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Two acidic polysaccharides from the flowers of *Chrysanthemum morifolium*

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Two new acidic polysaccharides, **F4** and **F5**, were isolated from the flowers of *Chrysanthemum morifolium*. Monosaccharide analysis indicated that **F4** contained arabinose, galactose and galacturonic acid units in a molar ratio of 1.0:2.3:6.8 and **F5** contained arabinose, rhamnose galactose and galacturonic acid units in a molar ratio of 1.0:3.2:1.0:4.3. The results of methylation analysis, partial acid hydrolysis and NMR spectral analysis indicated that **F4** had a homogalacturonan main chain with arabinogalactan side chain linked to 3 position of (1 → 3,4)-linked galacturonan and **F5** had a rhamnogalacturonan main chain with arabinogalactan side chain linked to 3 position of (1 → 3,4)-linked galacturonan or 4 position of (1 → 2,4)-linked rhamnose. Biological tests revealed that **F4** and **F5** could simulate the mitogen induced T and B lymphocyte proliferation *in vitro*.

Keywords: *Chrysanthemum morifolium*; Asteraceae; Pectin; Homogalactan; Rhamnogalacturonan

1. Introduction

Chrysanthemum morifolium Ramat. belongs to the family of Asteraceae and is widely used as a food, tea or drug in China and Japan. Up to now, a great diversity of low molecular weight constituents including flavonoids [1,2], triterpenes [3] and unsaturated fatty acids [4], etc., from *C. morifolium* have been elaborately characterised. Their anti-human immunodeficiency virus [1], anti-mutagenic [2] and anti-tumour [3] activities have also been reported. However, few reports have been concerned with the polysaccharide constituents from the flowers of *C. morifolium*. In this paper, we report the structural analysis and immunological activity of two pectic polysaccharides (**F4** and **F5**) from the flowers of this plant.

2. Results and discussion

The native polysaccharide **F4** was eluted as a single symmetrical peak corresponding to an average-molecular weight of 1.1×10^5 Da as determined by the HPLC method, and gave

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a single band on PAGE (polyacrylamide gel electrophoresis). It had $[\alpha]_D + 170.8°$ (*c* 1.0, water). A negative response to the Lowry method indicated **F4** contained no protein. The characteristic absorption band at 1741.4 cm^{-1} in the IR spectrum of **F4** indicated the presence of uronic acid. **F4** was partially hydrolysed with 0.2 mol l^{-1} trifluoroacetic acid (TFA) for 4 h and obtained a sub-polysaccharide **F4L**. **F4** and **F4L** were reduced by the 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate—sodium borohydride method [5] and obtained carboxyl reduced polysaccharides **F4R** and **F4LR**. Monosaccharide analysis of **F4** and **F4R** indicated that **F4** contained arabinose, galactose and galacturonic acid units in a molar ratio of 1.0:2.3:6.8. The results of methylation analysis are shown in table 1. The methylation analysis on native **F4** (data not shown) could not give quantitative results because of the poor solubility of the polysaccharide in dimethyl sulfoxide. However, no (1 → 4) or (1 → 3,4)-linked galactose residues appeared, suggesting that (1 → 4) and (1 → 3,4)-linked galactose residues in **F4R** all come from reduced galacturonic acid. Uronic acid content of **F4L** was determined by the *m*-hydroxydiphenyl method and was approximately 100% [6]. The GC-MS of **F4LR** only showed (1 → 4)-linked hexose, which originated from (1 → 4)-linked galacturonic acid. So **F4L** was composed of (1 → 4)-linked galacturonan and the branching point in **F4** was the 3 position of galacturonic acid. The signals at δ 54.4 in ^{13}C NMR and δ 3.8 in ^{1}H NMR of **F4** originated from methyl ester groups. The signals between δ 2.2 and 2.3 in ^{1}H NMR and the weak signal at δ 22.0 in ^{13}C NMR corresponded to acetyl groups. The degree of methylation was 67% and the degree of acetylation was 4%. Signals in the ^{1}H and ^{13}C NMR were assigned as completely as possible, based on monosaccharide analysis, linkage analysis, HSQC, HMBC and literature data [7] as shown in table 2. Biological tests revealed that **F4** could enhance the mitogen-induced T and B lymphocyte proliferation in vitro even at 1 $\mu g\ ml^{-1}$ (table 3).

The native polysaccharide **F5** was eluted as a single symmetrical peak corresponding to an average-molecular weight of 7.5×10^5 Da as determined by the HPLC method and gave a single band on PAGE. It had $[\alpha]_D + 105.0°$ (*c* 1.0, water). A negative response to the Lowry method indicated **F5** contained no protein. The strong band at 1739.5 cm^{-1} in IR spectrum indicated it contained uronic acid. **F5** was partially hydrolysed by 0.2 mol l^{-1} TFA for 4 h and obtained a sub-polysaccharide **F5L**. **F5** and **F5L** were reduced by the 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate-sodium borohydride method [5] and obtained carboxyl reduced polysaccharides **F5R** and **F5LR**. Monosaccharide analysis of **F5** and **F5R** indicated that **F5** contained arabinose, rhamnose, galactose and galacturonic acid units in a molar ratio of 1.0:3.2:1.0:4.3. The results of methylation analysis are shown in table 1. The methylation analysis on native **F5** also could not give quantitative

Table 1. Methylation analysis data of **F4**, **F4L**, **F5** and **F5L**.

Deduced linkages	*F4*	*F4L*	*F5*	*F5L*	*Methylated sugars*
Araf	0.3		4.5		2,3,5-Me_3-Ara
1,5Araf	1.0				2,3-Me_2-Ara
1,2Rhap			9.7	1.0	3,4-Me_2-Rha
1,2,4Rhap			5.1		3-Me-Rha
Galp	1.0		3.6		2,3,4,6-Me_4-Gal
1,3Galp	2.0				2,4,6-Me_3-Gal
1,3,6Galp			1.0		2,4-Me_2-Gal
1,4GalAp	7.4	1.0	17.7	1.3	2,3,6-Me_3-Gal
1,3,4GalAp	1.5		2.3		2,6-Me_2-Gal

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Table 2. ^{13}C and ^{1}H chemical shifts (δ in ppm) of **F4** and **F5**.

		C-1/H-1	*C-2/H-2*	*C-3/H-3*	*C-4/H-4*	*C-5/H-5*	*C-6/H-6*
F4	α-(1 → 4)-GalA	101.8/4.97	69.7/3.77	70.8/3.84	79.8/4.44	72.3/4.31	176.3[a]
	α-(1 → 3,4)-GalA	101.3/5.03	69.7/3.84	75.0/4.12	80.0/4.47	72.3/4.31	176.3[a]
	β-T-Gal	104.8/4.47	72.5/3.50	73.8/3.82	69.7/3.84	75.0/4.12	62.5/3.80
	β-T-(1 → 3)-Gal	104.8/4.47	72.5/3.50	78.2/3.94	69.7/3.84	75.0/4.12	62.5/3.80
F5	α-(1 → 4)-GalA	102.0/4.93	68.3/3.81	70.5/4.00	79.5/4.41	72.2/4.29	173.5
	α-(1 → 3,4)-GalA	101.5/5.08	68.3/3.85	78.1/4.11	79.5/4.41	72.2/4.29	173.5
	α-(1 → 2)-Rha	100.4/5.22	78.5/4.01	70.9/3.90	73.4/3.49	69.9/3.71	19.2/1.24
	α-(1 → 2,4)-Rha	99.2/5.00	78.5/4.01	70.9/3.90	77.0/3.66	68.3/3.81	19.2/1.20
	α-T-Ara	109.8/5.18	84.0/4.20	78.3/3.93	85.6/4.02	62.8/3.75	

[a]The signal of methylated galacturonic acid is at δ 173.2 ppm.

results and no (1 → 4) or (1 → 3,4)-linked galactose residue appeared in them, suggesting that (1 → 4) and (1 → 3,4)-linked galactose residues in **F5R** all come from reduced galacturonic acid. Uronic acid content of **F5L** determined by the *m*-hydroxydiphenyl method is 56% [6]. The GC-MS of **F5** only contained (1 → 4)-linked hexose and (1 → 2)-linked rhamnose in a molar ratio of 1.3:1.0. **F5L** was hydrolysed by 0.4 mol l^{-1} TFA and dialysed. The electrospray ionisation (ESI) of the dialysate gave five pseudomolecular ions at *m/z* $[M-H]^-$ 339.2 (23), 661.2 (100), 983.2 (74), 1305.2 (29) and 1627.3 (8), which could be attributed to $[(HexA)1(6deoxy)1\text{-}1]^-$, $[(HexA)2(6deoxy)2\text{-}1]^-$, $[(HexA)3(6deoxy)3\text{-}1]^-$, [(HexA)4(6deoxy)4-1], $[(HexA)5(6deoxy)5\text{-}1]^-$, respectively. Only (1 → 2)-linked rhamnose and (1 → 4)-linked galactose appeared in the methylation result of **F5LR**, so **F5L** is composed of repeating units of →)-GalAp-(1 → 2)-Rhap-(1 → . ^{1}H and ^{13}C NMR resonances of **F5** were assigned so far as possible based on monosaccharide analysis, linkage analysis, HSQC, HMBC and the literature values [7]. The signal at δ 54.2 indicated the presence of methyl ester. The signals at δ 22.1 and 21.8 in ^{13}C NMR and signals between δ 2.33 and 2.43 in ^{1}H NMR of **F5** originated from acetyl groups. The degree of methylation and acetylation was 19% and 11%, respectively. In the NMR spectrum of **F5L** these signals

Table 3. Effect of **F4** and **F5** on mitogenic activity of lymphocytes in mouse splenocytes *in vitro*.

		MTT test[b]	*^{3}H-TdR test*				
				T cell[c]		*B cell*[d]	
Fraction	*Conc. ($\mu g\ ml^{-1}$)*	*Mean ± SD (OD_{570nm})*	*Control*	*Mean ± SD (cpm)*	*Prolif. (%)*[a]	*Mean ± SD (cpm)*	*Prolif. (%)*[a]
			Negative	1966 ± 341		2031 ± 184	
Control		0.395 ± 0.001	Positive	29450 ± 1429		24409 ± 1367	
F4	1	0.380 ± 0.010		33905 ± 2015	15%	31071 ± 1721	27%
	10	0.396 ± 0.006		36082 ± 1458	23%	33292 ± 3400	36%
	100	0.464 ± 0.004**		40420 ± 1738	37%	38678 ± 1671	58%
			Negative	9817 ± 2007		10875 ± 144	
Control		0.249 ± 0.002	Positive	30381 ± 1962		22470 ± 416	
F5	1	0.229 ± 0.009		32092 ± 1182	6%	18692 ± 1398	−17%
	10	0.318 ± 0.064**		31759 ± 2480	5%	20976 ± 691	−7%
	100	0.408 ± 0.008***		36196 ± 3952	19%	25791 ± 1791	15%

[a] Minus sign shows samples had inhibition activity, ≥ 15% shows the sample was effective.
[b] Effect on activity of lymphocyte without any induction.
[c] Effect on ConA-induced mitogenic activity of T-lymphocyte.
[d] Effect on LPS-induced mitogenic activity of B-lymphocyte.
[e] $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

disappeared, indicating acetyl groups were hydrolysed by TFA treatment. Biological tests revealed that **F5** could enhance the mitogen-induced T and B lymphocyte proliferation *in vitro* at high concentration (100 $\mu g\ ml^{-1}$) and inhibit B lymphocyte proliferation at low concentration (1 $\mu g\ ml^{-1}$) (table 3).

3. Experimental

3.1 General experimental procedures

The optical rotation was measured with a Shanghai Spoif W22-1S automatic polarimeter. GLC analyses were performed on a Shimadzu GC-14B instrument, equipped with a FID detector and a 5% OV-225 column (2.1 m $\times$ 3.2 mm i.d.) and the column temperature was 210°C. GC-MS was conducted with a Finnigan MD-800 combined with GC-MS spectrometry equipped with an HP-1 capillary column. ESI-MS was recorded with a VG Quattro MS/MS spectrometer. ^{13}C and ^{1}H NMR spectra were recorded at room temperature with a Bruker AM 400 instrument. All the chemical shifts are reported relative to Me_4Si as external standard.

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Uronic acid content was determined by the *m*-hydroxydiphenyl method [6], carbohydrate by the H_2SO_4-phenol method [8] and protein by the Lowry method [9]. The degree of methyl esterification (DM) was determined by the GLC and colorimetric methods as described previously [10] and the degree of acetylation (DA) was calculated according to the ^{1}H NMR spectrum [11].

3.2 Plant material

Plant material was collected at Boxian (Anhui Province, China) in July 2001. It was identified as *Chrysanthemum morifolium* Ramat. by Dr X.L. Huang and the voucher specimen is deposited in the Herbarium of the Shanghai Institute of Materia Medica.

3.3 Homogeneity and molecular weight

HPLC of polysaccharides was done on a Waters Ultrahydrogel™ 1000 and Ultrahydrogel™ 500 tandem column equipped with a Waters 515 HPLC pump and a Waters 2410 refractive index detector, eluted with 0.003 M NaAc. The column was pre-calibrated using standard T-Dextrans (T-500, T-110, T-80, T-70, T-40 and T-9.3). All samples were prepared as 1% (w/v) aqueous solutions and 20 μl of solution was injected in each run.

PAGE was carried out in a Bio-Rad vertical slab electrophoresis apparatus with gel (7.5%) and Tris-glycine buffer (pH 8.3) at 8 mA for 5 h. Gels were stained by the periodate-Schiff (PAS) procedure.

3.4 Separation and purification of F4 and F5

The flowers (4.5 kg) of *Chrysanthemum morifolium* were extracted with hot water three times then filtered. The filtrate was dialysed against running water for 3 days. The non-dialysate was concentrated and precipitated with 4 volumes of EtOH, then centrifuged. The precipitate

was washed successively with EtOH and acetone then dried in vacuo, giving the crude polysaccharide **CMA** (378 g). **CMA** (10 g) was deproteinated five times by the Sevag method [12], then applied to a DEAE-cellulose column (70 × 6.0 cm) in two runs, eluted first with water and then with step gradient 0.1, 0.2, 0.3 mol l^{-1} NaCl (H_2SO_4-phenol monitoring). **CMA1** and **CMA3** were obtained from the 0.1 and 0.3 mol l^{-1} NaCl eluate, respectively. They were purified by repeated gel-permeation chromatography on a Sephacryl S-300 column (90 × 2.6 cm), giving **F4** (0.48 g) and **F5** (0.13 g).

3.5 Monosaccharide and methylation analysis

Polysaccharides (4 mg) were dissolved in 2 mol l^{-1} TFA (4 ml) and hydrolysed at 110°C for 4 h [13]. TFA was removed by repeated co-evaporation with the addition of MeOH. The hydrolysate was reduced with $NaBH_4$ (25 mg) at room temperature for 3 h, neutralised with AcOH, evaporated to dryness, and then acetylated with Ac_2O (100°C, 1 h). The consequent alditol acetates were analysed by GLC.

Methylation was carried out by the modified Ciucanu method as described by Needs. The permethylated polysaccharide was hydrolysed in 90% formic acid (100°C, 2 h) and then in 2 mol l^{-1} TFA (100°C, 4 h). The partially methylated sugars were reduced and acetylated as described in monosaccharide analysis, then analysed by GC-MS [14].

3.6 Partial acid hydrolysis

F4 (500 mg) was hydrolysed with 0.2 mol l^{-1} TFA at 100°C for 4 h. The mixture was evaporated to dryness, and the residue was dialysed against distilled water. The non-dialysate was lyophilised and yield **F4L** (210.0 mg). The dialysate was concentrated and separated on a Sephadex G-10 column (90 × 1.6 cm).

F5 (500 mg) was first hydrolysed as described above. The non-dialysate was lyophilised to yield **F5L** (231.5 mg). **F5L** (200 mg) was further hydrolysed with 0.4 mol l^{-1} TFA (100 ml) at 100°C for 4 h in sealed tubes. The mixture was evaporated to dryness, and the residue was dialysed against distilled water. The dialysate was concentrated and separated on a Sephadex G-10 column.

3.7 Lymphocyte proliferation test in vitro

The polysaccharide sample (10^{-3}-10^{-1} g l^{-1}) was incubated with mouse splenocytes in the presence of mitogen ConA (5 mg l^{-1}, for T lymphocyte proliferation) or LPS (20 mg l^{-1}, for B lymphocyte proliferation). After incubation for 44 h at 37°C in a humidified 5% CO_2 incubator, T and B lymphocytes proliferation was tested by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay. The absorption was measured by DG-3022 ELISA at 570 nm [15].

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