An Arabinogalactan-protein from Rape Leaves

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A L-fucose-containing arabinogalactan-protein that strongly inhibited hemagglutination by eel anti-H agglutinin of human O erythrocytes was purified from hot phosphate-buffered saline extracts of mature leaves of rape, *Brassica campestris*. The purified glycoconjugate consisted of 90% of the polysaccharide moiety comprising L-fucose, L-arabinose, D-galactose, 4-O-methyl-D-glucuronic acid, and D-glucuronic acid, and 4% of the hydroxyproline-rich protein portion. Upon methylation, periodate oxidation, and enzymatic degradation, we found that consecutive β -(1 $\rightarrow$ 3)-linked D-galactopyranosyl residues constituted a backbone chain of the polysaccharide moiety, to which the side chains of β -(1 $\rightarrow$ 6)-linked D-galactopyranosyl residues were attached through O-6. Most of L-arabinofuranosyl residues were linked as single units through O-3 to the side chains while a small quantity of the sugar was present as (1 $\rightarrow$ 2)-, (1 $\rightarrow$ 3)-, or (1 $\rightarrow$ 5)-linked inter-chain residues. Single residues of α -L-fucopyranose, apparently attached to (1 $\rightarrow$ 2)-linked L-arabinofuranosyl residues, reacted with eel anti-H precipitin and *Aleuria aurantia* L-fucose-specific lectin, and were assumed to be crucial in the expression of the H-like activity. The uronosyl residues were also located at the non-reducing terminal ends. Reductive alkaline degradation of the arabinogalactan-protein provided indications that the polysaccharide chains were mainly conjugated through serine-O-glycosidic linkages to the polypeptide core. In an immunoprecipitation test, the rape leaf arabinogalactan-protein cross-reacted with antisera raised against radish leaf arabinogalactan-protein, indicating that these cruciferous arabinogalactan-proteins share common immunodeterminant(s) in their molecules.

The occurrence of blood group H-active substances in the plant kingdom has first been reported by Springer *et al.*, who found the serological activity in complex polysaccharides isolated from aqueous extracts of twigs of *Taxus cuspidata* and *Sassafras albidum*, and demonstrated that 2-O-methyl-L-fucose and 3-O-methyl-D-galactose acted as the immunodeterminants, respectively.^{1,2)} In our previous work on H-like substances in leaf tissues of cruciferous plants, we have characterized several L-fucose-containing arabinogalactan-proteins (AGPs) that strongly inhibit on the agglutination of human O erythrocytes with eel anti-H agglutinin.³⁾ Subsequent structural investigations of radish leaf AGPs provided evidence that their polysaccharide moieties

could be regarded as a typical arabino-3,6-galactan having α -L-fucopyranosyl-(1 $\rightarrow$ 2)- α -L-arabinofuranosyl-(1 $\rightarrow$), as a possible immunodeterminants group for their H-like activity.^{4,5)} This paper is concerned with structural features and serological properties of a rape leaf AGP in comparison with those of radish leaf AGPs.

EXPERIMENTAL

Materials. An AGP with H-like activity was isolated from hot phosphate-buffered saline (PBS, 0.0145 M phosphate buffer, pH 7.2, containing 0.13 M NaCl) extracts of mature leaves of the rape, *Brassica campestris* L. subsp. *Napus* Hook.³⁾ An α -L-arabinofuranosidase purified from the culture filtrate of *Rhodotorula flava*⁶⁾ was provided by Dr. N. Shibuya, National Food Research Institute,

Tsukuba, Ibaraki, Japan. A partially purified preparation of a β -galactanase was prepared from Driselase, a commercial cellulase product as previously described.⁴⁾ A L-fucose-specific lectin of orange peel fungus, *Aleuria aurantia*,⁷⁾ was a generous gift of Dr. N. Kochibe, Gunma University, Gunma, Japan.

Analytical methods. The contents of total sugar, reducing sugar, uronic acid, and protein were measured colorimetrically.^{3,4)} Qualitative analysis of sugars was done by paper chromatography (PC) on Whatman No. 1 and 3MM papers in a solvent system of 1-butanol–pyridine–water (6:4:3, v/v/v). The spots of sugars on paper chromatograms were detected by alkaline silver nitrate or *p*-anisidine hydrochloride reagent. For the detection of non-reducing sugars, the paper after the run was sprayed with 1% NaIO₄ in 50% aqueous acetone before silver nitrate treatment. Analysis of sugars by gas liquid chromatography (GLC) was done in a Shimadzu GC-5A gas chromatograph with a flame ionization detector. Monosaccharides and their partially methylated derivatives were converted to their corresponding alditol acetates and separated isothermally on a glass column (3 mm $\times$ 2 m) of 3% ECNSS-M on Gas Chrom Q at 180°C or 190°C, and on a glass capillary column (0.28 mm $\times$ 50 m) of G-SCOT with Silar-10C at 180°C. The samples (1–2 mg) of the rape leaf AGP and its degradation products were hydrolyzed with M H₂SO₄ (0.6 ml) for 4 hr at 100°C. After neutralization (BaCO₃) and centrifugation, the hydrolyzates were deionized with a short column of Dowex 50W (H⁺) and analyzed by PC or GLC.

Methylation analysis. Samples (1–5 mg) of the rape AGP and its modified or degraded products were methylated in dimethylsulfoxide (2 ml) with methylsulfinyl anion and methyl iodide.⁸⁾ The methylated polysaccharides were suspended in 72% H₂SO₄ (0.1 ml), left for 1 hr at 4°C, then diluted with water (0.8 ml), and hydrolyzed by heating for 4 hr at 100°C. Hydrolysis of the permethylated oligosaccharides was made with 2 M trifluoroacetic acid (0.6 ml) for 4 hr at 100°C.

Periodate oxidation. The AGP (50 mg) was dissolved in 0.05 M NaIO₄ (50 ml) and left to react at 4°C in the dark. When the oxidation was completed after 96 hr, the formic acid produced and periodate consumed were 0.18 and 0.83 mol per sugar residue, respectively. Excess periodate was decomposed by adding 1,2-ethanediol (2 ml) and the mixture was dialyzed against water. Reduction with NaBH₄ of the oxidized product gave the corresponding glycan-polyalcohol (30.1 mg), which was used for Smith degradation⁹⁾ with M trifluoroacetic acid (4 ml). After standing for 48 hr at 25°C, the resulting products were fractionated with 75% methanol yielding a polymer (7.2 mg) and methanol-soluble sugar fraction (19.1 mg) containing 27% of total sugar. From the low-molecular-

weight fraction, oligosaccharides were separated by chromatography on a column of Dowex 1 (formate) followed by preparative PC. Three components having R_{GLC} of 0.80, 0.22, and 0.03 were obtained with the respective yields of 2.2, 0.7, and 0.3 mg.

Carboxyl-reduction. The carboxyl groups of uronic acid residues in the AGP (10 mg) were reduced by the method of Taylor and Conrad,¹⁰⁾ involving reaction with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and then reduction with NaBH₄.

Enzymatic degradation. The AGP (30 mg) was incubated under toluene with *R. flava* α -L-arabinofuranosidase (6 units) in 0.01 M citrate–phosphate buffer, pH 3.0 (4 ml) at 37°C. After incubation for 10 hr, additional enzyme (3 units) was added to the reaction mixture which was left standing for 20 more hours. When liberation of reducing sugars (25.9% as L-arabinose) reached a constant, the reaction was ended by heating in a boiling water bath for 5 min and then the mixture was cooled. The digest was deionized with Dowex 50W (H⁺) and filtered on a column (3 $\times$ 80 cm) of Sephadex G-75 gel. Elution with water from the column made it possible to separate a polymer (21 mg) from the L-arabinose released. A portion (10 mg) of the polymer was then degraded with β -galactanase (0.1 mg) in 0.05 M acetate buffer, pH 4.0 (4 ml) for 24 hr at 37°C under toluene. After heat-inactivation of the enzyme, the digest (apparent conversion to D-galactose, 16.3%) was separated on a column of Sephadex G-75 into three sugar fractions, designated I (2.5 mg), II (2.3 mg), and III (5.3 mg), which were collected in accordance with their respective partition coefficient $K_{av}((V_e - V_o)/(V_i - V_o))$ ranges of 0–0.2, 0.3–0.65, and 0.75–1.0.

Reductive alkaline degradation. The AGP (9 mg) was dissolved in 0.5 M NaOH (1 ml) containing 0.5 M NaBH₄ and incubated for 24 hr at 25°C. The reaction mixture was then treated successively with PdCl₂–HCl and alkaline borohydride by the method of Downs *et al.*,¹¹⁾ and deionized by using a column (2 $\times$ 28 cm) of Sephadex G-15. The product thus obtained was hydrolyzed for the analysis of amino acid composition.³⁾ Another sample obtained from the AGP (1.5 mg) by reductive alkaline treatment was neutralized with 6 M acetic acid, evaporated to dryness, and dissolved in water (1 ml). The change in the molecular size of the AGP was examined on a Sepharose CL-6B column (1 $\times$ 85 cm) by following the elution profiles of sugar and protein.

Serological method. The hemagglutination-inhibiting activity of the AGP and its chemically and enzymatically modified products was assayed by using serial 2-fold dilutions (25 μ l) of the samples in PBS or 0.85% NaCl (saline) plus 25 μ l of eel anti-H agglutinin or L-fucose-specific lectin (titer 1:8). The activity was measured by the addition of human O erythrocytes in saline and expressed

as the minimum concentration ($\mu\text{g/ml}$) or as the reciprocal values of the lowest dilution (2^n) causing complete inhibition of hemagglutination. The precipitation reactions of the samples against eel anti-H precipitin, rabbit anti-radiash leaf AGP sera,⁵⁾ and *A. aurantia* L-fucose-specific lectin were examined by the Ouchterlony double diffusion method¹²⁾ on a 1.5% Difco agar plate containing 0.025 M barbital buffer, pH 8.4, or PBS in the presence of 0.01% trypan blue. The diffusion was allowed to occur in moist atmosphere for 24 hr at room temperature.

RESULTS AND DISCUSSION

From hot PBS-extracts of mature rape leaves, an AGP having blood group H-like activity was purified by ethanol precipitation followed by successive chromatographic fractionations using columns of DEAE-cellulose and Sepharose 6B.³⁾ The apparent homogeneity of the purified AGP was verified by high voltage electrophoresis and ultracentrifugal analysis. The rape leaf AGP having an apparent molecular weight of 50,000 was composed of a large part (90.3%) of a polysaccharide moiety associated with a small quantity (3.8%) of hydroxyproline-rich protein.³⁾ Upon examination of the monosaccharide components by PC and GLC on a silar-10C column, one of the carboxyl-reduced products

was confirmed to be 4-*O*-methyl-D-glucose since the R_{Glc} (1.54) and T_{Glc} (0.87) of the derivative were in good agreement with those of the authentic specimen.³⁾ The monosaccharide composition of the carboxyl-reduced AGP revealed that the native AGP consisted of L-fucose, L-arabinose, D-galactose, 4-*O*-methyl-D-glucuronic acid, and D-glucuronic acid in molar proportions of 4.3:37.0:53.7:3.8:1.2. Upon testing for hemagglutination inhibition, this L-fucose-containing glycoconjugate was shown to completely inhibit the hemagglutination of human O erythrocytes by eel anti-H agglutinin at concentration of 10 $\mu\text{g/ml}$.³⁾

Results of methylation analysis of the native AGP are summarized in Table I (column A), that revealed high molar proportions of 2,4,6-tri-, 2,3,4-tri-, and 2,4-di-*O*-methyl-D-galactose, indicating that the polysaccharide moiety is a typical arabino-3,6-galactan.¹³⁾ A preponderance of 2,3,5-tri-*O*-methyl-L-arabinose, together with small amounts of 3,5-, 2,5-, and 2,3-di-*O*-methyl-L-arabinose could be taken as indications that a majority of the L-arabinofuranosyl residues were located at the non-reducing terminal end while some of the

TABLE I. METHYLATION ANALYSES OF NATIVE AGP AND ITS MODIFIED OR DEGRADED PRODUCTS

Sugar component ^a	Mode of linkage	T ^b	Molar percent					
			A	B	C	D	E	F
2,3,5-Me ₃ -L-Ara	L-Araf-(1→	0.55	23.1	17.9	7.0	1.0	— ^c	—
2,3,4-Me ₃ -L-Fuc	L-Fucp-(1→	0.67	4.0	2.9	—	5.1	18.1	11.2
3,5-Me ₂ -L-Ara	→2)-L-Araf-(1→	0.93	5.3	5.9	—	6.7	11.5	19.8
2,3,4,6-Me ₄ -D-Glc	D-Glcp-(1→	1.00	—	4.8	—	—	—	—
2,5-Me ₂ -L-Ara	→3)-L-Araf-(1→	1.09	1.0	0.9	—	—	—	—
2,3,4,6-Me ₄ -D-Gal	D-Galp-(1→	1.21	4.1	3.5	16.9	9.5	25.9	4.7
2,3-Me ₂ -L-Ara	→5)-L-Araf-(1→	1.31	6.8	6.9	—	—	—	—
2,4,6-Me ₃ -D-Gal	→3)-D-Galp-(1→	2.04	12.9	9.4	21.8	9.8	—	2.0
2,3,4-Me ₃ -D-Gal	→6)-D-Galp-(1→	3.17	10.9	12.3	32.4	26.0	35.8	46.8
2,4-Me ₂ -D-Gal	→3,6)-D-Galp-(1→	5.40	31.9	35.5	21.9	16.0	8.7	15.5
Free L-Arabinose ^d						25.9		

^a 2,3,5-Me₃-L-Ara = 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methyl-L-arabinitol, etc.

^b Retention time relative to 2,3,4,6-Me₄-D-Glc on a Silar-10C column.

^c Not detectable.

^d Reducing sugar in an α -L-arabinofuranosidase-digest of the AGP.

A, native AGP; B, carboxyl-reduced AGP; C, a polymer resistant to Smith degradation; D, an α -L-arabinofuranosidase-digested product; E, Fraction I (a β -galactanase-digested product) and F, Fraction II (a β -galactanase-digested product).

Upon digestion with α -L-arabinofuranosidase, liberation of a large amount of L-arabinose occurred from the AGP, leaving an enzyme-modified polymer. The degraded polymer was recovered by gel permeation chromatography on a Sephadex G-75 column, and was found to consist of L-fucose, L-arabinose, and D-galactose (molar percent, 7:13:80), together with uronic acid. Data of the methylation analysis of the enzyme-modified product compared with that of the native AGP in Table I (columns D and A) confirmed that the enzyme treatment was highly effective for the removal of (1 $\rightarrow$ 3)-, (1 $\rightarrow$ 5)-, or non-reducing terminal L-arabinosyl residues, resulting in a concomitant increase in molar proportions of non-reducing terminal and (1 $\rightarrow$ 6)-linked D-galactosyl residues. In contrast, molar proportions of non-reducing

terminal L-fucosyl and (1→2)-linked L-arabinosyl residues remained unaffected even after the enzymatic degradation. These results led to the assumption that L-arabinofuranosyl residues in the native AGP might be attached through O-3 to the terminal or the inter-chain β -(1→6)-linked D-galactosyl residues of the side chains (2). For further structural analysis, β -galactanase was used effectively to degrade the α -L-arabinofuranosidase-treated AGP. After prolonged digestion, the resultant products were separated on a Sephadex G-75 column into three fractions depending on their respective K_{av} ranges. Fraction III consisted of L-fucose, L-arabinose, and D-galactose (molar ratio, 0.03:0.04:1.0), and uronic acid. Analysis by PC of this fraction revealed that the major constituents were D-galactose and two galacto-oligosaccharides having R_{Glc} values (0.20 and 0.04) corresponding to those of β -D-Galp-(1→6)-D-Galp and β -D-Galp-(1→6)- β -D-Galp-(1→6)-D-Galp, which were obtained from radish leaf AGP.⁴⁾ Upon acid hydrolysis, fraction II gave L-fucose, L-arabinose, and D-galactose (molar ratio, 0.15:0.23:1.0). Structural analysis of the oligosaccharides on the whole by methylation (Table I, column F) indicates a high content of (1→6)-linked D-galactosyl residues substituted at O-3 with L-fucosyl or L-arabinosyl residues. These oligosaccharides could be fragments of intermediate size liberated from the side chains of rape leaf AGP by the action of β -galactanase. The high-molecular-weight fraction I had a monosaccharide composition of L-fucose, L-arabinose, and D-galactose (molar ratio, 0.26:0.38:1.0), and uronic acid. It is noteworthy that this fraction appears to retain a large part of the protein portion since it contains a higher proportion of protein (9%) than those of the native AGP (3.8%) and α -L-arabinofuranosidase-treated AGP (4.9%). In view of methylation analyses of these large fragments (Table I, columns E and F), it is highly probable that a common feature of their structure is a disaccharide unit of an equimolar proportion of non-reducing terminal L-fucopyranosyl and (1→2)-linked L-arabino-

TABLE II. CHANGES IN SERINE, THREONINE, AND ALANINE CONTENTS IN THE AGP BEFORE AND AFTER REDUCTIVE ALKALI TREATMENT

Amino acid	Before	After	(%)
Hydroxyproline	120.8	128.5	+ 6.4
Serine	58.1	45.8	-21.2
Threonine	40.5	38.0	- 6.2
Alanine	45.8	53.8	+17.5

The residues of amino acids are given in $\mu\text{mol/g}$ of the AGP.

furanosyl residues. This unit might be attached at O-3 to D-galactosyl residues of the side chains (3).

Reductive alkaline degradation of the AGP was done in 0.5M NaOH containing 0.5M NaBH₄ to analyze carbohydrate-protein linkages susceptible to alkaline β -elimination reaction. An elution profile of the products by gel permeation chromatography showed a large reduction in the molecular weight of the native AGP and its conversion to a polydisperse form, possibly as a result of cleavage in alkali-labile carbohydrate-protein linkages. In Table II are summarized the results of amino acid analysis that showed a decrease in serine content with an increase in conversion to alanine as a reduced β -elimination product of serine, although their stoichiometric recovery could not be obtained. Thus, this finding suggested that the polysaccharide chains of rape leaf AGP are linked through O-glycosidic linkage to serine residues in the polypeptide portion.

In a double diffusion test, rape leaf AGP reacted with eel anti-H precipitin to form a single precipitin line that fused completely with those of L-fucose-containing AGPs, purified from the leaves of radish or turnip.³⁾ This indicates that these cruciferous AGPs share a common serologically active structure involving L-fucosyl residues. Of the chemically and enzymatically modified AGPs tested, the carboxyl-reduced and α -L-arabinofuranosidase-treated rape leaf AGP maintained the serological response, apparently due to the presence of intact L-fucosyl residues.

FIG. 1. Immunodiffusion Patterns Showing the Reactions of the Native AGP and Its Modified Products against (A) Eel Anti-H Precipitin, (B) *A. aurantia* L-Fucose-Specific Lectin and (C) Rabbit Anti-radish AGP Antiserum.

The surrounding wells in each plate contained, clockwise from the top, 20 μ l of 0.5% solution in PBS of the native AGP, α -L-arabinofuranosidase-digested AGP, a product of the Smith degradation, none, saline and the carboxyl-reduced AGP. The substances diffused in a moist atmosphere for 24 hr at room temperature.

TABLE III. HEMAGGLUTINATION INHIBITION ACTIVITIES OF NATIVE AGP AND ITS MODIFIED OR DEGRADED PRODUCTS

Sample	Inhibitory activity (μ g/ml) against	
	Eel Anti-H agglutinin	<i>A. aurantia</i> lectin
Native AGP	10	40
Carboxyl-reduced AGP	20	160
α -L-Arabinosidase-digested AGP	10	80
Smith-degraded AGP	2500	1250
β -D-Galactanase-digested products		
Fraction I	20	135
Fraction II	125	190
Fraction III	5000	> 5000
L-Fucose	200	500

Titer of eel agglutinin or *A. aurantia* lectin was 1:8.

Hence, the Smith degradation product lacking L-fucose residues failed to form any precipitin line in response to eel anti-H precipitin and lost the H-like activity as shown in Fig. 1A and Table III, respectively. These serological results agree with the conclusion that the L-fucosyl residues in radish leaf AGPs are essential to the expression of the H-like activity.⁵⁾ Further, the formation of sharp precipitin lines in an agar plate and the inhibition of human O erythrocyte hemagglutination were observed

with a rape leaf AGP and its modified products in a reaction with an *A. aurantia* L-fucose-specific lectin as shown in Fig. 1B and Table III. This observation strongly supports the hypothesis that the L-fucosyl residues are at the non-reducing terminal end, where they are sufficiently exposed to bind the lectin. In contrast to H-active substances of animal origin, a rape leaf AGP, like radish leaf AGPs, was unreactive with chicken anti-H serum or anti-H lectin from *Ulex europaeus* seeds.³⁾ It is likely that the anti-H antibodies and lectin are highly specific for the terminal L-fucosyl residues as well as the penultimate D-galactosyl residue in mammalian H-active substances, and the ability to discriminate α -L-arabinofuranosyl residue. In an immunoprecipitation test with rabbit antiserum raised from the immunization of radish leaf AGPs, the rape AGP had the same immunological response as that of other cruciferous leaf AGPs, yielding a precipitin line fused with the others. The immunological properties of rape leaf AGP toward the antiserum were not affected by chemical and enzymatic modifications as far as examined on an agar plate (Fig. 1C). With the Smith degradation products, a significant difference was found in the immunological response between rape and radish AGPs,⁵⁾ probably due to the distinct sugar sequences surviving the Smith degradation at the peripheral regions of

their polysaccharide chains, since they were similar in chemical composition.

Structural studies on rape leaf AGP provided evidence that a major part of the carbohydrate moiety was built up with a core of continuous β -(1 $\rightarrow$ 3)-linked D-galactosyl residues substituted at O-6 by side chains of β -(1 $\rightarrow$ 6)-linked D-galactosyl residues. α -L-Arabinofuranosyl residues were present as single or (1 $\rightarrow$ 2)-, (1 $\rightarrow$ 3)-, and (1 $\rightarrow$ 5)-linked units and attached through O-3 to the side chains. Single units of α -L-fucopyranosyl and glucuronosyl residues were also located at non-reducing terminal ends. Anomeric configurations of the glycosidic linkages were assigned by the susceptibility to specific glycosidases and serological responses. Interestingly, unlike the presence of two AGP components in radish leaves, only one AGP component was isolated from rape leaf tissues but these AGPs were similar structurally and serologically. Two arabinogalactans (AGs) have been isolated from seeds of *Brassica* species.¹⁴⁻¹⁶ An AG from *B. campestris* seeds was apparently distinguished from the AGP of leaf tissues although it was a typical arabinogalactan composed of L-arabinose, D-galactose, and D-glucuronic acid in a molar ratio of 1:1.05:0.13.^{14,15} Larm *et al.* working with *B. napus* seeds, have reported the isolation of a high-molecular-weight polysaccharide comprising L-arabinose and D-galactose in the molar ratio of 10:1 but its structural features remained undetermined.¹⁶ In view of the distribution of AGPs and AGs in various tissues of cruciferous plants, it would be desirable to investigate their qualitative and quantitative alterations during

germination and development by chemical and serological methods to understand the physiological roles of these polysaccharides.

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