detectable. [f]Reducing sugar in reaction mixture.

methyl-L-arabinose showed that the majority of L-arabinose residues was located as nonreducing groups in the furanose form. Small proportions of 3,5- and 2,3-di-*O*-methyl-L-arabinose revealed the presence of some interchain L-arabinofuranosyl residues substituted at O-2 or-5. The L-fucopyranosyl residues, a minor sugar constituent, were detected exclusively as 2,3,4-tri-*O*-methyl-L-fucose, and obviously originated from nonreducing end groups. The location of 4-*O*-methyl-D-glucopyranosyluronic and D-glucopyranosyluronic acid residues as nonreducing end groups was confirmed by the detection of 2,3,4,6-tetra-*O*-methyl-D-glucose after methylation of carboxyl-reduced R-I and R-II (Table I, columns B and F). Further, the presence of a small proportion of 2,4,6-tri-*O*-methyl-D-glucose (1–2% of total methylated sugars) can be ascribed to pyranosyluronic residues substituted at O-3. The increase of the proportion of 2,3,4-tri-*O*-methyl-D-galactose after carboxyl-group reduction most probably resulted from the D-galactosyl residues substituted at O-6 by the pyranosyluronic residues.

The resistance to acid of the linkage involving pyranosyluronic residues in native AGPs allowed the isolation of 6-*O*-(4-*O*-methyl-β-D-glucopyranosyluronic acid)-D-galactose from the hydrolyzate.

In the periodate oxidation of R-I and R-II, 0.93 and 0.70 mol of periodate were consumed with concomitant production of 0.33 and 0.32 mol of formic acid per sugar residue, respectively. Reduction of the oxidized polymers gave the polyalcohols in 85 and 87% yield, respectively. Upon acid hydrolysis of the polyalcohol, D-galactose and glycerol (and a small proportion of L-arabinose) were detected as the main products by p.c. and g.l.c., in the molar ratios of 1.0:0.5:0.1 for R-I, and 1.0:0.4:0.12 for R-II. Degradation of the glucosyluronic groups (80–90% of total uronic acids) by the periodate oxidation was confirmed by colorimetric determination. Glycerol may have arisen from the nonreducing terminal groups, as well as O-5-linked L-arabinosyl and O-6-linked D-galactopyranosyl residues,

TABLE II

CHARACTERIZATION OF POLYMERS AFTER FIRST AND SECOND SMITH DEGRADATION OF AGPs R-I AND R-II

Properties	*R-I*	*R-II*	
	1st	*1st*	*2nd*
Yield (% of parent polymer)	29	24	49
$[\alpha]_D^{20}$ (*c* 0.5–0.6, water) (degrees)	−22	−19	−12
Kjeldahl N (%)	[a]	2.05	2.77
M_r	19 000	29 000	21 000
Monosaccharide composition (mol/100 mol)			
L-Arabinose	10	7.2	
D-Galactose	90	92.8	100
^{1}H-N.m.r. spectrum (δ)		4.40–4.70, 5.24 ($J_{1,2}$ low)	4.63–4.68

[a]Not determined.

whereas O-2-linked L-arabinofuranosyl, O-3-linked D-galactopyranosyl, and D-galactopyranosyl residues at branching points remained unoxidized.

Oxidized R-I and R-II were precipitated with 75% methanol and R-II subjected to a second Smith degradation (see Table II). The recovery of methanol-insoluble products was 29% for R-I (one degradation), and 24 and 49% for R-II (first and second degradation, respectively). Each degradation product yielded a single carbohydrate peak by gel-permeation chromatography on a Sepharose CL-6B column. The marked reduction in the M_r value of these products could be accounted for by the elimination of sugar residues in the peripheral region of the native AGPs, although the existence of a subunit structure, such as those proposed for the arabinogalactans of exudates from *Acacia* species[19], cannot be ruled out. The Smith degradation resulted also in an increase of the nitrogen content (see Table II), as compared to the original value[8] (0.77%). Methylation of the twice-degraded R-II (Table I, column I) indicated a structure consisting mainly of (1→3)-linked D-galactopyranosyl residues with small proportions of D-galactosyl residues as nonreducing end groups and O-3,6-branched residues. As shown by the ^{1}H-n.m.r. spectrum (Table II) the D-galactopyranosyl residues are in the β configuration. Thus, the first degradation of R-II afforded a product that was a β-D-galactan containing L-arabinose as a minor constituent, and the structure of which resembled closely that of the first degradation product of R-I (see methylation analysis, Table I, columns C, G): a β-D-(1→3)-galactan with nonreducing terminal L-arabinofuranosyl groups and O-6-linked D-galactosyl residues. The α-L configuration of the arabinofuranosyl residues was assigned on the basis of the ^{1}H-n.m.r. spectrum[27] and the susceptibility to α-L-arabinofuranosidase.

Low-molecular-weight products were recovered in the 75% methanol-soluble fraction in the respective yields of 40 and 46% after one degradation of R-I and

TABLE III

CHARACTERIZATION OF OLIGOSACCHARIDES FROM THE SMITH-DEGRADATION PRODUCTS OF AGPs

Properties	*Oligosaccharide*		
	1	**2**	**3**
Yield (%)[a]	7.8	3.8	2.6
$[\alpha]_D^{20}$ (c 0.3, water) (degrees)	+2.4	0	[b]
R_{Glc} (solvent A)	0.71	0.17	0.04
M_{Glc}	0.47	0.47	[b]
Sugar composition (molar ratio)			
D-Galactose	1.0	2.0	3.0
Glycerol	0.8	1.0	0.7
Methylated sugar (molar ratio)			
2,3,4,6-Me_4-D-Gal	only	1.0	1.0
2,3,4-Me_3-D-Gal		1.1	2.1
β-D-Galactosidase digest	D-Gal + glycerol	D-Gal + glycerol	D-Gal + glycerol

[a]Not determined. [b]Percentage of methanol-soluble fraction.

1
2
3
4
5
6
α-L-Araf
α-L-Fucp

R-II, and 43% after two degradations of R-II. The sugar content of the two products from R-II was 23 and 14%, respectively, and the product of the first degradation contained oligosaccharides besides a large proportion of glycerol. After Bio-Gel P-2 chromatography and preparative p.c., three oligosaccharides were obtained (Table III). The first oligosaccharide (**1**), a major component, migrated as a single spot on p.c., and paper electrophoresis, and was composed of an equimolar amount of D-galactose and glycerol. Methylation analysis, susceptibility to β-D-galactosidase, and a signal at δ 4.41 ($J_{1,2}$ 6.9 Hz) in the ^{1}H-n.m.r. spectrum suggested a β-D-galactopyranosyl group and structure **1** for the oligosaccharide. Similarly, structures **2** and **3** were assigned to the other two oligosaccharides. Similar oligosaccharides were found by p.c. in the methanol-soluble fractions obtained from the other products of degradation. These results suggest that a part of the side chains of the AGPs is composed of one to three consecutive β-(1→6)-D-galactosyl residues substituted at O-3 with sugar residues susceptible to periodate oxidation, and these sequence are located between unbranched, O-6-substituted β-D-galactopyranosyl residues (**4**).

Action of enzymes. — α-L-Arabinofuranosidase hydrolyzed 21 and 3% of R-II and the product obtained after the first degradation, respectively (Table I, columns E and H). Methylation analysis of the enzyme-modified R-II showed the complete removal of the nonreducing terminal and O-5-linked L-arabinofuranosyl residues, an increase of the nonreducing terminal and O-6-linked D-galactopyranosyl residues, a decrease of the O-3- and O-3,6-linked D-galactopyranosyl residues, and no change of O-2-linked L-arabinofuranosyl residues. A slight but significant alteration in the molar proportion of glycosidic linkages was observed with the Smith-degraded R-II (Table I, columns G and H). From the results of enzymic and Smith degradations, partial structure **5** could be

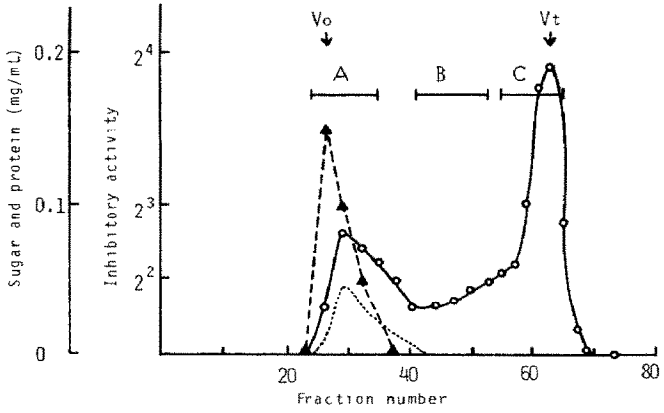

Fig. 1. Separation, on a column of Sephadex G-75, of the reaction products of AGP R-II after β-D-galactanase digestion: (—○—○—) carbohydrate, (------) protein, and (—▲—▲—) hemagglutination-inhibition activity. Arrows represent elution peaks of Blue Dextran (V_o) and D-glucose (V_t). Fractions A, B, and C were pooled as indicated.

deduced. This branched structure **5** might primarily represent the side chains that are attached to the β-(1→3)-D-galactan.

No measurable, reducing sugar was released by digestion of enzyme-modified R-II with β-D-galactosidase or α-L-fucosidase, and the serological activity remained unaffected.

R-II was extensively degraded with β-D-galactanase to give a mixture of D-galactose and oligosaccharides (apparent conversion into D-galactose, 14%), which were resolved into several components (Fig. 1). The higher-molecular-weight saccharides in Fraction A (28% of total sugars) contained L-fucose, L-arabinose, D-galactose (in the molar ratio of 15:38:100), and uronic acid. This fraction alone exhibited a potent H-like activity and contained almost the entire polypeptide of R-II. Fraction B (32% of total sugars) contained a mixture of oligosaccharides consisting of L-fucose, L-arabinose, and D-galactose (in a molar proportion of 7:7:100).

Fraction C (40% of total sugars) was composed of L-fucose, L-arabinose, D-galactose (in a molar ratio of 1:1:25) and of uronic acid, and gave two major oligosaccharides (C-I and C-II) having R_{Glc} 0.26 and 0.05 (solvent *A*). Based on methylation analysis and action of β-D-galactosidase, C-I and C-II were identified as *O*-β-D-galactopyranosyl-(1→6)-D-galactopyranose, and *O*-β-D-galactopyranosyl-(1→6)-*O*-β-D-galactopyranosyl-(1→6)-galactopyranose, respectively. Isolation of these di- and tri-saccharides suggests that the β-D-galactanase from Driselase splits off the O-6-linked β-D-galactosyl residues of the side chain, but the susceptibility of the O-3-linked D-galactosyl residues is still unclear. A third, higher oligosaccharide (C-III) migrated very little on p.c., and was hydrolyzed by α-L-fucosidase to give Fraction C-III-F.

Both the native and digested samples were subjected to methylation analysis (see Table IV). Fraction C-III-F showed a decrease in nonreducing terminal L-fucopyranosyl groups and of O-2-linked L-arabinosyl residues, and the appearance

TABLE IV

METHYLATION ANALYSES OF CARBOHYDRATES ISOLATED FROM THE β-D-GALACTANASE DIGEST OF AGP R-II

Sugar component[a]	*Mode of linkage*	*Proportion (mol/100 mol)*			
		A	*B*	*C-III*	*C-III-F*
2,3,5-Me_3-L-Ara	L-Ara*f*-(1→				4
2,3,4-Me_3-L-Fuc	L-Fuc*p*-(1→	11	6	5	2
3,5-Me_2-L-Ara	→2)-L-Ara*f*-(1→	15	6	4	1
2,3,4,6-Me_4-D-Gal	D-Gal*p*-(1→	1	3	6	6
2,4,6-Me_3-D-Gal	→3)-D-Gal*p*-(1→	6			
2,3,4-Me_3-D-Gal	→6)-D-Gal*p*-(1→	37	78	82	84
2,4-Me_2-D-Gal	→6)- →3)-D-Gal*p*-(1→	20	7	3	3

[a]Determined as *O*-acetylalditol.

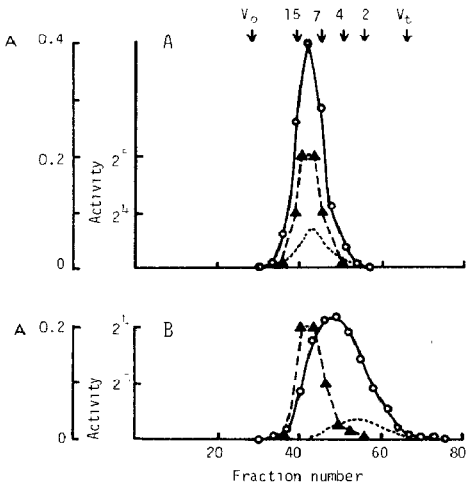

Fig. 2. Chromatography, on a column of Sepharose CL-6B, of AGP R-I (A) before, and (B) after treatment with alkaline borohydride. Arrows represent apparent M_r ($\times 10^4$) estimated by markers. See legend to Fig. 1 for symbols.

TABLE V

CHANGES IN SERINE, THREONINE, AND ALANINE CONTENT IN AGP R-I BEFORE AND AFTER TREATMENT WITH ALKALINE BOROHYDRIDE

Amino acid (Residues/100 residues)	*Before*	*After*	*Difference (%)*
Hydroxyproline	28.9	31.0	+7.3
Serine	14.8	10.9	−26.4
Threonine	8.4	8.3	−1.2
Alanine	10.7	13.0	+21.5

of nonreducing, terminal L-arabinosyl residues, indicating that the terminal α-L-fucopyranosyl groups had been linked to O-2 of the adjacent α-L-arabinofuranosyl residues. Methylation analysis of Fraction B suggested substitution at O-3 of consecutively linked β-D-(1→6)-galactosyl residues by side chains containing terminal L-fucosyl groups and O-2-linked L-arabinosyl residues (**6**). Such side chains are a common feature of the L-fucose-containing oligosaccharides and their presence was further ascertained by the isolation, after mild acid hydrolysis of α-L-arabinofuranosidase-treated R-II, exclusively of L-fucose and L-arabinose with a concomitant loss of H-like activity.

Carbohydrate–protein linkage[23]. — R-I was incubated with 0.5M NaOH containing 0.5M $NaBH_4$ for 24 h at 25°. The elution profile of alkaline sodium borohydride-treated R-I (Fig. 2) indicated high polydispersity and reduction of the M_r value suggesting the cleavage of the carbohydrate–protein linkage. The serine content was decreased by 26% and the alanine content was increased by 22%, the other

amino acids being unchanged (Table V). G.l.c. of the acetate derivatives of the acid hydrolyzate showed galactitol as the sole alditol, suggesting that D-galactosyl residues at the (potentially) reducing end of the chains (M_r ~24 000–30 000) are linked through an *O*-glycosyl bond to serine residues. Based on M_r value, amino acid analysis, and protein and serine content, four or five serine residues per peptide chain would be involved in the carbohydrate–protein linkage.

Conclusion. — From the aforementioned results, it may be concluded that the carbohydrate portion of the radish leaf AGPs could be classified, as L-arabino-(1→3,6)-D-galactan, into type II of Aspinall[28], which is represented by AGPs from wheat seeds and mucilages of *Gladiolus* and *Lilium longiflorum*[29–31]. Concerning the distribution of L-fucose among arabinogalactans and AGPs, Langhammer *et al.*[32] have reported the presence of nonreducing L-fucopyranosyl end groups (6% of total sugars) in an arabinogalactan from leaves of a desert plant, *Welwitschia mirabilis*, but the sequence of the sugar residues was not given. The present study provides evidence for a unique sugar group, α-L-fucopyranosyl-(1→2)-α-L-arabinofuranosyl, which is similar to α-L-fucopyranosyl-(1→2)-β-D-galactopyranosyl, the serological H-determinant group of blood-group H substances of animal origin[4]. The analogy between this two groups furnish an interesting model for understanding the required spatial arrangement of sugar residues for serological activity.

An O-galactosyl→serine linkage has been demonstrated in water-soluble glycoproteins from leaves of *Cannabis sativus*[33,34], and an AGP from hypocotyls of *Phaseolus vulgaris*[35], both of which also contain alkali-resistant, sugar–amino acid linkages[36]. The radish AGPs also seem mainly to contain the same linkage.

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