Isolation and Characterization of Mitogenic Pectic Polysaccharides from *Cistanche deserticola* Y. C. Ma.

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Three pectic polysaccharide fractions isolated from the methanol- and water-insoluble cell-wall material of the *Cistanche deserticola* drug by fractional extraction have been investigated using chemical, enzymic, and NMR spectroscopic methods. The pectins differed in the galacturonic acid content which varied between 30 % and 80 %, degree of methyl esterification and acetylation, composition of the neutral carbohydrate components, and molecular properties. They contained homogalacturonan and rhamnogalacturonan RG-I sequences in different proportions, and neutral polysaccharides represented mainly by a highly branched (1→3,5)-α-arabinan. All pectin fractions exhibited immunomodulating properties as indicated by their significant activities in mitogenic and comitogenic tests.

Cistanche deserticola Y. C. Ma. is a holoparasite grown on the roots of the hardwood *Haloxylon ammodendron* which is widely distributed in the Gobi desert. The underground part is a known Mongolian herbal medicine used to cure kidney pain, gynecological diseases and tropical intestinal infections. In our previous papers [1—3], we reported on the isolation and characterization of the low- and high-molecular carbohydrate components of the drug. The aim of the present paper was to investigate, in more detail, the pectin component, particularly in relation to its biological activity.

EXPERIMENTAL

Materials and Methods

The *Cistanche* drug was prepared by grounding the underground part of the parasite which had been collected in the Baian-Chongor (Mongolia) province in 1990. The starting material in this study was the insoluble cell-wall material (CWM) obtained by successive extractions of the drug with 95 % methanol, cold water, and hot water, as previously described [3]. α-Amylase was purchased from Fluka AG, γ-amylase from Merck, and pronase from *Streptomyces griseus* was a product of Koch-Light Laboratories. Young adult Wistar rats were used in the experiments. 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 (Aerosporin) 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). HPGPC was performed on Tessek Separon HEMA Bio-1000 and 100 columns (Laboratorní přístroje, Prague, Czech Republic) calibrated with pullulan standards (Shodex Standard, P-82, Macherey-Nagel, Germany). A computing procedure [4] was applied to obtain the molecular mass distribution and relative molecular mass.

Moisture content was determined by drying the sample at 105 °C to constant mass. Protein was calculated from the nitrogen content (w(N)/% × 6.25) assayed using an elemental analyzer (Perkin—Elmer, Model 240). Paper chromatography (PC) was performed by the descending method on Whatman No. 1 paper in the systems S_1 ethyl acetate—acetic acid—water (φ_r = 19:8:7) and S_2 ethyl acetate—pyridine—water (φ_r = 8:2:1) and reducing sugars were detected with anilinium hydrogen phthalate. The pectin samples were hydrolyzed with 2 M-TFA under reflux for 2 h. The neutral sugars isolated from the hydrolyzate by means of ion-exchange technique on Dowex 2 × 8 (acetate form) were analyzed by PC (S_2) as well as by GLC of their alditol trifluoroacetates, as previously described [5]. GLC was carried out on a Hewlett—Packard instrument, Model HP 5890. Uronic acids

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were determined by the 3-hydroxydiphenyl assay [6] and identified by PC in S_1 as galacturonic acid and traces of glucuronic acid. The degree of methyl esterification (d.e., $n(OCH_3)$ per 100 mol of galacturonic acid) was calculated from the methoxyl group content [7]. Optical rotations were measured with Perkin—Elmer Model 141 polarimeter in 0.5 % aqueous solution at 22 °C.

Methylation analysis was performed on 50 mg samples by the slightly modified method of *Ciucanu* and *Kerek* [8]. A part of the permethylated sample was reduced with $LiAlD_4$ in methanol and then subjected to hydrolysis, followed by GLC and GLC-MS analyses of the partially methylated alditol acetates as previously described [3, 9]. GLC-MS analyses were effected on a Finnigan MAT SSQ 710 spectrometer and CI spectral data were obtained using protonated pyridine [3]. Proton-decoupled ^{13}C and ^{1}H NMR spectra were recorded with a Bruker AM-300 spectrometer at 75 MHz for solutions in D_2O at 40 °C. ^{13}C chemical shifts are referenced to internal MeOH (δ = 50.15), and ^{1}H chemical shifts to HOD (δ = 4.80). The molar ratio of the acetyl and methoxyl groups x_r(O-acetyl—OCH_3) = 1:5.5 was estimated from the intensities of the corresponding proton signals at δ = 2.1 and 3.8, respectively.

Isolation of Pectic Fractions

A sample of CWM (50 g) was treated twice with 0.01 M-EDTA (800 cm^3, pH 6.8) at room temperature for 2 h. The combined extracts after concentration *in vacuo* were dialyzed against distilled water. After removal of the insoluble material by centrifugation, the supernatant was lyophilized, yielding fraction P1 (1.8 g) with 3.6 % of methoxyl groups. Another sample of CWM (100 g) was treated with 1 % NaOH (1000 cm^3) at room temperature for 2 h. After removal of the extract by filtration, the insoluble residue (AIR) was washed with 0.5 M acetic acid (100 cm^3), then with distilled water (2000 cm^3), and dried on air (yield = 62 g). A part of AIR (20 g) was subsequently extracted twice with 0.01 M-EDTA (800 cm^3, pH 6.8). From the combined extracts, the nondialyzable part was separated and lyophilized to give fraction P2 (3.4 g). A second portion of AIR (30 g) was treated with aqueous solution of sodium chlorite (500 cm^3, containing 60 % of active chlorine related to AIR), acidified with acetic acid (pH 4.8), at 40 °C for 8 h. The spent liquor separated from the delignified material by filtration was neutralized with 1 % NaOH and dialyzed. The nondialyzable part was lyophilized to yield fraction P3 (2.2 g).

Enzymic Purification of Pectic Fractions

A sample of the pectin fraction (300 mg) was dissolved in distilled water (30 cm^3) and the pH was adjusted to 7.5 with NaOH. Pronase (12 mg) was added and the solution was incubated at 37 °C for 48 h. The enzyme was then inactivated by heating the mixture at 100 °C for 10 min. The insoluble part was removed by centrifugation. The soluble part was concentrated *in vacuo* to 1 cm^3 and diluted with acetate buffer of pH 6.1 (30 cm^3). α-Amylase (15 mg) and γ-amylase (8 mg) were added and the solution was incubated at 37 °C for 36 h. After inactivation and centrifugation of the enzymes, the supernatant was dialyzed and lyophilized, yielding the purified pectin.

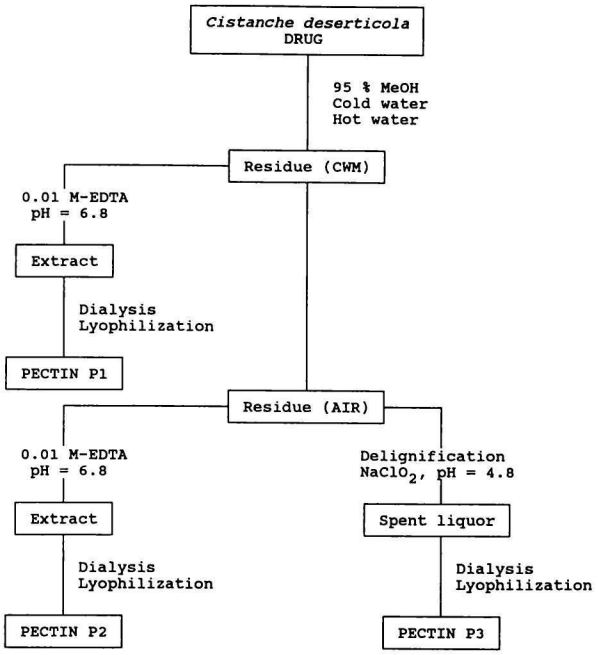
Mitogenic and Comitogenic Tests

Mitogenic and comitogenic tests were performed according to *Iribe* and *Koga* [10] with slight modification. Wistar rats thymocytes in modified Parker medium E-199 supplemented with 10 % fetal calf serum were cultivated at $1.5 \times 10^6/(0.2\ cm^3)$per well with 25 $\mu g\ cm^{-3}$ PHA. Test compounds were added to the final concentrations 1 $\mu g\ cm^{-3}$, 10 $\mu g\ cm^{-3}$, 100 $\mu g\ cm^{-3}$, and 200 $\mu g\ cm^{-3}$ After 72 h cultivation, the incorporation of ^{3}H-thymidine was measured. In each experiment, arithmetic means of counts per min (cpm) for each set of 3—4 replicas were used for calculation of the stimulation indices (SI). The direct mitogenic effect of the pectin samples 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 control.

Eventual contamination of the pectins by endotoxins was checked by cultivation in the presence of polymyxin (20 $\mu g\ cm^{-3}$) which inhibits, dose-dependently, the biological effects of endotoxin, including its mitogenic activity [11].

RESULTS AND DISCUSSION

The insoluble cell-wall material (CWM) of *Cistanche deserticola* obtained after subsequent extractions of the drug with methanol, cold and hot water represents 25.3 mass % of the original dry drug [3]. The isolation of pectic polysaccaride fractions from CWM is illustrated on Scheme 1. The yield and general characteristics of the obtained fractions are presented in Table 1. As has been reported in a previous paper [3], the cold and hot water treatments of the *Cistanche* drug afforded mainly a pectic arabinogalactan and starch, contaminated with minor amount of methyl esterified pectin. A larger part of pectic polysaccharides (P1, 0.9 mass % on dry drug basis), held in the cell-wall complex by divalent ions, was released from it with the chelating agent EDTA. P1 contained a high percentage of galacturonic acid (79.2 mass %) with a low degree of methyl esterification (d.e. = 28) and *O*-acetylation ($\approx$ 6, expressed as n(acetyl groups)/100 mol of galacturonic acid). When



Scheme 1. Extraction scheme of pectic polysaccharides from *Cistanche deserticola*.

Table 1. General Characteristics of Pectin Fractions Isolated from *Cistanche deserticola*

	P1	P2	P3
w(Yield)/ %[a]	0.9	2.7	1.2
w(Protein)/%	8.1	5.6	17.5
w(Uronic acid)/%[b]	79.2 (89.1)[c]	40.8 (49.5)	32.3 (74.0)
Neutral sugars, x_i/mole %			
Glucose	45.2 (16.9)	23.3 (4.3)	44.6 (8.5)
Galactose	11.6 (16.9)	7.5 (9.2)	15.7 (23.9)
Mannose	2.7 (3.1)	0 (0.6)	4.3 (6.9)
Arabinose	32.7 (50.8)	59.7 (73.5)	24.7 (41.3)
Xylose	4.2 (5.6)	0 (0.5)	4.3 (7.1)
Rhamnose	3.6 (6.7)	9.5 (11.8)	6.4 (10.5)
x_r(GalA—Rha)	165:1	6:1	4:1
[α] (D, 22 °C, ρ = 0.03 g cm^{-3}, water)	+ 2.8	+ 1.7	−3.0

a) Based on the dry original drug; *b*) More than 95 mass % is represented by galacturonic acid; *c*) In parentheses are data for the enzymically purified pectins.

CWM was pretreated with dilute alkali, EDTA extraction gave three times higher amount of pectic polysaccharides (P2, 2.7 % on dry drug basis). This is due to the increased accessibility of CWM, promoted by the swelling effect of the sodium hydroxide solution. P2 contains less galacturonic acid (40.8 mass %) and is free of methyl ester and acetyl groups. Another pectin fraction (P3) was obtained in a lower yield from the alkali-insoluble CWM (AIR) by the acidic sodium chlorite treatment which is usually used to remove lignin from the cell walls. P3 contained the lowest amount of galacturonic acid (32.3 mass %). On the other hand, the high content of neutral sugars and protein is in accord with the suggested [12] scission of cross-links between the neutral and acidic matrix polymers in higher plants during sodium chlorite delignification. In all fractions minor amounts of fucose (< 0.2 mole %) were detected by GC.

All pectin preparations were contaminated with co-extracted starch (detected by the positive I_2/KI test) and proteins. As seen in Table 1, the enzymic removal of starch and proteins changed substantially the sugar composition of the pectin fractions. In particular, the galacturonic acid content of P3 increased from 32.3 to 74 mass %. Of the neutral sugar components, arabinose and galactose are prevailing in all purified pectins. Their mole ratios x_r ranged from 1.6 to 8.0. The mole ratio of galacturonic acid to rhamnose is used to characterize the presence of homogalacturonan and rhamnogalacturonan (RG-I) blocks [13]; the last are built-up from the alternating disaccharide →4)-α-D-GalpA-(1→2)-α-L-Rhap-(1→. Whereas for the pu-

Table 2. Molecular Mass Distribution of the Pectin Fractions of *Cistanche deserticola*

Sample	$M_{m,r} \times 10^{-3}$	M_m/M_N	Area/%
P1	16	1.28	5
	187	2.13	75
	730	1.06	20
P2	20	1.25	3
	125	1.71	71
	> 800	nd	26
P3	8	1.45	74
	97	2.00	15
	> 800	1.09	11

nd = Not determined.

rified P1 x_r was 165:1, for P2 and P3 it was 6:1 and 4:1, respectively. The results indicate that P1 consists of almost but homogalacturonan chains, whereas both P2 and P3 contain a higher amount of RG-I structures.

HPGPC analysis revealed a high molecular heterogeneity of all pectin fractions (Table 2). P1 and P2 showed the main relative molecular mass ($M_{m,r}$) peak at 187000 and 125000, respectively, as well as large proprotions (20—26 %) of an extremely high $M_{m,r}$-component ($M_{m,r}$ > 700000), present also in commercial pectins [14]. In contrast, the main component of P3 was of low $M_{m,r}$ (≈ 8000). This distribution was most probably due to the hydrolytic effects of the sodium chlorite treatment.

Fractions P1 and P3 were analyzed for their glycosidic linkages by methylation analysis. As seen from the results in Table 3, the neutral sugar composition of the pectins corresponds well with the results obtained by GC (Table 1). In both pectins, the arabinose component was represented mainly by terminal, 5- and 3,5-linked arabinofuranosyl residues. The variety of galactose derivatives indicates the involvement of this sugar in different galactan structures, including both the (1→4)-galactan and (1→3,6)-galactan types. The predominance of 4- and 4,6-linked glucopyranosyl residues agreed with the presence of starch. However, a minor part of glucose was involved in (1→3)- and/or (1→6)-glucan chains and the detected fucose might originate from RG-I and/or a xyloglucan component [15]. The prevalence of (1→4)-galacturonan chains in

Table 3. Methylation Analysis Data of Pectin Fractions P1, $LiAlD_4$-reduced[a] P1, and P3

Derivatives[b]	Linkage type indicated	Glycosidic linkage (x_r/mole %)		
		P1	P1[a]	P3
2,3,4-Me_3-Rha	Rha*p*-(1→	3.9	2.4	4.2
3,4,-Me_2-Rha	→2)-Rha*p*-(1→	0.8	0.7	3.5
3-Me-Rha	→2,4)-Rha*p*-(1→	0.1		2.5
Total		4.8	3.1	10.2
2,3,5-Me_3-Ara	Ara*f*-(1→	10.9	6.5	12.4
3,5-Me_2-Ara	→2)-Ara*f*-(1→	1.8	0.5	1.5
2,5-Me_2-Ara	→3)-Ara*f*-(1→	1.1	tr	0.9
2,3-Me_2-Ara	→5)-Ara*f*-(1→	5.3	2.5	3.7
2-Me-Ara	→3,5)-Ara*f*-(1→	8.8	5.1	9.5
Total		27.9	14.6	28.0
2,3,4-Me_3-Xyl	Xyl*p*-(1→	1.3		0.3
2,3-Me_2-Xyl	→4)-Xyl*p*-(1→	1.1	0.4	
3-Me-Xyl	→2,4)-Xyl*p*-(1→	2.7	1.6	0.6
Total		5.1	2.0	0.9
2,3,4,6-Me_4-Glc	Glc*p*-(1→	10.7	5.0[c]	11.9
2,4,6-Me_3-Glc	→3)-Glc*p*-(1→	1.0	0.5	0.1
2,3,6-Me_3-Glc	→4)-Glc*p*-(1→	24.2	17.4	17.9
2,3,4-Me_3-Glc	→6)-Glc*p*-(1→	0.7	0.2	0.7
2,6-Me_2-Glc	→3,4)-Glc*p*-(1→	0.1	tr	0.1
2,4-Me_2-Glc	→3,6)-Glc*p*-(1→	4.2	3.0	5.0
2,3-Me_2-Glc	→4,6)-Glc*p*-(1→	5.0	3.5	12.0
Total		45.9	29.6	47.7
2,3,4,6-Me_4-Gal	Gal*p*-(1→	1.2	0.9	1.3
2,4,6-Me_3-Gal	→3)-Gal*p*-(1→	1.2	0.9	1.2
2,3,6-Me_3-Gal	→4)-Gal*p*-(1→	7.4	43.5[c]	6.4
2,6-Me_2-Gal	→3,4)-Gal*p*-(1→	0.9	0.6	0.6
2,3-Me_2-Gal	→4,6)-Gal*p*-(1→	1.9	1.9	0.9
2,4-Me_2-Gal	→3,6)-Gal*p*-(1→	3.7	2.7	2.1
2-Me-Gal	→3,4,6)-Gal*p*-(1→	–	–	0.7
Total		16.3	50.7	13.2

a) The methylated P1 was reduced with $LiAlD_4$; *b*) 2,3,4-Me_3-Rha =1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylrhamnose, etc.; *c*) deuterated units.

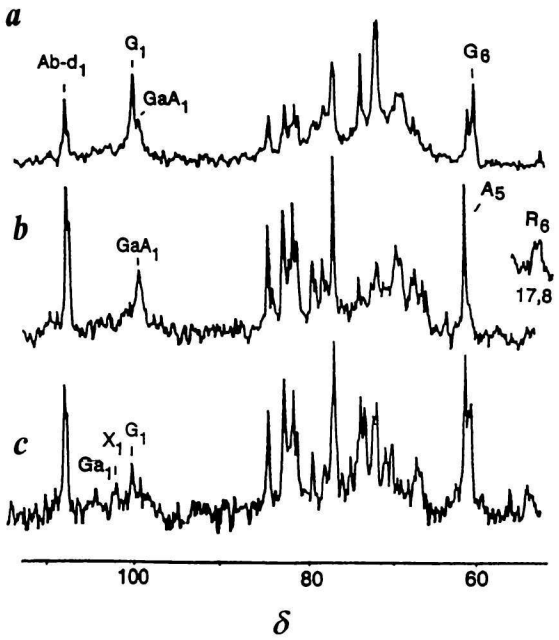


Fig. 1. ^{13}C NMR spectra (in D_2O) of pectin (*a*) P1, (*b*) enzymically purified P2, and (*c*) P3. G – glucose; X – xylose; Ga – galactose; GaA – galacturonic acid; R – rhamnose; the number denotes the carbon atom of the sugar unit.

P1 was confirmed by the increased proportion of 4-linked galactopyranosyl residues which showed incorporation of deuterium at C-6 after reduction of the permethylated P1 with $LiAlBD_4$.

The ^{13}C NMR spectra of P1, the purified P2, and P3, are shown in Fig. 1. The signals were assigned (Table 4) on the basis of previously reported data [9, 16—21] for various plant polysaccharides. In particular, the anomeric region of the spectra reveals information about the present polysaccharide types. The main signals at $\delta = 108.2$—108.5 and 100.8 correspond to C-1 of α-L-arabinofuranosyl [3, 9, 16] and α-D-glucosyl [17] residues, respectively. The minor signals at $\delta = 104.5$—105.5, 103.6, 102.8, and 101.8 were assigned to C-1 of variously linked β-D-galactosyl [9, 18], β-D-glucosyl [19], β-D-xylosyl [20], and α-L-rhamnosyl [19] residues. The complex character of the neutral carbohydrate components is in accord with the linkage analysis. The discrepancy between the galacturonic acid content and the intensity of C-1 and C-6 signals of α-D-galacturonosyl units [9, 21], which appeared in the ^{13}C NMR spectra at $\delta = 99.8$—100.3 and 173—175, can be explained by the rigid structure of the galacturonan chains. This is evident, particularly, in the case of P1 which gave a gel in D_2O due to its methyl ester (MeO: $\delta = 54.1$) and *O*-acetyl groups (OAc: $\delta = 171.4$ and 21.4). The corresponding signals of these groups in the ^1H NMR spectrum of P1 were found at $\delta = 3.8$ and 2.1, respectively.

It can be concluded that P1 represents a partially methyl esterified and *O*-acetylated polygalacturonan of high molecular mass. An acetylated homogalacturonan backbone was suggested also for the recently reported sugar beet pectin [22] and CDTA-soluble pectin fractions from flax hypocotyls [23]. The mean galacturonic acid/rhamnose mole ratio x_r of approximately 5:1, and the high proportion of 2,4-linked rhamnosyl residues in both P2 and P3 suggest that they contain RG-I-like polymers or sequences alternating with homogalacturonan blocks in the pectin chains. In all pectins, the 3,5-arabinan chains prevailed and are suggested to be associated with the rhamnogalacturonan backbone, similarly as in pectins isolated from higher plants [15, 24]. The different extractibility of the studied pectin fractions indicate that they might be located in different domains of the cell-

Table 4. Assignment of the Main Signals in the ^{13}C NMR Spectra of *Cistanche* Pectins

δ	Assignment
175.9—173.3	C-6 (GalA, GlcA)
171.4	CH_3CO
108.5	C-1 (t-α-Ara*f*)
	C-1 (5-linked α-Ara*f*)
108.2	C-1 (3,5-linked α-Ara*f*)
105.1—104.5	C-1 (β-Gal*p*)
103.6	C-1 (β-Glc*p*)
102.8	C-1 (4-linked β-Xyl*p*)
101.9	C-1 (α-Rha*p*)
100.8	C-1 (4-linked α-Glc*p*)
100.3—100.0	C-1 (4-linked α-GalA*p*)
98.8	C-1 (4-*O*-Me-α-GlcA*p*)
85.1	C-4 (t-α-Ara*f*)
83.4	C-4 (5-linked α-Ara*f*)
	C-3 (3,5-linked α-Ara*f*)
83.1	C-4 (4-*O*-Me-α-GlcA*p*)
82.5	C-2 (t-α-Ara*f*)
	C-4 (3,5-linked α-Ara*f*)
82.0	C-2 (5-linked α-Ara*f*)
80.5	C-2 (3,5-linked α-Ara*f*)
79.2—79.0	C-4 (4-linked β-Xyl*p*)
	C-4 (4-linked α-GalA*p*)
77.9	C-3 (t-α-Ara*f*)
	C-3 (5-linked α-Ara*f*)
77.4	C-4 (4-linked β-Xyl*p*)
74.8	C-3 (4-linked β-Xyl*p*)
74.4	C-3 (4-linked α-Glc*p*)
73.8	C-2 (4-linked β-Xyl*p*)
72.6	C-2 (4-linked α-Glc*p*)
72.3	C-5 (4-linked α-Glc*p*)
72.1	C-5 (4-linked α-GalA*p*)
70.3	C-5 (4-*O*-Me-α-GlcA*p*)
69.8—69.3	C-3 (4-linked α-GalA*p*)
	C-2 (4-linked α-GalA*p*)
67.7	C-5 (5-linked α-Ara*f*)
67.4	C-5 (3,5-linked α-Ara*f*)
64.1	C-5 (4-linked β-Xyl*p*)
62.2	C-3 (t-α-Ara*f*)
61.6	C-6 (4-linked α-Glc*p*)
61.1	OCH_3 (4-*O*-Me-α-GlcA*p*)
54.1	$COOCH_3$ (α-GlcA*p*)
21.4	OCH_3

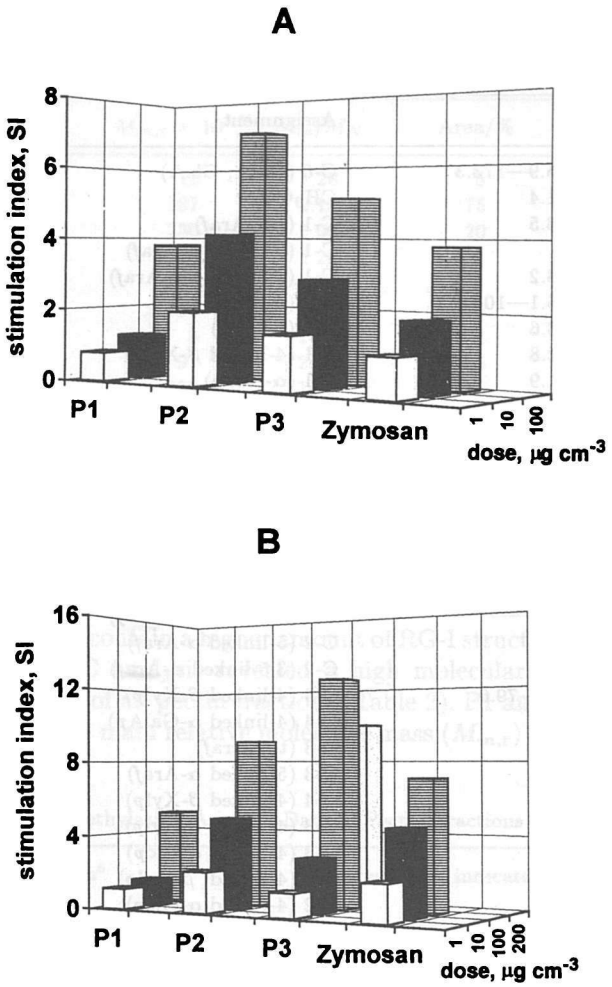


Fig. 2. Biological activities of the pectic polysaccharides from *Cistanche deserticola* and Zymosan; (A) mitogenic activity, (B) comitogenic acitivity.

wall matrix of the parasite plant and bound by various covalent and noncovalent linkages to the other CW components.

In the recent years, a variety of biological activities of pectic polymers isolated from herbal plants, has been reported [25, 26]. Fig. 2 illustrates the mitogenic and comitogenic activites of the *Cistanche* pectin fractions in comparison to those of the immunomodulator Zymosan. All fractions showed significant biological activities in both mitogenic and comitogenic tests. The direct mitogenic activity of P2 was about two times higher than that of Zymosan. Both P2 and P3 were more effective in the comitogenic test than Zymosan and P1. None of the investigated pectins was found to be contaminated with endotoxins. In accord with the results of recent studies [26, 27] on the mitogenic activity of pectic polysaccharides isolated from various Chinese medicinal plants [26, 27], for the expression of the immunomodulatory properties, the rhamnogalacturonan and α-3,5-arabinan moieties seem to be important also in the case of the *Cistanche* pectins.

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