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Structural characterization and immunological activity of two cold-water extractable polysaccharides from *Cistanche deserticola* Y. C. Ma

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Abstract—Two major polysaccharide fractions, CDA-1A and CDA-3B, were isolated from the cold-water extract of *Cistanche deserticola* Y. C. Ma, a holoparasitic plant and a valuable traditional Chinese medicine, using anion-exchange chromatography on DEAE-cellulose and gel-permeation chromatography on Sephacryl S-300 and Sephadex G-150. Their major structural features were elucidated using component and linkage analyses, periodate oxidation, Smith degradation, partial acid hydrolysis, and NMR spectroscopy. The results indicated that CDA-1A is an α-(1→4)-D-glucan with α-(1→6)-linked branches attached to the O-6 of branch points and that CDA-3B is an RG-I polysaccharide containing a typical rhamnogalacturonan backbone and arabinogalactan or arabinan branches. Bioactivity tests showed that CDA-1A is inert for T-cell proliferation stimulation but active for B-cell proliferation, while CDA-3B is potent for the stimulation of both T- and B-cell proliferation.

Keywords: Polysacchcaride; α-D-Glucan; Arabinogalactan; Rhamnogalacturonan I; *Cistanche deserticola*; Immunological activity

1. Introduction

Cistanche deserticola is a parasitic plant (Orobanchaceae) that is attached underground to the roots of the dicotyledonous plant *Haloxylon ammodendron* and grows by absorbing nutrients from the host plant. The parasite is distributed mainly in the desert region of northwestern China. As a rare traditional medicinal herb, the dried whole plant is used for the treatment of kidney pain, gynaecological diseases, intestinal infection and constipation.[1] Due to its wide use in folk medicine and consequent overexploitation, the natural source of this plant has become rare and has been listed in China as a class II endangered species. Various phenylethanoid glycosides have been isolated from *C. deserticola* and related species (*Cistanche tubolosa* and *Cistanche salsa*), and they were shown to exhibit anti-stress, antioxidant,[2,3] and hepatoprotective activity.[4] Naran et al. have carried out a preliminary study on its carbohydrate polymers[5] and have reported a starch-like α-(1→4)-glucan, an α-L-arabino-3,6-β-galactan, pectic polysaccharides and a 4-*O*-methyl-D-glucurono-D-xylan according to sugar composition and linkage analysis. A linear (1→4)(1→6)-α-D-glucan has also been reported from *C. deserticola*.[6] Pharmacological studies showed that the crude polysaccharide fractions from *C. deserticola* have anti-aging and immuno-stimulating effects,[7,8] but it was not clear which types of polysaccharides are responsible for such activities. In order to define the immunoactive principle, we made further studies on the structure and immunomodulatory activity of the polysaccharides isolated from *C. deserticola*. In this communication, we report on the structural features of two polysaccharides isolated from a cold-water extract and their immunostimulating activities.

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2. Results

2.1. Isolation and purification of polysaccharides

From 0.8 kg of EtOH-treated material (corresponding to 0.92 kg of material), 15.0 g (1.63%) of CDA was isolated. It contains neutral carbohydrate 49.6%, protein 44.0%, and uronic acid 6.3%, indicating the presence of a high content of water-soluble protein. Sugar analysis showed that CDA is composed of rhamnose, arabinose, galactose, and glucose, in a ratio of 22.5:10.0:8.3:59.2. CDA was treated with α-amylase, and the nondialysate fraction contained rhamnose, arabinose, xylose, galactose, and glucose, in a ratio of 20.6:16.4:1.4:14.6:46.9 (mol %). Although the amount of glucose decreased significantly, it remained as the predominant component, indicating that only a small fraction of glucose residues exists as starch-like glucan.

Upon anion-exchange chromatography on a DEAE-cellulose column, six fractions were obtained from CDA by stepwise elution with water, aqueous NaCl, and 0.1 M NaOH (Fig. 1). The sugar compositions of these fractions were determined and are shown in Table 1. All these fractions contain a significant amount of glucose. The fractions that eluted with low concentrations of NaCl (CDA-2 and CDA-3) contain arabinose and galactose as the major component, indicating the presence of arabinogalactan-type polysaccharides. CDA-4, eluted with 0.4 M NaCl, has a high proportion of rhamnose and uronic acid, indicating a rhamnogalacturonan I fraction, and also contains a substantial amount of glucose, probably from contaminating glucan. CDA-5 and CDA-6 both contain high amounts of proteins as their major component (75.5% for CDA-5; 68.1% for CDA-6), and both contain rhamnose and glucose as the major neutral sugars. Unexpectedly the 3-phenylphenol (*m*-hydroxybiphenyl) method showed a low uronic acid content for CDA-5 and CDA-6. This seems to indicate that their strong affinity on anion-exchanger is due to the high content of protein, which may associate tightly with the polysaccharide components via covalent or noncovalent interaction, and remains to be defined after interaction.

Table 1. Carbohydrate compositions of fractions isolated from CDA after anion-exchange chromatography

Fractions	Yield[a] (%)	Monosaccharide composition (mol %)					UA[b] (%)
		Rha	Ara	Xyl	Gal	Glc	
CDA-1	17.2	—	—	1.0	3.8	96.2	2.5
CDA-2	3.0	3.0	22.6	3.0	34.7	33.7	7.8
CDA-3	5.2	6.1	52.5	2.6	26.4	12.4	16.3
CDA-4	4.5	20.7	11.2	—	8.9	59.2	21.1
CDA-5	5.0	35.8	8.4	—	—	55.9	2.3
CDA-6	11.0	33.3	—	—	—	66.7	2.5

[a] Yield based on CDA applied to a DEAE-cellulose column.
[b] Uronic acid content was determined by the 3-phenylphenol (*m*-hydroxybiphenyl) method.

2.2. Structural investigation of CDA-1A

CDA-1A was the major polysaccharide isolated from the water-eluted fraction CDA-1. It showed a single symmetrical peak on high-performance gel-permeation chromatography (HPGPC) and was estimated to have a molecular weight of 1.0×10^4. Its specific rotation $[\alpha]_D$ is +150 (*c* 0.5, H_2O). Composition analysis showed that it is composed of only glucose. Methylation analysis showed that it consists of 1-, 1,4-, 1,6- 1,4,6-linked glucose, in the ratio of 1:5.8:3.5:0.8. It gave a purple color reaction to I_2–KI reagent, indicating a probable starch-like structure, with the backbone containing consecutively 1,4-linked α-D-glucosyl residues. The presence of terminal and 1,4,6-linked residues indicated a branched structure for the polysaccharide. The 1,6-linked residues are proposed to exist in the branches. If not, they may interfere with the formation of a helix conformation, which is a structural prerequisite for a positive iodine reaction.

Upon periodate oxidation the resulting polyhydroxyl derivative was hydrolyzed into glycerol and erythritol,

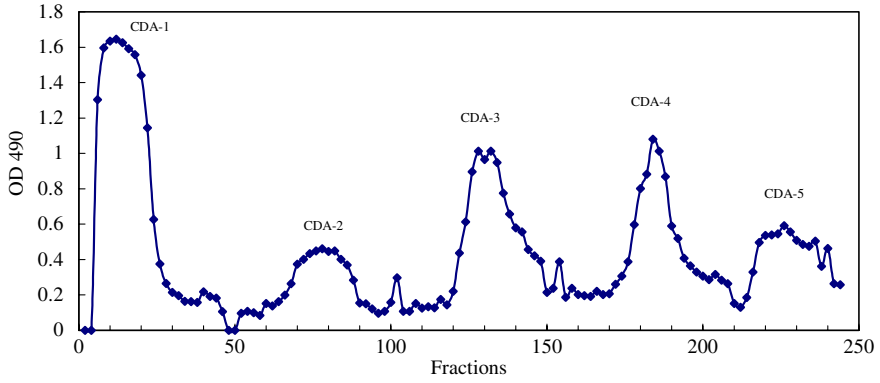


Figure 1. Elution profile of CDA on DEAE-cellulose column. Eluate was detected with phenol–sulfuric acid method. CDA-6 (0.1 M NaOH fraction) was not shown.

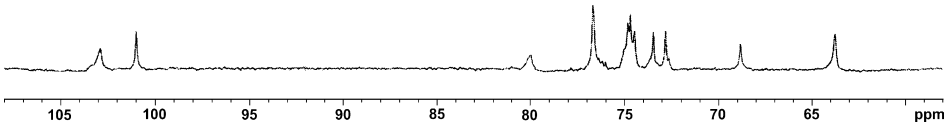


Figure 2. ^{13}C NMR spectrum of CDA-1A (in D_2O).

in a ratio of 0.80:1.00, in approximate agreement with the expected value (0.70:1.00) calculated based on methylation analysis. The ^{13}C NMR spectrum (Fig. 2) showed two strong signals at δ 102.89 and 100.98 ppm. The former was broad and assigned to 1,4- and 1,4,6-linked glucose, and the latter was sharp and assigned to 1,6-linked glucose due to the transglycosidic correlation of its corresponding proton signal (δ 5.02 ppm) to C-6 (δ 68.82 ppm) of 1,6-linked residues in HMBC (not shown). The chemical shifts of the anomeric signals also indicated an α anomeric configuration for all the glucosyl residues.

2.3. Structural investigation of CDA-3B

CDA-3B was separated from the 0.2 M NaCl-eluted fraction CDA-3 by gel-permeation chromatography. It was estimated to have an average molecular weight of 8.7×10^5, and its specific rotation $[\alpha]_D$ is -23.4 (c 0.5, H_2O). The native polysaccharide contains 74.0% neutral carbohydrate, 6.7% protein, and 12.3% uronic acid. The native CDA-3B was composed of rhamnose, arabinose, galactose, and glucose, in a ratio of 0.23:1.99:1.00:0.47. After carboxyl reduction, the reduced CDA-3B consists of rhamnose, arabinose, galactose, and glucose in a ratio of 0.31:1.99:1.28:0.22. Taken together, these data indicate that the polysaccharide should contain rhamnose, arabinose, galactose, glucose, and galacturonic acid in a ratio of 0.31:1.99:1.00:0.22:0.28.

Glycosidic linkages, as revealed by methylation analysis for native and carboxyl-reduced CDA-3B, are shown in Table 2. For the native polysaccharide, the arabinose residues are 1-, 1,5-, and 1,3,5-linked, and the galactose residues are 1,3-, 1,4-, 1,6-, and 1,3,6-linked. The high proportion of nonreducing terminals (1-linked arabinose) and branch points (1,3,5-linked arabinose and 1,3,6-linked galactose) indicate that the polysaccharide possesses a highly branched structure. In contrast with CDA-3B the carboxyl-reduced polysaccharide CDA-3BR showed an increased content of 1,4-linked galactose residues, indicating that the galacturonic acid is 1,4-linked, as reported in other pectic polysaccharides.

Table 2. Linkage analysis for native CDA-3B and carboxyl-reduced CDA-3BR

Methylated sugars	rt (min)	Molar ratios (mol %)		Linkages
		CDA-3B	CDA-3BR	
2,3,5-Me_3-Ara	9.11	21.4	25.2	1-
2,3-Me_2-Ara	12.82	14.4	16.7	1,5-
2-Me-Ara	15.33	14.8	14.5	1,3,5-
3-Me-Rha	15.76	2.1	8.1	1,2,4-
2,3,6-Me_3-Gal	17.07	1.7	10.3	1,4-
2,3,4-Me_3-Gal	17.93	5.1	5.4	1,6-
2,4,6-Me_3-Gal	17.81	2.0	3.8	1,3-
2,4-Me_2-Gal	21.78	11.3	10.5	1,3,6-
2,3,6-Me_3-Glc	14.84	2.2	5.5	1,4-

CDA-3B showed six anomeric signals in its ^1H NMR spectrum (not shown), including three strong resonances (δ 5.20, 5.16, 5.14 ppm) and three weak signals (δ 5.29, 5.02, and 4.55 ppm). Due to the high amount of arabinose in CDA-3B, the three strong signals were assigned to H-1 of the α-L-arabinose residues. The signal at δ 5.29 ppm may arise from the contaminating α-D-glucose residues, and the one at δ 5.02 was ascribed to α-D-GalA. The broad signal at δ 4.55 ppm corresponds to galactose residues, indicating their β anomeric configuration. The two signals at δ 1.35 and 1.30 ppm could be attributed to the H-6 of rhamnose residues under different chemical environments. The ^{13}C NMR spectrum of CDA-3B, as shown in Figure 2, also showed six signals in the anomeric region. Two strong signals at δ 106.66 and 106.33 ppm and a weak signal at δ 108.49 ppm were assigned to α-L-arabinofuranose residues with 1-, 1,5-, and 1,3,5-linkages, respectively. The two signals at δ 103.61 and 102.71 ppm could be ascribed to 1,6-, 1,3-, and 1,3,6-linked β-D-galactose, and the signal at δ 96.93 ppm may arise from 1,4-linked GalA (Fig. 3).

Upon periodate oxidation of CDA-3B (30 mg), the reaction was complete in 96 h. The resulting polyhydroxylated compound (27 mg), after hydrolysis, released glycerol, erythritol, rhamnose, arabinose, and galactose, in a ratio of 1.25:0.61:0.51:1.56:1.00. The high content of glycerol arose from terminal and 1,5-linked arabinose. Glucose residues were completely removed, in agreement with the 1,4-glycosidic linkage as shown by methylation analysis. The polyhydroxylated compound (27 mg) was subjected to Smith degradation, and the nondialysable fraction (CDA-3B-SDI: 5.2 mg) was found to contain rhamnose, arabinose, and galactose in a ratio of 0.20:0.08:1.00, corresponding to a 1,3-linked galactan core attached to a backbone of rhamnose residues. The arabinose residues were almost completely removed, suggesting that 1,3,5-linked arabinose was probably not directly attached to 1,3-linked galactosyl side chains, and may be distributed in a separate arabinan side chain as the branch point.

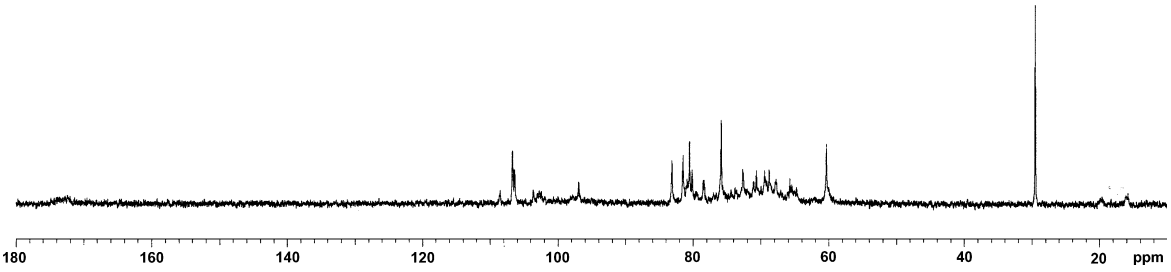


Figure 3. ^{13}C NMR spectrum of CDA-3B (in D_2O, with acetone as internal standard).

In order to determine the backbone structure, CDA-3B was subjected to mild acid hydrolysis that released dialysate PHIA and nondialysate PHIB. PHIA was fractionated into PHIA-1, PHIA-2, and PHIA-3 by gel-permeation chromatography on Sephadex G-10, and their glycosyl compositions are shown in Table 3. PHIA-3, corresponding to a monosaccharide fraction by its retention time, consisted almost exclusively of arabinose. PHIA-2, the disaccharide fraction, also contained mainly arabinose as the component sugar, indicating that it is a mixture of disaccharides, Ara–Ara and Ara–Gal. PHIB remained the major portion as a modified polysaccharide, with a MW of 5.0×10^5. These results indicate that arabinose residues are distributed in branches, so their removal did not break the backbone, leading to a significant, but not a great reduction in MW.

PHIB was subjected to a second mild hydrolysis. The dialysate was separated into three fractions, PHIIA-1, PHIIA-2, PHIIA-3, which were all shown to contain a significant amount of rhamnose, besides a high content of arabinose and galactose, indicating the stronger acidic conditions (0.1 M TFA) had not only led to the removal of more arabinose and galactose, but also hydrolyzed some backbone rhamnose as well. The derived polysaccharide PHIIB showed a high content of rhamnose, galactose, and galacturonic acid and small amount of arabinose, indicating the presence of a rhamnogalacturonan backbone, to which is attached arabinogalactan or arabinan branches.

Table 3. Yield and sugar compositions of the fractions released from partial acid hydrolysis of CDA-3B

Fractions	Yield (mg)	Monosaccharide composition (mol %)			
		Rha	Ara	Gal	Glc
PHIA-1	8	15.8	33.7	33.7	16.8
PHIA-2	2	—	89.7	10.3	—
PHIA-3	4	—	98.0	2.0	—
PHIB	21	10.0	51.2	30.3	8.5
PHIIA-1	3	13.8	5.0	62.5	18.8
PHIIA-2	1	8.6	10.1	71.9	9.4
PHIIA-3	2	7.6	33.5	58.5	—
PHIIB	9	19.2	2.6	51.8	26.4

2.4. Immunological activity

CDA-1A and CDA-3B were both subjected to immune tests to evaluate their effect on ConA-induced T-cell proliferation and LPS-induced B-cell proliferation. The results (Table 4) show that CDA-1A is inactive for T-cell proliferation but active for B-cell proliferation, while CDA-3B is effective both for T- and B-cell proliferation when the concentration is 10 μg/mL or higher.

3. Discussion

As a desert-distributed parasitic plant, *C. deserticola*, in order to make itself adaptable to the peculiar environment, must be different in physiology from the general green plants that grow by photosynthesis. One aim of this study is to find a possible difference in polysaccharide composition or structure. Our results indicate some distinct structural features for the polysaccharides of *C. deserticola*. Starch is the common α-D-glucan that serves as an energy reserve in different organs of a variety of green plants. As an α-D-glucan, CDA-1A has a 1,4-linked backbone with branches that are different from those of common starches by a 1, 6-glycosidic linkage, although it shows a positive iodine reaction. RG-I type pectic polysaccharides with repeating →α-L-Rha*p*-(1→4)-α-D-Gal*p*-(1→ backbone and arabinogalactan branches have often been isolated from a variety of green plants. CDA-3B shows some structural features that are the same as those reported for pectic arabinogalactans from other plant sources,[9,10] indicating a similarity in its biosynthesis to the pectic polysaccharides of other sources. Distinct from other reported arabinogalactan fractions, CDA-3B shows a high ratio (2:1) of arabinose to galactose, and all of its rhamnose residues are substituted with branches.

Many different types of polysaccharides with various immunomodulatory activities have been isolated and characterized. Among those most frequently reported are the branched pectic polysaccharides such as rhamnogalacturonan I and arabinogalactans.[9,10] In the in vitro immune tests on mitogen-induced lymphocyte proliferation, CDA-3B, a typical highly branched rhamnogalac-

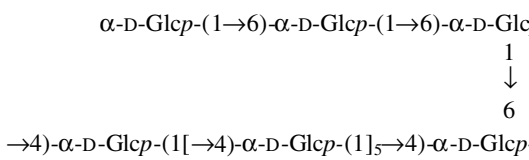
Table 4. Effects of CDA-1A and CDA-3B on ConA-induced T-cell proliferation and LPS-induced B-cell proliferation

Samples	Dose (μg/mL)	ConA (T-cell)		LPS (B-cell)	
		Mean ± SD	Percent[a]	Mean ± SD	Percent[a]
Control	Medium	2860 ± 170		2898 ± 41	
	ConA, LPS	99,926 ± 3789		27,765 ± 642	
CDA-1A	1	108,996 ± 5926	9	28,528 ± 1240	3
	10	109,910 ± 3670	10	33,438 ± 1204	20
	100	111,429 ± 9715	12	40,163 ± 3547	45
CDA-3B	1	122,331 ± 8782	22	28,627 ± 7	3
	10	126,503 ± 3167	27	40,756 ± 3531	47
	100	156,159 ± 3721	56	53,975 ± 12,434	94

[a] The sample is effective when the percentage is greater than 15%.

turonan I, showed significant stimulating activity to both T- and B-cell proliferation. CDA-1A, a neutral α-D-glucan with 1,6-linked branches, showed stimulating activity toward LPS-induced B-cell proliferation but no effect in T-cell proliferation. Moreover we cannot exclude the possibility that the lymphocyte proliferation effect of the polysaccharide on LPS-stimulated proliferation may be mediated by stimulation of T-cells, as the whole population of splenocytes was used in the experiments. As reported for other polysaccharides,[11,12] the peripheral parts of the branched structure of the two polysaccharides probably play an important role in the expression of immunological activity.

In this study we have isolated two different polysaccharides, namely CDA-1A and CDA-3B, from CDA, the cold-water extractable preparation of the roots of *C. deserticola*. CDA-1A was obtained from the water eluate on anion-exchange chromatography and found to be an α-D-glucan that has a 1,4-linked glucosyl backbone, with a 1,6-linked trisaccharide as branch, attached to O-6 of every seventh backbone residue, which is shown below. The structure we propose here is different from the linear glucan as reported by Wu and Tu,[6] and thus is a novel polysaccharide isolated from *C. deserticola* as in the following:



The in vitro lymphocyte proliferation stimulating effect of the two polysaccharides was tested. CDA-1A showed a stimulating effect to LPS-induced B-cell proliferation, while CDA-3B is effective both for T- and B-cell proliferation at the higher concentration ($\geqslant$10 μg/mL).

CDA-3B was isolated from 0.2 M NaCl eluate on anion-exchange chromatography, and structural investigation showed that it possesses a rhamnogalacturonan backbone as the core. To the O-4 of the 1,2,4-linked rhamnose are attached the arabinogalactan branches. The arabinogalactan moiety has a type II branching pattern, possessing a 1,3-linked β-D-galactan backbone, with 1- and 1,5-linked arabinosyl or 1,6-linked galactosyl branches attached to O-6 of 1,3,6-linked galactose residues. It is also probable that some arabinose residues exist as separate O-3-branched (1→5)-α-L-arabinan side chains.

4. Experimental

4.1. Materials

The crude drug of *C. deserticola* was purchased from the local market as dried slices (Shanghai Xuhui Herb Slices Co. Ltd.), and the original plant was collected in the Inner Mongolia region of China. A voucher specimen was deposited in the herbarium of Shanghai Institute of Materia Medica. Sephacryl S-300, Sephadex G-150, and Sephadex G-10 were purchased from Amersham Biosciences. DEAE-cellulose 32 was from Whatman Co. Standard monosaccharides, sodium borohydride, iodomethane, and *N*-cyclohexyl-*N*′-(2-morpholinoethyl)-carbodiimide methyl-*p*-toluenesulfonate (CMC) were all Fluka products. Dimethyl sulfoxide and PEI-cellulose TLC plates were E. Merck products. α-Amylase (E.C. 3.2.1.1, from *Aspergillus oryzae*) was purchased from Sigma. All the reagents used were of analytical grade.

4.2. General methods

All evaporations were carried out at <40 °C under reduced pressure. Protein was determined by the Lowry method with bovine serum albumin as standard.[13] Total carbohydrates were determined by the phenol–sulfuric acid method,[14] with glucose as standard. Uronic acid content was determined by the 3-phenylphenol (*m*-hydroxybiphenyl) method,[15] with D-glucuronic acid as standard. TLC was performed by the ascending method on PEI-cellulose plates (E. Merck Co.), developed with 5:5:1:3 EtOAc–pyridine–HOAc–H_2O. Reducing sugars were visualized with aniline–*o*-phthalic acid reagent.[16]

IR spectra were determined with a Perkin–Elmer 591B spectrophotometer as KBr pellets (native polysaccharides) or Nujol films (permethylated polysaccharides). The optical rotations were determined with a WZZ-1S polarimeter (Shanghai Physical Optics Co.). GLC analyses were conducted on a Shimadzu GC-14B instrument equipped with a 3% OV-225-packed glass column (3.2 mm × 2 m) and an FID detector. The column temperature was kept at 210 °C for sugar analysis and at 190 °C for methylation analysis, and the carrier gas was N_2 at a flow rate of 25 mL/min. The injection and detection temperatures were 250 °C and 240 °C, respectively.

4.3. Extraction and fractionation

The dried slices of *C. deserticola* (2.3 kg) were refluxed in a Soxhlet extraction apparatus for 16 h to remove lipids. The residue was air-dried (2.0 kg), and 0.8 kg of the residue was extracted at rt with distilled water (25 L) for 6 h under intermittent stirring. The extract was concentrated to 5 L and dialyzed against running water. The nondialysate was concentrated to 2 L, centrifuged, and three volumes of 95% EtOH were added to the supernatant under vigorous stirring. The precipitate was collected by centrifugation, washed successively with absolute EtOH and CH_3COCH_3 and dried in a vacuum at 40 °C, giving a grayish powder of CDA (15.0 g). CDA (6.0 g) was dissolved in distilled water (60 mL) and applied to a DEAE-cellulose 32 column (50 cm × 5.0 cm, Cl^-), which was eluted stepwise with H_2O, 0.1, 0.2, 0.4, and 0.8 M NaCl, and then with 0.1 M NaOH, detected by the phenol–sulfuric acid method, giving CDA-1 (1.03 g), CDA-2 (0.18 g), CDA-3 (0.31 g), CDA-4 (0.27 g), CDA-5 (0.30 g), and CDA-6 (0.66 g), respectively.

CDA-1 (500 mg) was rechromatographed on a DEAE-cellulose column (60 cm × 2.6 cm), eluted with distilled water, and the resulting fraction was applied to a Sephacryl S-300 column (90 × 2.6 cm), equilibrated and eluted with 0.1 M NaCl, giving only one major fraction CDA-1S. CDA-1S was separated on a Sephadex G-150 column (90 × 2.6 cm), giving two fractions CDA-1A (87 mg) and CDA-1B (102 mg). CDA-3 (310 mg) was applied to a DEAE-cellulose column, eluted successively with distilled water and 0.2 M NaCl. The NaCl-elutable fraction was applied to a Sephadex G-150 column, giving CDA-3A (20 mg), CDA-3B (110 mg), and CDA-3C (53 mg).

4.4. Homogeneity and molecular weight

The homogeneity and molecular weight of polysaccharides were estimated using the HPGPC method,[12] which was performed on a Waters HPLC module consisting of a Model 515 pump, an RI detector (Model 2410) and a dual-wavelength UV detector (Model 2487). The column was a serially linked combination of an Ultrahydrogel™ 2000 and an Ultrahydrogel™ 500 column. 0.003 M NaOAc was used as the solvent for samples and as the eluent, with the flow rate kept at 0.5 mL/min. The column temperature was 30 ± 0.1 °C. The column was calibrated by reference to the MW-known T-series dextrans (T-700, T-580, T-110, T-80, T-40, T-11). Data were processed by GPC software (Millennium[32]).

4.5. Composition analysis

The polysaccharide (2 mg) was hydrolyzed in 2 M TFA (2 mL) at 110 °C for 1.8 h in a sealed test tube. After evaporation to completely remove TFA, the hydrolysate was dissolved in distilled water (0.15 mL), and 4 μL was analyzed by TLC on a precoated PEI-cellulose plate. The remaining hydrolysate was dissolved in H_2O (2 mL) and reduced with $NaBH_4$ (30 mg) at rt for 3 h. After neutralization with HOAc and evaporation to dryness, the residue was acetylated with Ac_2O at 100 °C for 1 h. The alditol acetates were subjected to GLC analysis. For the acidic polysaccharide the sample was carboxyl-reduced by CMC-mediated $NaBH_4$.[17]

4.6. NMR spectra

The sample (40 mg) was deuterium-exchanged and dissolved in 0.5 mL of D_2O (99.8% D). The ^1H and ^{13}C NMR spectra were measured at rt with a Bruker AM 400 NMR spectrometer, with acetone as internal standard (δ 31.50 ppm for carbon). All chemical shifts are reported downfield relative to Me_4Si.

4.7. Methylation analysis

Samples (5 mg) were dried overnight in vacuo (P_2O_5) at rt, then dissolved in 4 Å molecular sieve-dried dimethyl sulfoxide (1.5 mL), and methylated by the method of Ciucanu and Kerek as described by Needs.[18] The completeness of methylation was confirmed by the disappearance of the hydroxyl absorption in IR spectrum (Nujol). The permethylated polysaccharides were hydrolyzed, converted into the partially methylated alditol acetates and analyzed by GC–MS.[19]

4.8. Periodate oxidation and Smith degradation

The polysaccharide was dissolved in 0.015 M $NaIO_4$ and kept at 4 °C in the dark. The oxidation was monitored spectrometrically at 223 nm.[20] $NaIO_4$ consumption was calculated according to the change of absorbance at 223 nm. The production of HCO_2H was determined by titration with 0.01 N NaOH. The excess $NaIO_4$ was decomposed with ethylene glycol (0.2 mL), and the mix-

ture was dialyzed. The nondialysate was reduced with $NaBH_4$ and dialyzed. The nondialysate was freeze-dried, giving the polyhydroxylated derivative. For Smith degradation, a portion of the polyhydroxylated compound was hydrolyzed in 0.05 M TFA at rt for 24 h. The hydrolysate was dialyzed, and the dialysate (SDO) and nondialysate (SDI) were both freeze-dried.

4.9. Partial acid hydrolysis

The polysaccharide CDA-3B (35 mg) was dissolved in 0.05 M TFA, hydrolyzed at 100 °C for 1 h, then dialyzed against distilled water (500 mL × 3). The dialysate and nondialysate were recovered, giving PHIA and PHIB (21 mg), respectively. PHIA was separated on a column of Sephadex G-10 (1.6 cm × 90 cm), eluted with distilled water, giving PHIA-1, 2, and 3. PHIB was further hydrolyzed in 0.1 M TFA at 100 °C for 1 h. The hydrolysate was dialyzed and separated in the same manner as described for PHIA, giving PHIIA-1, 2, and 3 as the dialysate fractions. PHIIB was obtained as the nondialysate.

4.10. Lymphocyte proliferation tests in vitro

The female BALB/c mice (6–8 weeks old) were sacrificed by cervical dislocation, and the spleens were removed aseptically. The splenocytes were prepared according to reported procedure.[21] The proliferation of cells was determined by the ^{3}H-TdR incorporation method.[22,23] The mouse splenocytes (4×10^5 cells/well) were incubated in the presence of mitogen ConA (5.0 μg/mL) or LPS (10.0 μg/mL) or in the absence of them, but with medium, only. The polysaccharide samples in different concentrations (1, 10, 100 μg/mL) were incubated with mouse splenocytes in the presence of ConA or LPS, respectively. After incubation for 44 h at 37 °C in a humidified 5% CO_2 incubator, each well was pulsed with 0.25 μCi/well ^{3}H-TdR (thymidine, [methyl-^{3}H]). The plate was further incubated for another 4 h. The cultured cells were harvested onto glass fiber filters. The radioactivity incorporated was determined with a Beta Scintillation Counter. ConA or LPS was used as a positive control, and the wells without either polysaccharide and ConA or LPS were the negative controls.

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References

1. Jiangsu Medical College *Dictionary of Traditional Chinese Medicine*; Shanghai People Publisher: Shanghai, 1977; p 895.
2. Xiong, Q.; Kadota, S.; Tani, T.; Namba, T. *Biol. Pharm. Bull.* **1996**, *19*, 1580–1585.
3. Xiong, Q.; Tezuka, Y.; Kaneko, T.; Li, H. Y.; Tran, L. Q.; Hase, K.; Namba, T.; Kadota, S. *Eur. J. Pharmacol.* **2000**, *400*, 133–144.
4. Xiong, Q.; Hase, K.; Tezuka, Y.; Tani, T.; Namba, T.; Kadota, S. *Planta Med.* **1998**, *64*, 120–125.
5. Naran, R.; Ebringerova, A.; Hromàdková, Z.; Patoprstý, V. *Phytochemistry* **1995**, *40*, 709–715.
6. Wu, X. M.; Tu, P. F. *J. Peking Univ. (Health Sciences)* **2004**, *36*, 24–26.
7. Sun, Y.; Wang, D. J.; Liu, X. M.; Shi, L.; Zhang, H. Q. *Chin. Pharmacol. Bull.* **2002**, *18*, 84–87.
8. Zeng, Q. L.; Mao, J. H.; Lu, Z. L. *J. Zhejiang Med. Univ.* **1998**, *27*, 108–111.
9. Nergard, C. S.; Matsumoto, T.; Inngjerdingen, M.; Inngjerdingen, K.; Hokputsa, S., et al. *Carbohydr. Res.* **2005**, *340*, 115–130.
10. Duan, J. Y.; Wang, X. S.; Dong, Q.; Fang, J. N.; Li, X. Y. *Carbohydr. Res.* **2003**, *338*, 1291–1297.
11. Nergard, C. S.; Kiyohara, H.; Reynolds, J. C.; Thomas-Oates, J. E.; Matsumoto, T., et al. *Carbohydr. Res.* **2005**, *340*, 1789–1801.
12. Sakulai, M. H.; Matsumoto, T.; Kiyohara, H.; Yamada, H. *Immunology* **1999**, *97*, 540–547.
13. Bensadoun, A.; Weinstein, D. *Anal. Biochem.* **1976**, *70*, 241–250.
14. Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. *Anal. Chem.* **1956**, *28*, 350–356.
15. Blumencrantz, N.; Asboe-Hansen, G. *Anal. Biochem.* **1973**, *54*, 484–489.
16. Zhang, W. J. *Biochemical Techniques in Complex Carbohydrates*, 2nd ed.; Zhejiang University Press: Hangzhou, 1996, p 23.
17. Taylor, R. L.; Conrad, H. E. *Biochemistry* **1972**, *11*, 1383–1388.
18. Needs, P. W.; Sevendran, R. R. *Carbohydr. Res.* **1993**, *245*, 1–10.
19. Dong, Q.; Ding, S. W.; Yang, X.; Fang, J. N. *Phytochemistry* **1999**, *50*, 81–84.
20. Dixon, J. S.; Lipkin, D. *Anal. Chem.* **1954**, *26*, 1092–1093.
21. Price, R. J.; Skilleter, D. N. *Arch. Toxicol.* **1985**, *56*, 207–211.
22. Heeg, K.; Reimann, J.; Kabelitz, D.; Hardt, C.; Wagner, H. *J. Immunol. Meth.* **1985**, *77*, 237–246.
23. Li, X. Y.; Wang, J. F.; Zhu, P. P.; Lin, L.; Ge, J. B.; Yang, S. X. *Acta Pharm. Sin.* **1990**, *11*, 542–545.