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Studies on the Structure of Polysaccharide from *Tetragonia tetragonoides*. I

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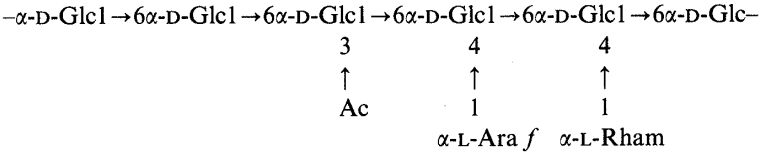
Water-soluble polysaccharides were isolated from *Tetragonia tetragonoides* (Aizoaceae), and their anti-inflammatory effects on carrageenan-induced edema and adjuvant arthritis were tested. One of the active fractions TII_c, gave a single peak on high-performance liquid chromatography and gel filtration. Methylation, periodate oxidation and carbon-13 nuclear magnetic reasonance spectroscopic studies suggested that TII_c is composed of the following repeating unit:



Keywords——*Tetragonia tetragonoides*; heteropolysaccharide; anti-inflammatory; carrageenan-induced edema; adjuvant arthritis; methylation analysis; ^{13}C-NMR

Tetragonia tetragonoides (Japanese name: Hamajisha, Tsuruna) is used as a substitute for vegetables in certain districts in Japan, and is said to be effective against stomach cancer and ulcer of the stomach.[1] Yamazaki and Okuyama[2] isolated a steryl glucoside as one of the principles which inhibited ulcer formation in mice under conditions of restraint and water immersion. Suga *et al.*[3] reported on the chemical constituents of this plant; they isolated an acyclic diterpene aldehyde and a norditerpene aldehyde. However, there has been no report concerning the structures of the polysaccharides of the plant.

We examined the structures and anti-inflammatory activities of polysaccharides isolated from the aerial parts of *Tetragonia tetragonoides* (Aizoaceae) in this study. Crude polysaccharides were obtained by adding ethanol to the hot water extract. The crude polysaccharides were dialyzed and the non-dialyzable fraction was chromatographed on a column of Sephadex G-50 to give three fractions. The fractions, TI, TII, and TIII, were collected separately and lyophilized (TI 12%, TII 36%, TIII 52% yield). The TII fraction was further purified by chromatography on coupled Sephadex G-100 and Sephadex G-50 columns to give three fractions (TII_a, TII_b, TII_c), as shown in Fig. 1. Each fraction yielded a single peak on ultracentrifugation and on high-performance liquid chromatography (HPLC).

The above polysaccharide preparations were subjected to bioassay to investigate their inhibitory effects on carrageenan-induced hind foot edema in mice according to the method reported in our previous paper.[4] The experimental results are shown in Fig. 2; treatment with the crude polysaccharide preparation and TII at a dose of 50 mg/kg *p.o.* produced 29.5% and 37.1% decreases, respectively, in foot edema as compared with the control group at 7 h after induction of the edema. On the other hand, TI and TIII were ineffective. TII_a, TII_b and TII_c

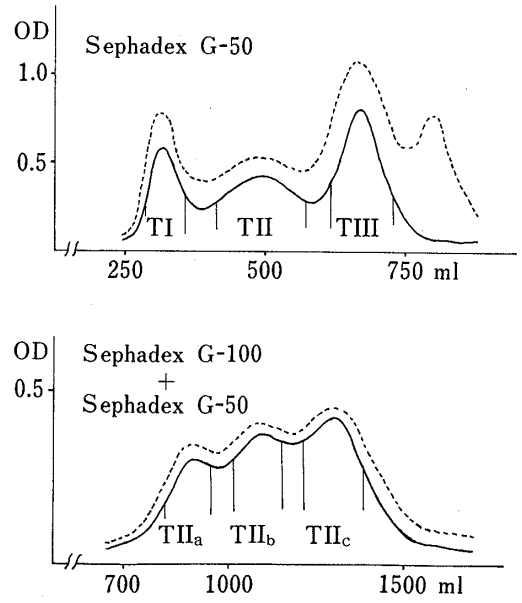


Fig. 1. Gel Filtration on Sephadex
------; 280 nm, ——; 620 nm.

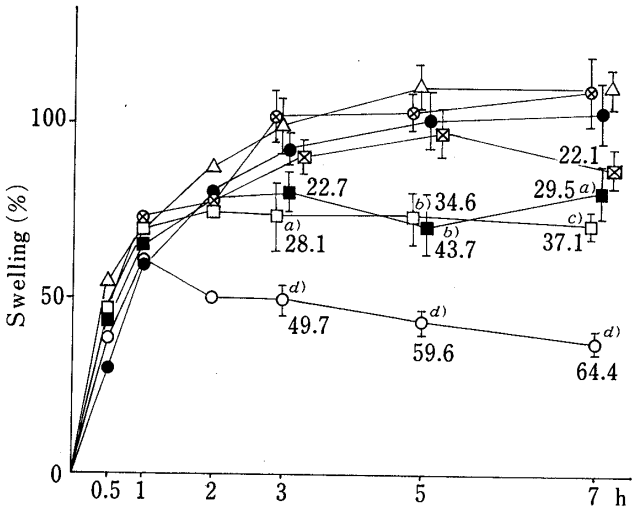


Fig. 2. Inhibitory Effects of Polysaccharide Preparations on the Swelling of Mouse Hind Paw Induced by Carrageenan

⊗, TI 25 mg/kg; △, TIII 50 mg/kg; ●, control; ⊠, TII 25 mg/kg; ■, ppt. 50 mg/kg; □, TII 50 mg/kg; ○, prednisolone 25 mg/kg. Numbers indicate the inhibition (%). Each value is the mean ± S.E.M. from 6 male mice. *a*) $p<0.05$. *b*) $p<0.025$. *c*) $p<0.005$. *d*) $p<0.001$.

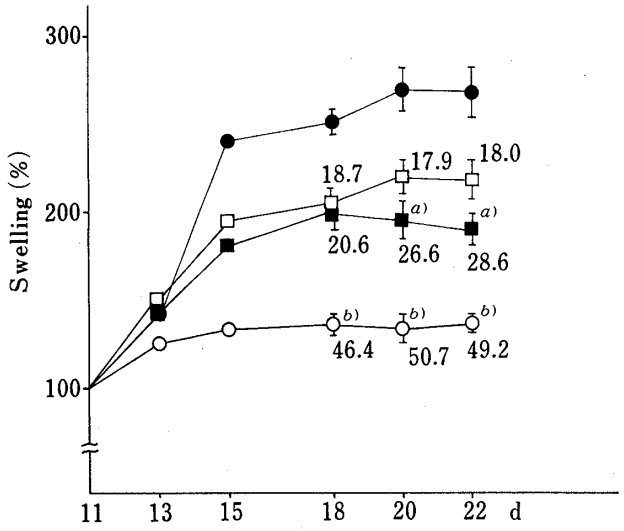


Fig. 3. Inhibitory Effects of Crude Polysaccharide (ppt.) on the Swelling of Rat Hind Paw Induced by Adjuvant

●, control; □, ppt. 5 mg/kg; ■, ppt. 50 mg/kg; ○, indomethacin 2.5 mg/kg. Numbers indicate the inhibition (%). Each value is the mean ± S.E.M. from 8 male rats. *a*) $p<0.05$. *b*) $p<0.001$.

caused 30.9%, 36.6%, and 34.7% inhibition, respectively, at a dose of 25 mg/kg *p.o.* These polysaccharides thus seem to be the active principles inhibiting carrageenan-induced edema in *Tetragonia tetragonoides*. Their inhibitory effect on adjuvant arthritis in rats was also investigated by the method of Pearson *et al.*[5]; the inhibitory effects of administration for 12 d (from day 11 to day 22 after treatment) of the crude polysaccharide (5 and 50 mg/kg *p.o.*) and indomethacin (2.5 mg/kg *p.o.*) on the edema formation in the rat hind paw were determined. The results are illustrated in Fig. 3. The crude polysaccharide inhibited the edema by 28.6% on the 22nd day ($p<0.05$). Further investigation on the active principle is in progress.

The component sugars of TII_c ($[\alpha]_D$ +130.4°) were identified as glucose, arabinose and rhamnose by thin layer and gas liquid chromatographies (TLC and GLC) of the acid hydrolysate, and their molar ratio was estimated to be approximately 6 : 1 : 1 by GLC.

In the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum of TII_c, all of the carbon signals were elucidated, and their chemical shifts are recorded in Table I. By comparison of the TII_c spectrum with those of dextran (α-1→6 glucan),[6] amylose (α-1→4

TABLE I. ^{13}C-Chemical Shifts of TII_c Examined in D_2O

Carbon assignment	Chemical shifts (δ)			
	TII_c		References	
Glucose				
(1→6)		(1→6) (1→4)	Dextran[6]	Amylose[6]
C-1	99.7	101.0	99.0	100.9
C-2	72.0		72.5	72.7
C-3	75.2		74.5	74.5
C-4	72.0	78.7	71.3	78.4
C-5	70.5		70.7	72.4
C-6	67.7		66.7	61.8
Arabinose			Methyl α-L-arabinofuranoside[7]	
C-1	108.8		109.3	
C-2	82.6		81.9	
C-3	78.3		77.5	
C-4	84.0		84.9	
C-5	62.6		62.4	
Rhamnose			Methyl α-L-rhamnopyranoside[7]	
C-1	101.8		101.9	
C-2	70.5		71.0	
C-3	71.5		71.3	
C-4	73.3		73.1	
C-5	69.8		69.4	
C-6	18.5		17.7	

glucan),[6] methyl α-L-arabinofuranoside and methyl α-L-rhamnopyranoside,[7] a complete assignment of TII_c resonances could be made. As the chemical shift for C-1 in dextran is δ 99.0 ppm, the signal at 99.7 ppm in the TII_c spectra might be regarded as due to C-1 linked α-1→6. The resonance at 101.0 ppm is clearly due to C-1 of an α-1→4 link by comparison with the data for amylose. Accordingly, the peaks at 67.7 and 78.7 ppm can be attributed to C-6 in an α-1→6 link and C-4 in an α-1→4 link, respectively, located in glycosidic bonds.

GLC of the methanolysis products of TII_c methyl ether prepared by the Hakomori method[8] revealed the liberation of methyl 2,3,5-tri-*O*-methylarabinofuranoside, methyl 2,3,4-tri-*O*-methylrhamnopyranoside, methyl 2,3,4-tri-*O*-methylglucopyranoside, and methyl 2,3-di-*O*-methylglucopyranoside in a 1:1:4:2 ratio; these products were characterized by comparison with authentic samples. The methylated sugars were further characterized by GLC-mass spectrum (MS) analysis, using an XE-60 column. When TII_c was subjected to periodate oxidation,[9] 1.30 mol of periodate per one anhydro-sugar unit was consumed. Periodate oxidation followed by reduction and acid hydrolysis gave 0.13 mol of propylene glycol, 0.78 mol of glycerol and 0.31 mol of erythritol, in good agreement with the methylation data.

The infrared (IR) spectrum of TII_c has absorption bands at 1240 and 1735 cm^{-1}, suggesting the presence of ester linkages, and the absorption at 845 cm^{-1} is due to α-glycosidic linkages. The ^{13}C-NMR spectrum showed acetyl signals at δ 172.3, and 21.7 ppm, and the acetyl content of TII_c was determined to be 6.48% according to the method of Tomoda *et al.*[10] In order to elucidate the location of *O*-acetyl groups,[10,11] TII_c was exhaustively treated with methyl vinyl ether in the presence of *p*-toluenesulfonic acid in dimethylsulfoxide. After conversion of the hydroxyl groups into 1-methoxyethyl ethers, the product was de-*O*-acetylated with sodium methoxide, and then methylated with methyl iodide and silver oxide. The resulting product was hydrolyzed and analyzed by GLC. A hexose methyl ether was detected and identified as 3-*O*-methyl-D-glucose. This result indicates that a 3-*O*-acetyl-D-

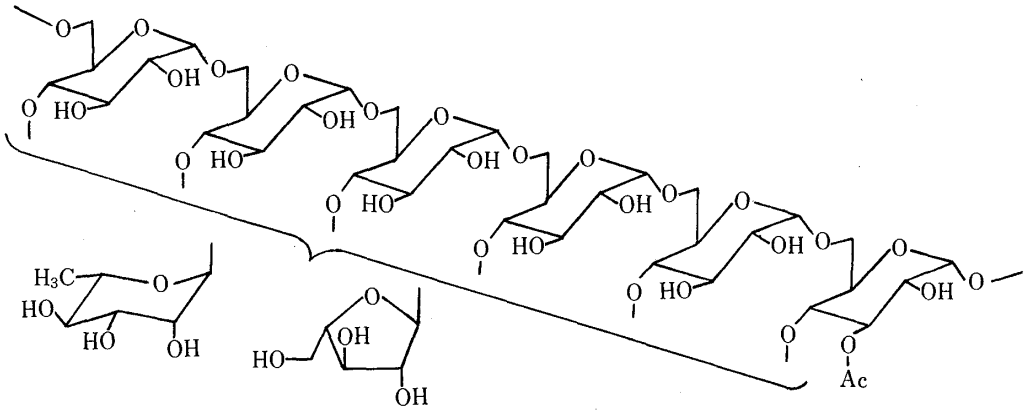


Chart 1. Proposed Structure of TII_c

glucose unit is present in the repeating unit.

Based on the above evidence, it is proposed that TII_c is composed of repeating units of an octasaccharide having a main chain consisting principallyl of (1→6)-linked α-D-glucopyranosyl units containing one mol of 3-*O*-acetyl-D-glucose, substituted in some of the 4-positions by side chains of α-L-arabinofuranose and α-L-rhamnopyranose (Chart 1). The molecular weight of TII_c was determined to be about 24000 by the Park–Johnson method.[12] The results of detailed analysis of the structures of TII_a and TII_b will be reported in subsequent papers.

Experimental

Optical rotations were measured with a JASCO DIP-4 digital polarimeter. The IR spectra were measured with a JASCO IRA-2 spectrometer. GLC analyses were carried out with a Shimadzu GC-6A gas chromatograph equipped with a hydrogen flame ionization detector. Sedimentation analysis was performed at 58000 rpm with a Beckman Spinco ultracentrifuge equipped with a schlieren optical system. The ^{13}C-NMR spectra were obtained with a JEOL FX-100 spectrometer operating at 25.0 MHz in the pulsed Fourier-transform mode. Free-induction decays were accumulated with a 45° pulse. All spectra were recorded in D_2O at 70 °C using 8000 data points and a spectral width of 5 kHz. ^{13}C-Chemical shifts are expressed in ppm downfield from external tetramethylsilane. HPLC was carried out on a Shimadzu LC-5A instrument equipped with an RI detector. The eluent (H_2O) flow rate was 1 ml/min at 40 °C on a column of G 3000 SW.

Isolation and Purification——The whole plant of *Tetragonia tetragonoides* (2 kg) was cut into pieces and extracted with benzene and then with methanol in order to remove organic solvent-soluble components, and the residue was extracted further with distilled water on a boiling water-bath for 24 h. Ethanol was added to the hot filtered extract to form precipitates, which were collected by centrifugation, washed with ethanol and ether, and dried to yield a pale brownish water-soluble powder (400 g). The crude polysaccharide was dialyzed. The non-dialyzable fraction (139 g) was chromatographed on a column of Sephadex G-50 (4.5 × 60 cm) to give three fractions (TI 16.6 g, TII 49.8 g, TIII 72.6 g). TII (49.8 g) was further purified on coupled columns of Sephadex G-50 and Sephadex G-100 (4.4 × 110 cm each) to give three fractions (TII_a 3.7 g, TII_b 9.7 g, TII_c 21.8 g), as shown in Fig. 1. Fractions of 0.5 ml were collected and analyzed by the anthrone–sulfuric acid method. TII_c: $[\alpha]_D^{22}$ +130.4° (c=0.48, H_2O); retention time on HPLC, 10.2 min.

Anti-inflammatory Activity on Carrageenan-Induced Hind Foot Edema in Mice——Male mice of ddy strain, 5 weeks old and weighing 18—22 g, were used as experimental animals, and foot edema was induced with carrageenan according to the method reported in our previous paper.[4] Six mice per group were used. λ-Carrageenan (1.0% in physiological saline solution, 0.05 ml) was injected *s.c.* under the plantar surface of the right hind paw at 1 h after oral administration of a test drug dissolved in water. Increase in the foot volume was measured as decribed previously[13] and expressed as percent of the foot volume measured before the injection of carrageenan. The anti-edema effect of the test drugs was expressed in terms of percent inhibition of the foot edema in the drug-treated group compared with the foot edema in the control group treated with the vehicle.

Adjuvant Arthritis in Rats——According to the method described in our previous paper,[13] adjuvant arthritis was induced in 5-week-old male Wistar rats, weighing 160—200 g at the start of the experiment. A subplantar injection of 0.05 ml of a 0.6% suspension of heat-killed *Mycobacterium butyricum* in liquid paraffin was made into the right hind

TABLE II. Results of Methylation Analysis of the Polysaccharide

Methylated sugars	Retention time	Molar proportion	Linkage pattern	Fragment obtained in GLC-MS analysis
2,3,4-Me-rhamnopyranoside	1.78	1	Rham *p*-(1→	45, 73, 83, 88, 101, 161
2,3,5-Me-arabinofuranoside	3.41, 4.68	1	Ara*f*-(1→	45, 73, 87, 101, 161
2,3,4-Me-glucopyranoside	8.27, 11.29	4	→6)-Glc*p*-(1→	45, 73, 75, 88, 89, 101
2,3-Me-glucopyranoside	14.60, 20.40	2	→4,6)-Glc*p*-(1→	45, 73, 87, 88, 101

Abbreviations: Me = methyl (*e.g.* 2,3,4-Me- = 2,3,4-tri-*O*-methyl-).

paw; the paw volume was measured prior to the injection, and on 11, 13, 15, 18, 20, and 22 d after the injection. The animals which had the same degree of edema volume on day 11 were selected and used. They were administered the test drug *p.o.* on day 11 after the injection and then once a day for 12 d. The difference in paw volume between day 11 and each succeeding day (days 13, 15, 18, 20 and 22) was designated as the edema volume. The percent inhibition of edema produced was calculated for each animal group with respect to the vehicle-treated control group.

Sugar Components of TII_c——TII_c was hydrolyzed with 1 N H_2SO_4 for 8 h at 90 °C. The hydrolyzate was neutralized with ion-exchange resin (IR-410) and concentrated. Trimethylsilylation of a half of the concentrate followed by GLC [2% OV-17 on Chromosorb WAN-DMCS (3 mm × 2 m): column temperature 160 °C; N_2 flow rate 50 ml min^{-1}] showed the presence of arabinose, rhamnose and glucose in a ratio of 1 : 1 : 6. Relative retention times: arabinose 0.28, 0.34; rhamnose 0.28, 0.36; glucose 1.00, 1.57.

The other half was reduced, acetylated, and analyzed by GLC [ECNSS-M (3 mm × 2 m): column temperature 190 °C, N_2 flow rate 40 ml min^{-1}]. Relative retention times: arabinitol 0.28; rhamnitol 0.16; glucitol 1.00.

Methylation Analysis of TII_c——1,1,3,3-Tetramethylurea (2 ml)[14] and methylsulfinylcarbanion solution (4 ml) were added to a dimethyl sulfoxide (2 ml) solution of TII_c (50 mg), and after 3 h a large excess of methyl iodide (6 ml) was added dropwise to the mixture under vigorous stirring, with cooling to keep the reaction temperature below 50 °C. When the methylation was completed, the mixture was diluted with water, and dialyzed against running water to remove excess methyl iodide. The dialyzate was extracted with chloroform, and the extract was washed with water, dried, and evaporated. The product showed no hydroxyl absorption in the IR spectrum. The methylated product (10 mg) was methanolyzed with 5% HCl–MeOH (2 ml) in a sealed ampoule at 100 °C for 5 h. The resulting methyl glycosides were analyzed by GLC and GLC-MS.

GLC was carried out using a column (3 mm × 2 m) packed with Silicone XE-60 at 155 °C with a nitrogen flow of 50 ml min^{-1}. In the sample from TII_c, methyl 2,3,5-tri-*O*-methylarabinofuranoside (3.41, 4.68), methyl-2,3,4-tri-*O*-methylrhamnopyranoside (1.78), methyl-2,3,4-tri-*O*-methylglucopyranoside (8.27, 11.29) and methyl-2,3-di-*O*-methylglucopyranoside (14.60, 20.40) were identified by comparison with authentic samples; they were present in a ratio of 1 : 1 : 4 : 2.

GLC-MS was carried out under the same conditions, but with helium as a carrier gas. The main fragments in the MS are listed in Table II.

Periodate Oxidation, Smith Degradation and Analysis of Products——TII_c (50 mg) was added to a solution of 0.05 M sodium periodate (20 ml). Oxidation was carried out in the dark at 7 °C. Aliquots (1 ml) were removed from the solution at intervals for estimation of iodate. When the oxidation was complete (after 24 h), the oxidized TII_c was reduced with sodium borohydride (20 mg), and hydrolyzed with 1 N sulfuric acid (5 ml) at 90 °C for 6 h; 0.13 mol of propylene glycol (3.66 min), 0.78 mol of glycerol (12.49 min) and 0.31 mol of erythritol (22.12 min) per anhydroglucose unit were identified as their trimethylsilylated derivatives by GLC [SE-30, column temperature 70—150 °C (3 °C/min); N_2 flow rate 30 ml min^{-1}].

Determination of *O*-Acetyl Groups——The sample (5 mg) was hydrolyzed with 1 N H_2SO_4 (0.5 ml) containing propionic acid (0.1 mg) as an internal standard at 100 °C for 2.5 h. The hydrolyzate was directly subjected to GLC [Unisole F-200 (3 mm × 2 m); column temperature 120 °C; N_2 flow rate 30 ml min^{-1}]. Acetic acid (4.1 min) was identified and the content corresponded to 6.48% in TII_c.

Analysis of the *O*-Methyl Derivative——TII_c (50 mg) was methoxyethylated with methyl vinyl ether (5 ml) in the presence of *p*-toluenesulfonic acid (6 mg) in dimethyl sulfoxide (2 ml). The IR spectrum of the final residue had no absorption near 3400 cm^{-1}. The *O*-acetyl-*O*-(1-methoxyethyl)-derivative (60 mg) was deacetylated with 0.2 M methanolic sodium methaoxide (5 ml). The solution was refluxed at 75 °C and applied to a column (4 × 25 cm) of Sephade LH-20. The *O*-(1-methoxyethyl)-derivative (20 mg) was methylated three times with methyl iodide (5 ml) and silver oxide (0.1 g) in *N*,*N*-dimethylformamide (2 ml). The *O*-methyl-(1-methoxyethyl)derivative (5 mg) was treated with 90% formic acid (1 ml) and 0.5 N sulfuric acid (1 ml). The hydrolyzate was trimethylsilylated, and GLC was carried out as described above. Relative retention times of the products: arabinose (0.28, 0.34), rhamnose (0.28, 0.36), glucose (1.00, 1.57), 3-*O*-methylglucose (0.61, 0.84).

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