Structural Elucidation and Antitumor Activity of Polysaccharide AMP-1 from *Atractylodis macrocephalae K.*

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A polysaccharide named as AMP-1 was isolated from the root of *Atractylodis macrocephalae Koidz*, and purified by ethanol fractionation and gel filtration. The homogeneity of AMP-1 was determined by HPLC and capillary electrophoresis that gave a single peak. AMP-1 is composed of galactose and mannose in a molar ratio of 1.0:1.9. Its molecular weight is 3.8×10^3. The structure of glycan was elucidated by IR, ^{1}H NMR, ^{13}C NMR and methylation analysis, and on the basis of the results was suggested that AMP-1 contain a backbone of β-(1→2)-*D*-galactose residues with branches of single β-*D*-mannose residue being substituted at the *O*-6. Bioactivity assay of AMP-1 showed that it could inhibit growth of Sarcoma 180 and Lewis pulmonary carcinoma implanted in mice.

Keywords structure, polysaccharide, *Atractylodis macrocephalae K.*, antitumor

Introduction

Atractylodes macrocephala Koidz is a traditional Chinese medicine. It was reported that it has these activities: "(1) invigorating the spleen and benefiting vital energy; (2) depriving dampness and promoting diuresis; (3) strengthening superficies and antiperspiration".[1] Modern pharmacology studies showed that *Atractylodes macrocephala K.* can nutrient for the vital energy, stomachic and digestive disorder.[2] It is also used to release the quickening of womb and prevent abortion in traditional Chinese medicine.[3] Much attention has been paid to this herb and its constituents have been demonstrated to have the perfect effects on gastrointestinal movement.[4-7]

In order to confirm the above-mentioned functions, we report in this paper the results of chemical studies of a polysaccharide AMP-1 from *Atractylodis macrocephalae K.* and its antitumor activities.

Experimental

Material

Atractylodis macrocephalae K. was purchased from Xingchang in Zhejiang province, the roots of which were washed with water, sun-dried, and air-dried at room temperature. It was pulverized and the powder was passed through an 8 mm-mesh sieve. Sephadex-G-25 was purchased from Ammersham Pharmacia Biotech.

Isolation and purification of AMP-1

The dry powder of *Atractylodis macrocephalae K.* (200 g) was dispersed in 2000 mL of distilled water for 1 h, and then boiled for 1 h, and filtrated. The residue was further extracted with 400 mL of water at 100 ℃ for 1 h. The aqueous-extract was concentrated under diminished pressure at 50 ℃, and slowly added into 6 times volume of 95% ethanol to precipitate the crude polysaccharide. After centrifugation, the precipitate was dissolved in 400 mL of water, and dialyzed against water for 3 d. The supernatant was freeze-dried. The crude polysaccharide was named AMP-O_2, which was further fractionated by 2 times volume of 95% alcohol to precipitate (AMP-O_2-I). The supernatant was treated by 2 times volume of 95% alcohol again, and the precipitate was dissolved in a small quantity of water and dialyzed, named AMP-O_2-II. AMP-1 was obtained and purified by chromatography in a Sephadex-G-25 column (1.5 cm × 110 cm), which was eluted with a 0.1 mol/L NaCl at a flow of 0.3 mL/min.

Carbohydrate analysis

Carbohydrate content was determined by the phenol-sulfuric acid method. Component sugars were determined by capillary gas-liquid chromatography [3% OV-225 capillary column (0.32 mm × 30 m), Varian VISTA 402] of alditol acetate derivatives after complete hydrolysis by 1.0 mol/L H_2SO_4 at 100 ℃ for 4 h. The molar ratio was calculated from the peak area of each component. The molecular weight of the polysaccharide was estimated by ESI-MS and HPLC (Shimadzu LC-10AD, RI detector) with TSK exclusion column. The methylated polysaccharide was confirmed by an IR spectrometer (Bio-Rad FTS 185). Gas-liquid chromatography-mass spectrum (GC-MS) of the alditol acetates of partially methylated sugar was done with a Shimadzu QP 5000 sprectrometer equipped with OV-17 capillary column (0.30 mm ×

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Received March 4, 2002; revised June 4, 2002; accepted September 8, 2002.

25 m), at a temperature programmed from 140 ℃ to 200 ℃ at 5 ℃/min, increasing to 300 ℃ at 10 ℃ /min. The polysaccharide was dissolved in D_2O and ^1H NMR and ^{13}C NMR spectra were determined at room temperature (Bruker-MX-400 spectrometer).

Methylation analysis

The sample of polysaccharide (5 mg) was dissolved in dimethyl sulfoxide (2 mL) under nitrogen, and methylated by treatment with NaOH powder (100 mg), then with methyl iodide (1.5 mL) for 2 h at 25 ℃ by the method of Needs.[8] The reaction mixture was extracted with trichloromethane, then trichloromethane was removed by evaporation. The completeness of methylation was comfirmed by the disappearance of the OH (3300—3600 cm^{-1}) in the infrared spectrum. The fully methylated polysaccharide (5 mg) was hydrolyzed with 90% formic acid (2 mL, 3 h at 100 ℃) and with 3 mL of trifluoroacetic acid (TFA, 2 mol / L) for 6 h at 100 ℃. The partially methylated sugars in the hydrolysate reacted with sodium borohydride and acetylated by acetic anhydride, and the resulting mixture of alditol acetates was analyzed by GLC and GC-MS.[9]

Assay of antitumor activity

Assay of the antitumor activities of polysaccharide was done by the method of Yu.[10] $C_{57}BL$ /C mice weighting about 20 g were obtained from Shanghai Animal Center of Chinese Academy of Sciences, were used for the antitumor assay. Sarcoma 180 cells (5×10^6/mL) were transplanted subcutaneously into the right groins of the mice. The test samples were dissolved in 0.9% NaCl solution and injected intraperitoneally daily for 8 d (injection volume, 0.2 mL), starting 24 h after tumor implantation. All mice were kept under observation for 2 weeks and then killed for final evaluation of the effects of treatment on tumor growth. Tumors were excised and weighted. The growth inhibition ratio was calculated by the following equation:

$$\text{Inhibition ratio } (\%) = 100(A - B)/A$$

where A is the average tumor weight of the control group and B is that of the treated group. Furthermore, the effect of AMP-1 on Lewis pulmonary carcinoma was investigated by the same methods of Sarcoma 180.

Results and discussion

Isolation, purification and physico-chemical properties

The dry powder of *Atractylodis macrocephalae K.* (200 g) was treated with boiling water and ethanol to give the crude polysaccharide AMP-O_2 (28.36 g). AMP-O_2 (5 g) was fractionated by different concentration alcohol and produced AMP-O_2-I (yield, 1.97 g) and AMP-O_2-II (yield, 0.85 g). AMP-O_2-II (110 mg) was further purified on a Sephadex-G-25 column to afford a homogeneity polysaccharide named as AMP-1 (82 mg) (Fig. 1). The homogeneity of AMP-1 was detected by HPLC and CE that gave a single peak.

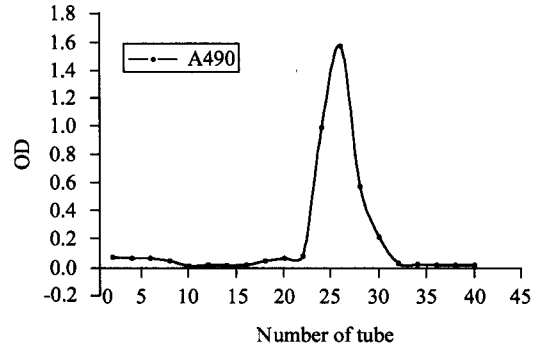

Fig. 1 Gel chromatography of AMP-1 on Sephadex-G-25 (1.5 × 110 cm) (eluted with 0.1 mol/L NaCl, and each eluted fraction was detected by the phenol-H_2SO_4 method).

The molecular weight of AMP-1 was 3.8 kDa, determined by ESI-MS and TSK column (using T-dextran as standard). Elemental analysis was C 40.06%, H 6.19% and N 0%. Monosaccharide composition of AMP-1 was determined by gas chromatography. It contains *D*-mannose and *D*-galactose in a molar ratio of 1.0:1.9.

^1H NMR, ^{13}C NMR *spectra of* AMP-1

In order to obtain more detailed structural information, ^1H NMR and ^{13}C NMR spectra of AMP-1 were measured, and the results are showed in Fig. 2 and Fig. 3. Anomeric H chemical shifts ($\delta < 5.0$) indicated that it should contain

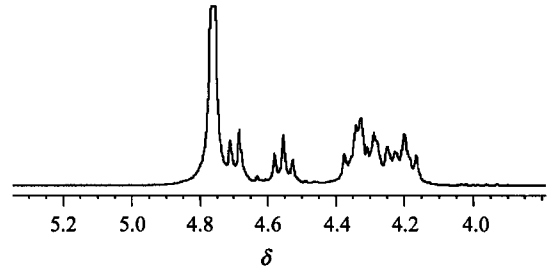

Fig. 2 ^1H NMR spectrum of AMP-1.

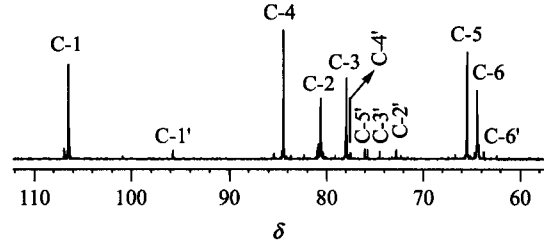

Fig. 3 ^{13}C NMR spectrum of AMP-1.

glycosidic linkages of β-type.[11] ^{13}C NMR spectra: δ_1 106.4 (C-1), 84.4 (C-4), 80.7 (C-2), 78.0 (C-3), 65.4 (C-5), 64.3 (C-6) and δ_2 95.7 (C-1′), 76.0 (C-4′), 75.7 (C-5′), 74.5 (C-3′), 72.8 (C-2′), 63.7 (C-6′) showed that AMP-1 was probably β-*D*-galactopyranosyl residues (δ_1) and a small amount of β-*D*-mannopyranosyl residues (δ_2).

Linkage analysis

The linkage of monosaccharides in AMP-1 was determined by GC-MS, and the results of linkage analysis are shown in Table 1.

The identification of 2, 3, 4, 6-tetra-*O*-methyl-mannose indicated that mannose residue was present as non-reducing terminal, and the analysis of 3, 4-di-*O*-methyl galactose showed the presence of branched galactopyranosyl residues. The results indicate that AMP-1 contain a backbone of β-(1→2)-*D*-galactopyranosyl residues with branches of single β-*D*-mannose residue being substituted at the *O*-6 (Scheme 1).

Effect of AMP-1 *on Sarcoma* 180 *and Lewis pulmonary carcinoma in* C57 BL/C *mice*

The ascites cells of Sarcoma 180 or Lewis pulmonary carcinoma (2×10^6 cells/0.2 mL) were inoculated into the right groin (or the right armpit) of mice subcutaneously. Two doses of AMP-1 were administered intraperitonearlly (ip) every other day for 8 d after the tumor inoculation. As shown in Table 2 and Table 3, this results show that AMP-1 has significant antitumor activity at the dose of 100 mg/kg and 200 mg/kg.

Acknowledgements

Anthors are greatfully thankful for supports of Dr. PENG, Xuemei, CHEN, Xiaoming and ZHANG, Jian.

Table 1 GC-MS of alditol acetate derivatives from the methylated product of AMP-1

GC peak (No.)	Mathylated sugar (as alditol acetates)	Primary mass fragment (*m/z*)	Molar percentage	Mode of linkage
1	2,3,4,6-Me_4Man	43, 71, 101, 117, 129, 145, 161, 205	2.0	[Manp] 1→
2	3,4,6-Me_3Gal	43, 87, 129, 145, 161, 189	2.9	→2[Galp]1→
3	3,4-Me_2Gal	43, 87, 129, 189,	2.0	→2,6[Galp]1→

Scheme 1

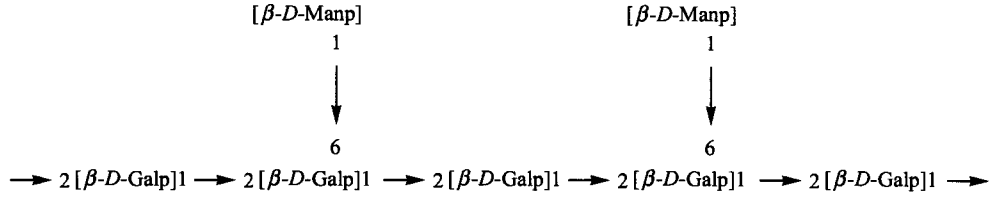

Table 2 Effect of AMP-1 on the inhibition of Sarcoma 180 growth in mice

Sample	Dose (mg/kg)	Administration method	No. of mice	Tumor weight (g)[a]	Inhibition ratio
AMP-1	100	ip × 8 d	10	1.53 ± 0.17	34.61[b]
AMP-1	200	ip × 8 d	10	1.41 ± 0.19	39.74[b]
NS (as control)		ip × 8 d	10	2.34 ± 0.29	

[a] Antitumor activity was expressed as mean ± SD. [b] $P < 0.01$: significantly different from AMP-1 with NS group.

Table 3 Effect of AMP-1 on the inhibition of Lewis pulmonary carcinoma growth in mice

Sample	Dose (mg/kg)	Administration method	No. of mice	Tumor weight (g)[a]	Inhibition ratio
AMP-1	100	ip × 8 d	10	0.51 ± 0.08	49.00[b]
AMP-1	200	ip × 8 d	10	0.48 ± 0.11	51.8[b]
NS (as control)		ip × 8 d	10	2.34 ± 0.29	

[a] Antitumor activity was expressed as mean ± SD. [b] $P < 0.01$: significantly different from AMP-1 with NS group.

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(E0203041 ZHAO, X. J.; FAN, Y. Y.)