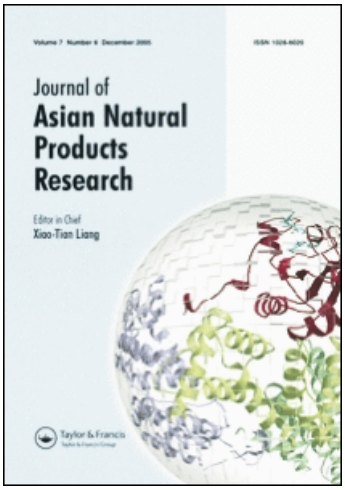

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Isolation and characterization of α-(1→6)-glucans from *Cistanche deserticola*

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Three unique polysaccharides (**1**–**3**) have been obtained from the 0.5 M NaOH extract of the stem of *Cistanche deserticola* Y. C. Ma. The results of methylation analysis, partial acid hydrolysis, ^{13}C, ^{1}H NMR, ^{1}H—^{1}H COSY, HMQC and HMBC spectroscopic analyses indicate that they are all composed of glucose, having a backbone of α-(1→6)-glucan, and have different molecular weights. Their structures differ from that of linear starch.

Keywords: *Cistanche deserticola*; Polysaccharide; α-(1→6)-Glucan

1. Introduction

Cistanche deserticola Y. C. Ma. belongs to the family Orobanchaceae. It is a holoparasite widely distributed in the northwest of China. The parasite is attached underground to the roots of the dicotyledonous plant *Haloxylon ammodendron* Bunge. As an important tonic in traditional Chinese medicine, the stem has long been used for kidney deficiency, female infertility, morbid leucorrhea, neurasthenin, and senile constipation due to colonic inertia, *etc*. in China and Japan. The major active components are reported to be lignans, phenylethanoid glycosides and iridoid glycosides [1,2], which have activities in regulating immunity function, anti-aging, curing constipation and so on.

We found the active polysaccharides in *C. deserticola* by pursuing the activity method. The results indicate that the 0.5 M NaOH extract of *C. deserticola* could stimulate the incrementation of the splenocyto of mice. In this paper, we report the isolation, properties, and structure elucidation of three polysaccharides from the 0.5 M NaOH extract of *C. deserticola*. The three polysaccharides were all α-(1→6)-glucan, but their molecular weights differ. Moreover, their spatial structures differ from that of starch.

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2. Results and discussion

A crude polysaccharide was obtained from the 0.5 M NaOH extract of the flesh stem of *C. deserticola*. After separation using DEAE-anion-exchange chromatography, a fraction was obtained from the water eluate. This was purified on Sephadex G-150 and Sephacry S-300 column to obtain three polysaccharides. Each of the three polysaccharides was eluted as a single symmetrical peak at different retention times by the HPSEC method. These data indicate that the three polysaccharides are homogeneous with different molecular weight (table 1). Their IR spectra all show an absorption at 840 cm^{-1} for the α configuration. After complete hydrolysis with 2 M trifluoroacetic acid (TFA), only glucose was observed on TLC. GLC analysis of the alditol acetates showed that **1**–**3** are only composed of glucose.

The three polysaccharides were first partially hydrolyzed with 0.04 M TFA at 100°C for 1 h. The products were then evaporated to dryness and dialyzed against distilled water. The nondialysate was determined as a single peak by HPSEC. Examination of alditol acetates of the product by GLC revealed only glucose. Methylation and spectral analysis indicate that the nondialysate is cyclic glucan. Its molecular weight is smaller than the original polysaccharide. The nondialysate was partially hydrolyzed with 0.1 M TFA at 100°C for 1 h, and the product was separated with Sephadex G-25 into three parts. Acid hydrolysis and methylation analysis indicate that they are linear (1→6)-glucans. Methylation analysis on **1**–**3** revealed only 1,6-linked glucopyranosyl residues and no terminal glucopyranosyl residue.

The ^{1}H and ^{13}C NMR spectra of the three polysaccharides are in good agreement with previous observations. The anomeric signals in the ^{1}H NMR spectrum have been assigned according to sugar composition and literature data [3–5]. A pair of doublets between δ 4.978 and 4.983 ($J = 2.5$ Hz) in ^{1}H NMR indicate that all D-Glcps residues have an α configuration. Furthermore, they all have a signal at δ 98.4 in the ^{13}C NMR spectra, confirming the α configuration of D-Glcp residues. The linkage at C-6 is confirmed by the signal at δ 66.2 for O-substituted C-6, while unsubstituted C-6 appears at δ 60.3 [6]. Furthermore, the HMBC spectrum shows three signals, confirming the correlation of C-6 (δ 66.2) and H-1 (δ 4.978–4.983). Other signals were assigned according to cross-peaks in the COSY and HMQC spectra or by comparison with literature values (tables 2 and 3). These data suggest that the three polysaccharides were α-(1→6)-glucan (figure 1). After reaction of the three polysaccharides with KI—I_2, no UV absorption at 500 nm was observed, indicating that their structures differ from linear starch, which may be related to linkage position and stereochemistry [7,8]. Nevertheless, the presence of only six resonances in the ^{13}C NMR and seven resonances in the ^{1}H NMR indicate that each glucosyl residue possesses the same average microenviroment [9].

Most α-glucans in natural plants or fungi have a short or long side-chain, and their backbones mainly consist of 1,4 or 1,3-linked glucosyl residues. The big molecular linear (1→6)-α-D-glucan without branching is infrequent. From *Cistanche deserticola* found in

Table 1. Molecular masses of polysaccharides **1**–**3**.

Compound	M_r
1	$>2 \times 10^6$
2	1.5×10^5
3	3.3×10^4

Table 2. ^{1}H NMR chemical shifts of polysaccharides **1**–**3** recorded in D_2O at room temperature.

Compound	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*	*H-6a,*	*H-6b*
1 1,6-α-glup	4.983, 4.978	3.589,3.583[a]	3.718, 3.699	3.541, 3.522	3.918, 3.902	3.997	3.763
		3.569,3.564[b]				3.982 d	3.738 d
	$J = 2.5$ d	$J^{a} = 3.0$	$J = 8.5$ d	3.503	$J = 8.0$ d		
		$J^{b} = 2.5$ dd		$J = 9.5$ t		$J = 7.5$	$J = 12.5$
2 1,6-α-glup	4.969 s	3.579, 3.574[a]	3.711, 3.693	3.532, 3.513	3.910, 3.897	3.756	
		3.561, 3.555[b]	$J = 9.0$ d	3.495 t	$J = 6.5$ d	3.989 d	3.731 d
		$J^{a} = 2.5$					
		$J^{b} = 3.0$ dd				$J = 8.0$	$J = 12.0$
3 1,6-α-glup	4.973, 4.967	3.577, 3.571[a]	3.709, 3.691	3.530, 3.511	3.910, 3.895	3.988	3.729
	$J = 3.0$ d	3.558, 3.552[b]	$J = 9.0$ d	3.493 t	$J = 7.5$ d	3.975 d	3.709 d
		$J^{a} = 3.0$					
		$J^{b} = 3.0$ dd				$J = 6.5$	$J = 10.0$

Table 3. ^{13}C NMR chemical shifts of polysaccharides **1**–**3** recorded in D_2O at room temperature.

Compound	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
1	98.410	72.117	74.137	70.252	70.905	66.212
2	98.410	72.102	74.106	70.221	70.890	66.212
3	98.425	72.117	74.122	70.221	70.890	66.228

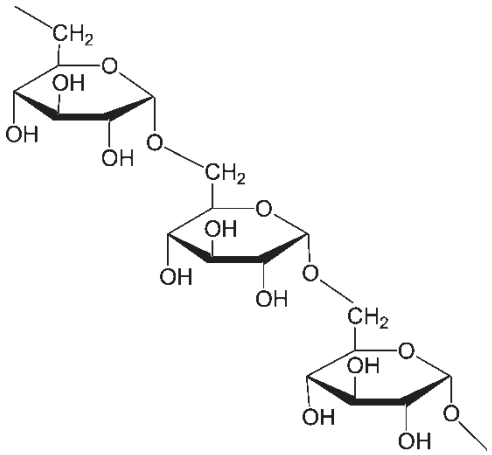

Figure 1. Possible repeating unit for cyclic α-(1 → 6)-glucan; each glucosyl residue refers to NMR assignment in Tables II and III.

Mongolia, the glucans are starch-like, having a (1→4)-α-D-glucan backbone, none contain (1→6)-α-D-glucan [10,11]. This indicates a notable effect of climate on the high molecular substances of *Cistanche deserticola*.

3. Experimental

3.1 General experimental procedures

Evaporations were performed below 40°C under reduced pressure. Optical rotation was measured with a W22-1S automatic polarimeter. IR spectra were determined with a Perkin–Elmer 599B spectrometer. GLC analyses were performed on an Agilent HP6890N instrument, equipped with a FID detector and a HP-5 column (30 m × 0.32 mm), and programmed from 160 to 250°C at 5°C min^{-1}. T-series Dextran, DEAE, and Sephadex G-150 were purchased from Pharmacia Co. All other reagents were of analytical grade, obtained in China. Uronic acid content was determined by the *m*-hydroxydiphenyl method. Protein contents were measured by Lowry's method.

3.2 Plant material

Stems of *Cistanche deserticola* Y. C. Ma. were cultivated in the desert of Xinjiang Province of China and purchased from TongRenTang Co. A voucher specimen of this plant has been deposited at the School of Pharmaceutical Sciences, Peking University.

3.3 Extraction and isolation

The stems (1500 g) of *Cistanche deserticola* were exhaustively extracted with 95% ethanol under reflux. The dried ethanol-insoluble residue was macerated (3 ×) with cold distilled water for 24 h. After extraction with cold water, the residue was extracted with hot water, and then macerated (3 ×) with 0.5 M NaOH. The 0.5 M NaOH extract was concentrated at 40°C under reduced pressure. This concentrated residue (46.5 g) was then extracted (3 ×) with 80% ethanol at ambient temperature. The resultant precipitate was dried at 40°C *in vacuo* (yield: 35.6 g). The protein of the precipitate was removed by the Sevags method [12]. After dissolution with distilled water, the residue (30.2 g) was then dialyzed with distilled water for three days. The nondialysate was concentrated and dried to yield the crude polysaccharide (25.6 g, 1.8% yield).

The crude polysaccharide (12 g) was dissolved in distilled water (160 mL, 8% w/v) and separated using a DEAE column (70 × 5 cm), eluting with a gradient 0–2 M NaCl, the water elute fraction (800 mg) of which was obtained. A sample (600 mg) was purified by column gel-permeation chromatography on a Sephadex G-150 column (80 × 4 cm, 1600 mL, 5 mL per fraction). Fractions of polysaccharide A and fractions of polysaccharide C were pooled, dialyzed and freeze-dried. After desalting by Sephadex G-25 (60 × 1 cm), polysaccharide 1 (98 mg) and polysaccharide (110 mg) were obtained and determined as a single peak by HPSGC. Fractions of polysaccharide B was purified by column gel-permeation chromatography on a Sephacry S-300 column (80 × 1 cm), and polysaccharide 2 (120 mg) was obtained.

3.4 Homogeneity and molecular weight

These were detected and determined by HPSEC performed on an Agilent 1100 series apparatus with a Shodex KS-805 column. The column was calibrated with T-series Dextran T-2000, T-500, T-200, T-70, T-40 and glucose (Pharmacia Co.). The sample concentration was 1% (w/v). Distilled water was used as solvent and eluent, and the flow rate was kept at 1.0 mL min^{-1}. A 20 μL aliquot was injected for each run.

3.5 Completed acid hydrolysis

The three polysaccharides (**1**–**3**, 5.6, 4.8 and 6.0 mg, respectively) were hydrolyzed with 2 M TFA (5 mL) at 121°C for 1 h. The hydrolysates were analyzed using TLC on a silica gel plate containing 5% sodium dihydrogen phosphate developed with 3.5:10:6:3.5:3:3 BuOH–EtOAc–isopropyl alcohol–HOAC–H_2O–pyridine. The plate was visualized by spraying with 1,3-naphthalenediol reagent and heating at 110°C for 10 min. The remaining hydrolysates were reduced with $NaBH_4$ (25 mg) at room temperature for 3 h, neutralized with AcOH, and evaporated to dryness, and then acetylated with Ac_2O (100°C, 1 h). The resulting alditol acetates were analyzed by GLC.

3.6 Methylation analyses

The three polysaccharides **1**–**3** (6.4, 6.5 and 6.8 mg) were each methylated three times by the Ciucanu method [13]. The completeness of methylation was confirmed by the disappearance

of the hydroxyl absorption in the IR spectrum. The permethylated polysaccharide was hydrolyzed in 90% formic acid, and then in 2 M TFA (100°C, 4 h). The partially methylated sugars were reduced and acetylated as described under composition analysis.

3.7 Partial acid hydrolysis

The three polysaccharides (25 mg) were each hydrolyzed with 0.04 M TFA (4 mL) at 100°C for 1 h. The products were then evaporated to dryness to remove TFA, and the residues dialyzed against distilled water (3 × 250 mL). The nondialysate was detected by HPLC. Subsequently, the nondialysate was hydrolyzed with 0.1 M TFA at 100°C for 1 h. The mixture was evaporated to dryness, and the residue was dialyzed against distilled water (3 × 250 mL). The dialysate was concentrated and separated on Sephadex G-25 (70 × 2.6 cm), giving two fractions. All fractions were analyzed by GLC as the alditol acetates. The nondialysate was separated on a Sephadex G-25 column (70 × 2.6 cm), giving two fractions that were then subjected to sugar analysis.

3.8 KI–I_2 reaction

Polysaccharides **1**–**3** (3.0, 3.1, 3.0 mg) were dissolved in distilled water (3 mL) and KI—I_2 was then added to the aqueous solution. UV absorption of three polysaccharides was determined at 500 nm.

3.9 NMR spectroscopy

Polysaccharides **1**–**3** (50, 48, 42 mg) were dissolved in D_2O (0.5 mL), left for 12 h, freeze-dried, and resolved in D_2O (0.5 mL). ^1H and ^{13}C NMR spectra were recorded at room temperature with an Inova-500 instrument.

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