An Immunomodulating Pectic Arabinogalactan from Roots of *Cistanche deserticola*

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The crude cold water-extractable polysaccharide isolated from the holoparasite *Cistanche deserticola* Y. C. Ma. was separated into several fractions by cetavlon precipitation and ion-exchange chromatography. The immunological properties of the fractions were characterized using mitogenic and comitogenic rat thymocytes tests. The most potent fraction after purification by gel filtration yielded the polysaccharide cistan A. It was composed of L-arabinose, D-galactose, L-rhamnose, and D-galacturonic acid in the mole ratio 6.3:10.0:1.0:0.8, in addition to minute amounts of D-xylose and D-glucose. The relative mean molecular mass was 201×10^3. The results of chemical and spectroscopic investigations indicated the cistan A to comprise a complex of pectic arabino-3,6-galactan type II with lowly-branched 3,5-α-L-arabinan. The polysaccharide exhibits remarkable immunomodulatory activities exceeding those of the commercial immunomodulator Zymosan.

The underground part of the holoparasite *Cistanche deserticola* Y. C. Ma. is known as "Cistanchis Herba" in the traditional medicine of Mongolia. The drug is used as a tonic and in curing kidney pain, gynecological diseases, and intestinal infections. In previous works [1—4], various polysaccharides have been isolated from the drug by sequential extraction methods. The pectin fractions isolated from *Cistanche* under different extraction conditions were rich in 3,5-α-L-arabinan side chains and contained minor amounts of galactan side chains as well as coextracted xylan. All pectin fractions were shown to exhibit remarkable immunomodulatory activities in mitogenic and comitogenic *in vitro* tests [5]. The aim of the present paper was to describe the structural and molecular properties of the most immunologically active component of the crude polysaccharide fraction [1] obtained from the *Cistanche* roots by cold water extraction.

EXPERIMENTAL

The underground part of *Cistanche deserticola* Y. C. Ma. was collected in 1990 in the Baian-Chongor province, Mongolia. A voucher specimen of the plant is deposited in the Herbarium of the Institute of Botany of the Mongolian Academy of Sciences, Ulan-Bator. Pronase from *Streptomyces griseus* and α-amylase were purchased from Koch-Light Laboratories (England) and amyloglucosidase (γ-amylase) from Merck (Germany). Zymosan was obtained from Likospol (Bratislava, Slovakia). The modified Parker medium E-199 was supplied by the Institute of Sera and Vaccines (Prague, Czech Republic) and fetal calf serum was purchased from Biocom (Brno, Czech Republic). Phytohaemagglutinin (PHA) and polymyxin B (Aesporin) were supplied by Wellcome Diagnostics, UK. ^{3}H-Thymidine (specific activity 960 GBq mmol^{-1}) was purchased from the Institute for Research, Production, and Application of Radioisotopes (Prague, Czech Republic).

Evaporations were carried out under reduced pressure below 45 °C. Moisture content of the polysaccharide fractions was determined by drying at 60 °C in vacuum for 12 h. The uronic acid content was determined by the 3-hydroxydiphenyl assay using galacturonic acid as the standard [6]. Polysaccharide fractions were hydrolyzed with 2 M-TFA under reflux for 2 h. The paper chromatography (PC) of the hydrolyzates and methods of quantitative determination of sugar components in the form of alditol trifluoroacetates and alditol acetates by GC and of GC-MS as well as the determination of the optical rotation were described in detail in previous papers [5, 7]. ^{1}H and ^{13}C NMR spectra were recorded in the inverse-gated decoupling mode with an FT NMR Bruker AVANCE DPX 300 spectrometer (^{1}H at 300.13 MHz and ^{13}C at 75.46 MHz) for solutions in D_2O at 40 °C. ^{13}C chemical shifts were referenced to internal MeOH (δ = 50.15), and

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^{1}H chemical shifts to HOD (δ = 4.80). FTIR spectra were recorded with a Nicolet Magna 750 spectrometer in KBr pellets (2 mg sample/300 mg KBr). The molecular mass distribution of the polysaccharides was determined by HPGPC using a commercial instrument (Laboratorní Přístroje, Prague, Czech Republic) equipped with Tessek Separon HEMA-BIO 100 and 1000 columns. The columns were calibrated with pullulan standards P20, P50, P100, P200, P400, and P800. All samples were prepared as 0.1 % solutions and 0.1 M-$NaNO_3$ was used as the mobile phase as previously reported [8].

Isolation and Purification of the Cold Water-Extractable Polysaccharides

Sequential extraction of the 95 % methanol-insoluble cell-wall material of the *Cistanche* drug with cold water, hot water, 0.5 M-NaOH, and 0.01 M-EDTA yielded a crude water-extractable polysaccharide fraction [1] used as the starting material. The fraction (3 g) was dispersed in water (200 cm^3) and dialyzed against distilled water for 4 days. After the nondialyzable portion was centrifuged to remove insoluble material, the supernatant was lyophilized yielding the crude water-soluble polysaccharide fraction C1 (2.2 g, 0.55 % of the original dry drug).

A 1 % solution of C1 in 0.2 M-NaOAc buffer (pH = 6.1) was treated with α-amylase (1 mg/10 mg sample) and amyloglucosidase (1 mg/20 mg sample) at 37 ℃ for 72 h. The digestion was checked using the I_2-KI test. After inactivation of enzymes by heating at 100 ℃ for 10 min, the solution was centrifuged and the supernatant subjected to dialysis against distilled water until negative by the phenol—sulfuric acid test. The pH of the nondialyzable portion after amylases digestion was adjusted to 7.5 with 0.5 M-NaOH and then the solution was treated with pronase (3 mg/50 mg sample) at 37 ℃ for 48 h. The digestion was terminated by neutralization with 0.1 M-HCl and subsequent heating at 100 ℃ for 10 min. Then the mixture was dialyzed and the soluble portion separated by centrifugation, dialyzed and lyophilized yielding the purified product C1p (yield 0.46 % of the dry original drug).

Fractionation of C1p

Polysaccharide C1p was fractionated using cetavlon precipitation [9]. Briefly, aqueous cetavlon (2 %) was added dropwise to incipient turbidity to aqueous solution (0.5 %) of C1p. After 6 h of stirring, the precipitate formed was collected by centrifugation, and the pellet dispersed in 4 M-NaCl to dissociate the complex. The polysaccharide was recovered after dialysis by lyophilization yielding fraction C1p-p (yield 36 % of C1p). The nonprecipitated fraction (C1p-s) was recovered from the supernatant after acidification with dilute acetic acid to pH = 4 by dialysis and lyophilization (yield 56 % of C1p).

C1p-p (1.5 g) was dissolved in water (50 cm^3) and loaded on a column (4 cm × 53 cm) of DEAE-Sephadex A-50 in acetate form and eluted with H_2O to obtain the unabsorbed fraction (C1p-p1, 70 mg). The retained material was washed stepwisely with 0.1 M-NaCl, 0.5 M-NaCl, and 0.1 M-NaOH solutions. The carbohydrate in the fractions (10 cm^3) was monitored by the phenol—sulfuric acid assay, and pooled appropriately yielding fractions C1p-p2 (390 mg), C1p-p3 (580 mg), and C1p-p4 (170 mg) which were analyzed for sugar composition and immunological activity. Rechromatography of C1p-p3 (250 mg in 2 cm^3) on a column (2.7 cm × 120 cm) of Sephadex G-75 gave the polysaccharide fraction (cistan A). Cistan A eluted as a single peak also on Sephadex G-100 and its monosaccharide composition was constant.

Methylation Analysis

The dry cistan A (50 mg) dissolved in DMSO (2 cm^3) was methylated by the DMSO-solid NaOH-CH_3I method [10] as previously described in detail [1]. The methylated product was extracted with $CHCl_3$ and dried over Na_2SO_4. The absence of free hydroxyl groups was checked by IR spectroscopy. A part of the permethylated sample was reduced with $LiAlD_4$ in methanol and then subjected to the two-step hydrolysis (90 % HCOOH, 1 h, 100 ℃; 2 M-TFA, 1 h, 100 ℃). Partially methylated alditol acetates were prepared by reduction of the hydrolyzed product with $NaBD_4$ in 1 M-NH_4OH (4 h) and analyzed by GC (Hewlett—Packard Model 5890 Series II chromatograph) and GC-MS (FINNIGAN MAT SSQ710 spectrometer).

Mitogenic and Comitogenic Tests

The assay of the mitogenic and comitogenic activities was based on method [11] with slight modification. Wistar rat thymocytes in modified Parker medium E-199 supplemented with 10 % fetal calf serum were cultivated at 1.5×10^6/0.2 cm^3 per well with 25 μg cm^{-3} PHA. Test compounds were added to the final concentrations 1 μg cm^{-3}, 10 μg cm^{-3}, 100 μg cm^{-3}, and 200 μg cm^{-3}. After 72 h cultivation, the thymocytes proliferation was measured by incorporation of ^{3}H-thymidine. In each experiment, arithmetic means of counts per minute (cpm) for each set of 3—4 replicas were used for calculation of the stimulation indices (SI). The direct mitogenic effect of the polysaccharides was expressed as SI_{mit} = mean cpm of test compound/mean cpm of control and the comitogenic effect was expressed as SI_{comit} = (mean cpm of test compound + PHA)/mean cpm of PHA. The mean cpm ± SD for control cultures without any addition was 912 ± 323 and for cultures incubated with PHA it was 1165 ± 622. Potential contamination of the polysac-

Table 1. Analytical Characteristics of Fractions Derived from the Cold Water-Extractable Polysaccharide C1p

Fraction	Neutral sugar composition (x_r/mole %)						UA[a]	x_r(Gal—Ara)
	Rha	Ara	Gal	Xyl	Glc	Man	%	
C1p	8.2	30.6	50.0	3.4	4.7	3.1	5.4	1.63
C1p-p	7.2	24.5	53.2	2.9	9.6	2.6	3.9	2.17
C1p-s	5.0	29.6	43.9	7.1	11.9	2.5	3.7	1.48
C1p-p1	4.0	26.8	50.2	3.2	8.2	7.6	0.9[b]	1.87
C1p-p2	3.5	25.1	49.8	6.2	6.8	8.6	3.2	1.98
C1p-p3	6.6	30.1	54.4	6.0	2.1	0.8	4.9	1.81
C1p-p4	7.0	30.4	33.8	15.3	9.8	3.7	5.6	1.08
Cistan A	5.6	35.2	56.5	0.9	1.8	0	5.5[b]	1.59

a) Uronic acid assayed by the 3-hydroxydiphenyl method; *b*) estimated as galacturonic acid by PC and NMR spectroscopy.

charide fractions by endotoxins was checked by cultivation in the presence of polymyxin (10 μg cm^{-3}) which inhibited, dose-dependently, the biological effects of endotoxin, including its mitogenic activity [12].

RESULTS AND DISCUSSION

The water-soluble fraction of the cold water-extractable polysaccharide (C1), obtained from the underground part of the holoparasite *Cistanche deserticola* Y. C. Ma. [1], was enzymically purified from starch and protein. The purified product C1p contained mainly galactose and arabinose amounting to about 80 % of the neutral sugar components, and minor amounts of rhamnose, xylose, glucose, mannose, and 5.4 % of galacturonic acid (Table 1). Cetavlon fractionation of C1p gave the precipitable C1p-p and nonprecipitable (C1p-s) fractions, the last in a higher yield. However, both fractions had comparable neutral and acidic sugar profiles. Interestingly, considerable differences were found in the molecular properties of the fractions determined by HPGPC on pullulan-calibrated Separon columns (Table 2). The fractions contained three molecular populations of different sizes in various proportions. However, polysaccharides of higher molecular mass were more accumulated in C1p-p than in C1p-s.

The immunomodulatory activities of the purified polysaccharide C1p and derived fractions C1p-p and C1p-s were compared to those of the commercial immunomodulator Zymosan as control using the *in vitro* mitogenic and comitogenic thymocyte tests. The polymyxin B test indicated the absence of endotoxin in the samples. As demonstrated in Fig. 1, the fractions showed dose-dependent immunomodulatory activities in both tests comparable to that of Zymosan. However, C1p-p in comparison to C1p-s gave a significantly higher biological response in the comitogenic test which was the most pronounced at dose 100 μg cm^{-3}.

Therefore, C1p-p was further fractionated on DEAE-Sephadex A-50 and four subfractions eluting

Table 2. Molecular Mass Distribution of C1p and its Fractions

Sample	$M_{m,r} \times 10^{-3}$	M_m/M_n	Area/%
C1p	16	1.28	5
	187	2.13	75
	730	1.06	20
C1p-p	182	2.47	81
	790	1.05	4
	>1000		15
C1p-s	27	1.28	40
	115	1.27	57
	<1000		3
C1p-p3	35	1.20	10
	226	1.75	90
Cistan A	201	1.54	100

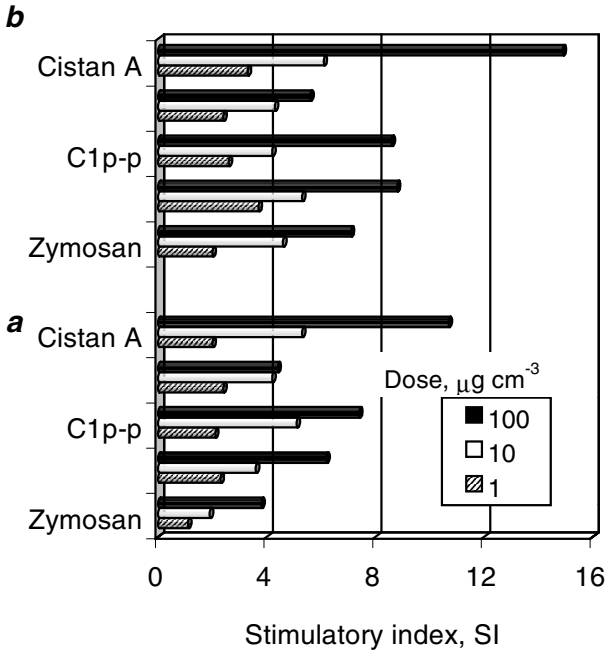

Fig. 1. Immunological activities of various cold water-extractable polysaccharide fractions from *Cistanche deserticola* and Zymosan; mitogenic activity (*a*), comitogenic activity (*b*).

Table 3. Methylation Analysis Data of Cistan A

Derivatives[a]	Linkage type indicated	Glycosidic linkage (x_r/mole %)
2,3,4-Me$_3$-Rha	Rha*p*-(1→	0.5
2,3-Me$_2$-Rha	→4)-Rha*p*-(1→	3.1
3-Me-Rha	→2,4)-Rha*p*-(1→	1.3
Total		*4.9*
2,3,5-Me$_3$-Ara	Ara*f*-(1→	9.2
2,3-Me$_2$-Ara	→5)-Ara*f*-(1→	20.0
2-Me-Ara	→3,5)-Ara*f*-(1→	4.1
3-Me-Ara	→2,5)-Ara*f*-(1→	0.8
Total		*34.1*
2,3,4,6-Me$_4$-Gal	Gal*p*-(1→	18.6
2,4,6-Me$_3$-Gal	→3)-Gal*p*-(1→	4.9
2,3,6-Me$_3$-Gal	→4)-Gal*p*-(1→	3.5
2,3,4-Me$_3$-Gal	→6)-Gal*p*-(1→	8.4
2,6-Me$_2$-Gal	→3,4)-Gal*p*-(1→	1.7
2,4-Me$_2$-Gal	→3,6)-Gal*p*-(1→	21.7
Total		*58.8*
2,3-Me$_2$-Xyl	→4)-Xyl*p*-(1→	0.8
Total		*0.8*
2,3,6-Me$_3$-Glc	→4)-Glc*p*-(1→	0.4
3,6-Me$_2$-Glc	→2,4)-Glc*p*-(1→	0.9
Total		*1.3*

a) 2,3,4-Me$_3$-Rha = 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylrhamnitol, *etc.*

with water, 0.1 M-NaCl, 0.5 M-NaCl, and 0.1 M-NaOH were recovered. They differed considerably in neutral sugar composition and uronic acid content (Table 1). Even the nonabsorbed subfraction C1p-p1 contained a very small amount of uronic acid (0.9 %) estimated as galacturonic acid by PC. Arabinose and galactose were the prevailing sugar components in all four subfractions. The first ones contained in addition mainly glucose and mannose, whereas the last one was enriched in xylose. Recently, this sugar has been reported to accompany pectic polysaccharides and arabinogalactan type II polysaccharides [13—15].

Screening of the immunomodulatory acitivity of the fractions revealed (results not shown) a pronounced increase of the biological response in both tests with fraction C1p-p3, eluted with 0.5 M-NaCl. When C1p-p3 was analyzed by HPGPC, it gave a component (about 90 %) with $M_{m,r} = 226 \times 10^3$ and a minor one with $M_{m,r} = 35 \times 10^3$ (Table 2). Rechromatography of C1p-p3 on Sephadex G-50 yielded a molecularly homogeneous polysaccharide fraction with $M_{m,r} = 201 \times 10^3$ named as cistan A. It consisted of L-arabinose, D-galactose, L-rhamnose, and D-galacturonic acid (estimated by PC) in the mole ratio 6.3:10.0:1.0:0.8 in addition to very small amounts of D-xylose and D-glucose. Its optical rotation ($[\alpha]_D$) was −10.0°. As shown in Fig. 1, cistan A exhibited the highest activities in both mitogenic and comitogenic tests at dose 100 μg cm^{-3} when compared to those of Zymosan and the starting purified cold water-extractable polysaccharide C1p.

The results of methylation analysis of cistan A, shown in Table 3, indicated a highly branched structure composed of a variety of sugars and linkages. Rhamnose occurred mainly as 4-linked residues typical of rhamnogalacturonan II sequences [16]. Arabinose in the furanose form was present mainly as terminal, 5-, and 3,5-linked residues. A minor amount of arabinofuranosyl residues was 2,5-linked. All these types of linkages are common in plant arabinogalactans [13, 17—19]. Galactose was found in 3- (8 %), 6- (14 %), and 3,6-linked (37 %) positions, which reflects a highly substituted arabinogalactan type II. The minor amounts of 4- and 4,6-linked galactopyranosyl residues were indicative of the presence of arabinogalactan type I. After reduction of the permethylated polysaccharide with $LiAlD_4$, only the 2,3,6-tri-*O*-methylgalactose showed incorporation of deuterium at C-6 what suggests the presence of (1→4)-linked galacturonic acid residues originating from a pectin backbone.

The ^{13}C NMR and partial ^{1}H NMR spectra of cistan A are shown in Fig. 2. The resonances were assigned on the basis of previously reported spectral data for pectic polysaccharides [1, 20—24]. The ^{13}C NMR spectrum of cistan A showed resonances of the anomeric carbons of α-arabinofuranosyl residues at δ = 110.3 and 108.2—108.5, β-galactopyranosyl residues at δ = 104.3—105.2, α-rhamnopyranosyl residues at δ = 101.8, and α-galactopyranosyl uronic acid residues at $\delta \approx 100.1$. The low and similar intensities of C-1 resonances assigned to rhamnopyranosyl and galacturonic acid residues were in accord with the proportion of these sugars in cistan A (Table 1). The results in addition to the occurrence of 2-linked rhamnopyranosyl units suggested that they occur as rhamnogalacturonan sequences substituted at position *O*-2 by neutral side chains. The ^{13}C chemical shifts of 5-linked, 3,5-linked, and nonreducing terminally linked α-arabinofuranosyl residues were tentatively assigned (Table 4) [25]. The signals of anomeric proton resonances (all had J= 1.5 Hz) were found at δ = 5.09—5.13, 5.18, and 5.26. The last corresponds to terminal α-arabinofuranosyl residues linked to the galactan backbone [26, 27], the others to α-arabinofuranosyl, α-rhamnopyranosyl, and α-galactopyranosyl uronic acid residues. The group of signals at δ = 4.65—4.76 (all had J = 7.8 Hz) indicated the β-galactopyranosyl residues to be involved in various linkages in accord with the methylation analysis data. Two C-6 resonances of the α-rhamnopyranosyl residues occurred at δ = 17.9 and 17.6 and two H-6 resonances at δ = 1.32 and 1.26 confirming the two main linkage types of this sugar (Table 3). The broad signals at δ = 100.1/5.02 and 79.9 corresponded to the C-1/H-1 and C-4 resonances of the 4-linked α-galactopyranosyl uronic acid residues, respectively.

The present study indicated that the most immunologically potent fraction of the cold-water ex-

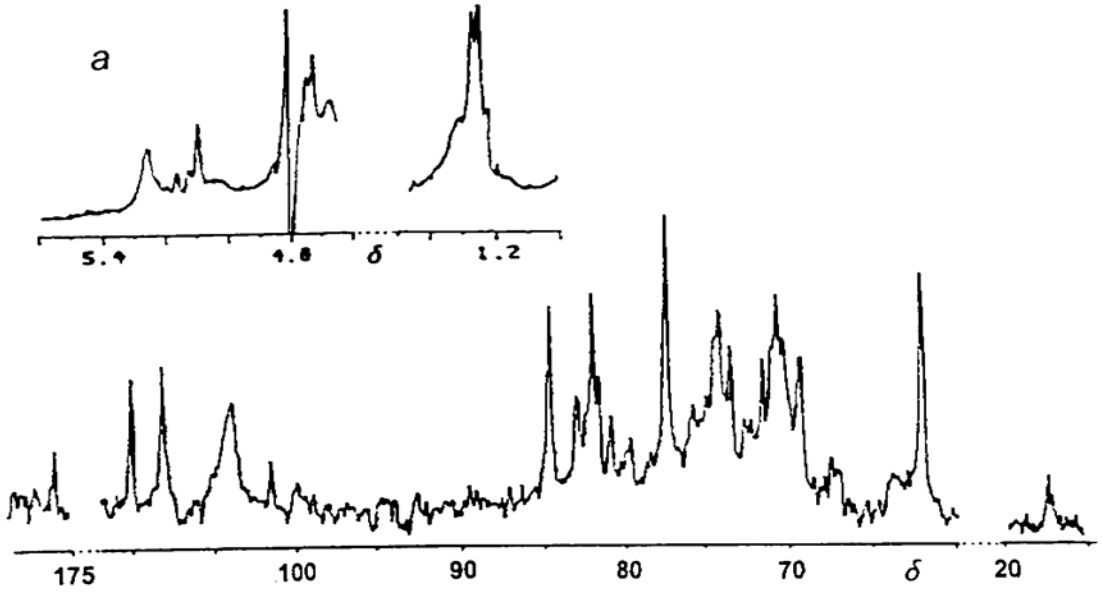

Fig. 2. ^{13}C NMR spectrum (in D_2O) and inserted partial ^{1}H NMR spectrum of cistan A (*a*).

Table 4. Assignment of the ^{13}C NMR Chemical Shifts (in D_2O) of the Arabinan Component of Cistan A

Linkage type	Chemical shifts, δ				
	C-1	C-2	C-3	C-4	C-5
α-Ara*f*-(1→ galactose	110.4	81.7	77.6	85.0	62.3
α-Ara*f*-(1→ arabinose	108.6	82.5	77.6	85.0	62.3
→5)-α-Ara*f*-(1→	108.6	82.0	77.8	83.4	67.8
→3,5)-α-Ara*f*-(1→	108.3	80.6	83.4	82.5	67.6

tractable polysaccharides from the *Cistanche* drug comprises a complex of arabino-3,6-galactan type II associated with branched 3,5-α-L-arabinan and a very low proportion of rhamnogalacturonan chains. However, covalent linkages between the different types of polysaccharides might exist and make the separation difficult. Pectic polysaccharides, commonly considered to possess galacturonan and/or rhamnogalacturonan cores with attached arabinan and arabinogalactan side chains, have been isolated from various medicinal plants and were reported to exhibit anticomplementary and mitogenic activities [14, 28—32]. The ramified regions of the galacturonan core and both the neutral side chains consisting of 3,6-β-D-galactan and 3,5-α-L-arabinan were suggested to be involved in the expression of the biological activities. The results confirmed the *Cistanche* drug to contain an immunomodulating pectic arabinogalactan exhibiting remarkable mitogenic and comitogenic activities.

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