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Note

Blood-group active proteoglycan containing 3-*O*-methylrhamnose (acofriose) from young plants of *Osmunda japonica**

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Polysaccharides from higher plants have been reported to exhibit potent inhibitory activity toward hemagglutination by eel anti-H serum[1–3]. Further screening of the hemagglutination-inhibition activity of macromolecules of plant origin revealed that a relatively wide range of plants have human blood-group active substances[4–9], the structure of some of which being closely similar to those of proteoarabinogalactans, an ubiquitous constituent of plant cell walls[10–13]. Furthermore, some of the proteoglycans obtained from plants could be efficient antigens for arising, in rabbits, antibodies that would react with specific blood-group substances on erythrocytes and in solution[14]. We report therein the partial characterization of a 3-*O*-methylrhamnose (acofriose)-containing proteoglycan that shows human blood-group activity.

EXPERIMENTAL

Materials. — Young plants of *Osmunda japonica* were collected in Fukushima, in early May, chopped into small pieces, and extracted with distilled water (five times their fresh weight) for 1 h at 100°. The crude extracts were centrifuged at 20 000*g* for 30 min. The supernatants were concentrated under vacuum to one third of the starting volume and the proteoglycans were precipitated from the solution by the addition of ethanol to 20, 50, and 80% (v/v) concentration. The precipitates were dialyzed against distilled water, lyophilized, and stored at −20° until use. They were redissolved in water and the undissolved material was removed by centrifugation. The water-soluble proteoglycan was partially purified by gel filtrations on a Toyopearl HW-55F (Toyosoda Manufacturing Co., Ltd.)

*Dedicated to Professor Walter T. J. Morgan.
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column (2.5 cm × 60 cm) and a Sepharose CL-6B (Pharmacia Fine Chemicals) column (0.9 cm × 100 cm) and equilibrated with distilled water, 0.015M Na phosphate buffer, pH 7.2, containing 0.15M NaCl.

General methods. — Neutral sugars were determined with a Shimadzu gas-liquid chromatograph Type GC-R1A, equipped with a flame-ionization detector. Alditol acetates of the corresponding neutral sugars were separated in a glass column (3 mm × 2.1 m) packed with 3% OV-225 on Gas-chrom Q, programmed from 170 to 210° (2°/min). The sample (2–5 mg) was hydrolyzed in a sealed glass tube with 2.5M trifluoroacetic acid (1 mL) for 8 h at 100°. After hydrolysis, the acid was removed from the hydrolyzate by repeated additions and evaporations of distilled water. The hydrolyzate was de-ionized by passage through columns of Amberlite IR-120 (H^+) and IRA-400 (AcO^-) ion-exchange resins. Descending paper chromatography was done on Whatman No. 1 paper in 6:4:3 (v/v) butanol–pyridine–water and the sugar spots were detected with the aniline hydrogen phthalate or $AgNO_3$–NaOH reagents. G.l.c.–m.s. was performed with a Hitachi M-80 spectrometer, equipped with a Hitachi 002B-8DK data-processing system; e.i. mass spectra were recorded under an electron energy of 70 eV and an ionizing current of 110 μA. Amino acids were determined with an Atto amino acid analyzer Type MLC-703, equipped with SIC Chromatocorder 11 for automatic integration. The sample was hydrolyzed according to Simpson *et al.*[16]. Total carbohydrate was determined by the phenol–H_2SO_4 method with D-glucose as standard[17].

Hemagglutination-inhibition assay. — The method employed was essentially the same as that published[15]. To a serial, two-fold dilution of a 0.5 or 1% (w/v) sample in 0.015M Na phosphate buffer, pH 7.2, containing 0.15M NaCl and mM NaN_3 (25 μL), was added the equal volume of agglutinins and the mixture was incubated for 2 h at 37°, and then overnight at 4°. At the end of incubation, a 2% (v/v) suspension of erythrocytes in 0.015M Na phosphate buffer, pH 7.2, containing 0.15M NaCl (25 μL) was added, and the hemagglutination-inhibition was observed, at room temperature (23–25°), 30 min after the addition of the cells. The hemagglutination-inhibition activity is given for the concentration of the sample (mg/mL) required for complete inhibition of the hemagglutination. The agglutinins employed were human anti-A and anti-B sera, eel (*Anguilla japonica*) anti-H serum, anti-H lectin from *Ulex europaeus* seeds and rabbit anti-Le^a and anti-Le^b sera (prepared by immunizing rabbits with human salivas of appropriate blood groups), and all titers of the agglutinins were adjusted to 1:4.

Synthesis of 3-O-methyl-L-rhamnose. — This compound was prepared from L-rhamnose by the method of Haque *et al.*[18]. The structure was confirmed by ^1H-n.m.r. spectroscopy performed with a JEOL GX-400FT NMR spectrometer using a 5-mm spinning tube at 20° for a solution in ($^2H_5$)pyridine at 400 MHz; tetramethylsilane was the internal standard (δ 0).

RESULTS AND DISCUSSION

A proteoglycan having potent inhibitory activity toward hemagglutination by human blood-group specific reagents was partially purified, by gel filtrations, from a fraction precipitated with 50–80% ethanol. The proteoglycan was eluted as a single symmetrical peak from the Sepharose CL-6B column, and the maximum inhibitory activity toward hemagglutination by eel anti-H serum was also eluted at the same elution volume. The apparent mol. wt. of the proteoglycan was estimated by gel permeation chromatography using dextrans as molecular weight standards as 51 000 (Fig. 1). The proteoglycan was characterized by a high mol. wt. carbohydrate and a low mol. wt. protein component. The amino acid composition of the protein component is shown in Table I and the amino acid residues accounted for 6% (w/w) of the proteoglycan. The hydrolyzate was analyzed for neutral sugars by g.l.c. of the corresponding alditol acetates, indicating molar proportions of galactose (60%), arabinose (12%), rhamnose (9%), and an unknown sugar (10%, as rhamnose) (see Fig. 2). In order to identify the unknown sugar, it was investigated by g.l.c.–m.s. The e.i. mass spectrum suggested a series of fragments of *m/z* 43, 87, 101, 129, 143, 189, and 203 (Fig. 3), corresponding to the mass spectrum of the 6-deoxy-3-*O*-methylhexose reported by Jansson *et al.*[19]. The biosynthesis of *O*-

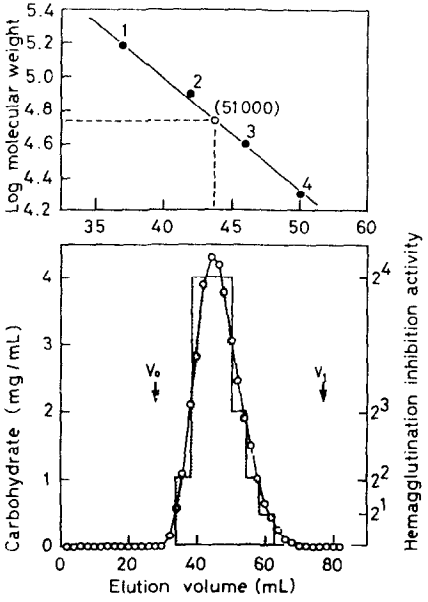

Fig. 1. Gel-filtration of the purified proteoglycan on a Sepharose CL-6B column: Carbohydrate (—○—), and inhibitory activity (dilutions) toward hemagglutination by eel anti-H serum (——). Arrows represent positions of peaks of Blue Dextran (Vo) and D-glucose (Vt). Molecular weight standards used were: (1) FITC-dextran 150 (154 000); (2) FITC-dextran 70 (68 500); (3) FITC-dextran 40 (39 000); and (4) FITC-dextran 20 (19 000).

TABLE I

AMINO ACID COMPOSITION OF THE PROTEOGLYCAN

Amino acid	*Mol. per 100 mol. of amino acids*	*Amino acid*	*Mol. per 100 mol. of amino acids*
Hyp	11.2	Val	5.6
Asn	11.7	Met	[a]
Thr	9.1	Ile	3.0
Ser	10.6	Leu	5.7
Glu	18.8	Tyr	1.3
Pro	4.3	Phe	1.9
Gly	8.0	Lys	4.6
Ala	1.4	His	0.6
Cys	0.5	Arg	1.7

[a]Trace.

methylated component sugars in polysaccharides[20,21] suggested L-rhamnose and L-fucose as possible precursors of the unknown 6-deoxy-3-*O*-methylhexose. 3-*O*-Methyl-L-rhamnose (acofriose) was synthesized from L-rhamnose, and its ^{1}H-n.m.r. spectrum recorded (Fig. 4). The paper chromatographic properties of the unknown 6-deoxy-3-*O*-methylhexose were compared to those of synthetic 3-*O*-methyl-L-rhamnose. The spots of the two sugar migrated identically and faster than that of L-rhamnose (R_{Rha} 1.20–1.23). The spots were not stained with the $AgNO_3$–NaOH reagent, but with the aniline hydrogen phthalate reagent. Furthermore, the alditol

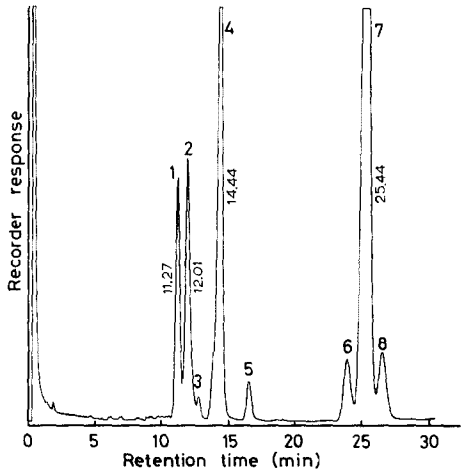

Fig. 2. G.l.c. of neutral sugars from the hydrolyzate of the proteoglycan. Alditol acetates of their corresponding sugars were separated on a column of 3% Silicone OV-225 on Gas-Chrom Q (170–210°, 2°/min): (1) Unknown sugar, (2) L-rhamnose, (3) L-fucose, (4) L-arabinose, (5) D-xylose, (6) D-mannose, (7) D-galactose, and (8) D-glucose.

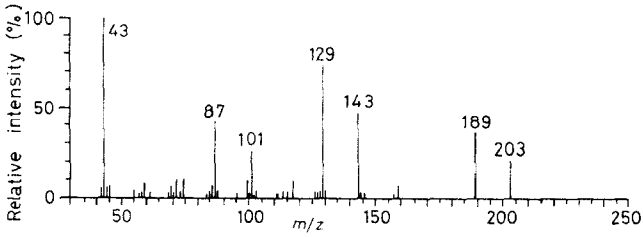

Fig. 3. E.i. mass spectrum of alditol acetate of the unknown sugar of the proteoglycan.

acetate derivatives of the two compounds exhibited an identical retention time on g.l.c. analysis.

Table II shows the human blood-group activity of the 3-*O*-methylrhamnose-containing proteoglycan as measured by the hemagglutination-inhibition method. Although hemagglutination by eel anti-H serum was completely inhibited by the proteoglycan at the concentration of 0.31 mg/mL, the proteoglycan was not an inhibitor for *Ulex* anti-H lectin. Neither human anti-A nor anti-B sera were inhibited by the proteoglycan. Of the two Lewis blood-group specific antisera employed, only rabbit anti-Le^a serum was completely inhibited by the proteoglycan at the concentration of 0.63 mg/mL; however, rabbit anti-Le^b serum was not inhibited by it at its maximum concentration tested. It has been established that an L-fucosyl group at the nonreducing end of human and animal blood-group active substances is a prerequisite for blood-group H activity[22,23]. The potent inhibition toward eel anti-H agglutinin by the proteoglycan containing 3-*O*-methylrhamnose, regardless of its low fucose content (<1 mol per cent), suggested that another blood-group H active sugar residue may be involved in the hemagglutination-inhibitory activity. Table III shows the inhibitory activity of the major neutral sugars of the proteoglycan toward hemagglutination by eel anti-H serum. Among those tested,

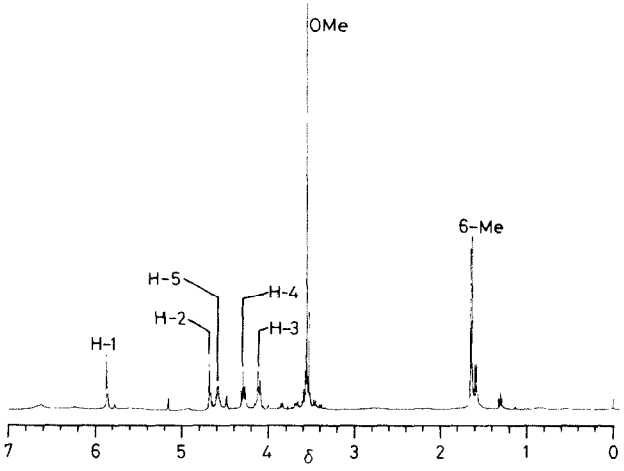

Fig. 4. ^{1}H-N.m.r. spectrum of synthetic 3-*O*-methyl-L-rhamnose (acofriose).

TABLE II

INHIBITORY ACTIVITY OF THE PROTEOGLYCAN TOWARD HEMAGGLUTINATION BY HUMAN BLOOD-GROUP SPECIFIC REAGENTS

Agglutinins	*Blood group of erythrocytes*	*Minimum amount of the proteoglycan required for complete inhibition of hemagglutinins (mg/mL)*[a]
Eel anti-H	O	0.31
Ulex anti-H	O	>10
Human anti-A	A	>10
Human anti-B	B	>10
Rabbit anti-Le[a]	Papainized OLe[a] [b]	0.63
Rabbit anti-Le[b]	Papainized OLe[b]	>10

[a]The inhibitory activity is measured as concentration (mg/mL) of the sample before addition of agglutinin and erythrocyte suspension. [b]The erythrocytes were treated with 0.1% (w/v) papain in PBS for 30 min at 37°, and then the cells were washed three times with PBS to remove papain.

TABLE III

INHIBITION BY NEUTRAL SUGARS PRESENT IN THE PROTEOGLYCAN OF HEMAGGLUTINATION BY EEL ANTI-H SERUM

Sugars[a]	*Minimum amount of sugar required for the complete inhibition of eel anti-H agglutinin*	
	mg/mL[b]	*μmol/mL*
L-Fucose[c]	0.08	0.47
3-*O*-Methyl-L-rhamnose	0.02	0.11
L-Rhamnose	>5	>30.5
L-Arabinose	>5	>33.3
D-Galactose	>5	>27.7

[a]3-*O*-Methyl-L-rhamnose was synthesized and other sugars were obtained from commercial sources. [b]The inhibitory activity is given as the concentration of sample (mg/mL) before addition of agglutinin and erythrocyte suspension. [c]L-Fucose was used as the positive control.

only 3-*O*-methyl-L-rhamnose was a potent inhibitor toward eel anti-H agglutinin, other than L-fucose; the latter was used as a positive control since its inhibitory activity toward the agglutinin has been reported by Springer and Desai[15]. Therefore, it is suggested that a 3-*O*-methyl-L-rhamnose-residue is at least involved in the blood-group H activity of the proteoglycan. Proteoglycans containing 3-*O*-methyl-rhamnose (acofriose) and showing blood-group activity have not hitherto been reported in nonflowering plants.

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