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Water-soluble polysaccharides from *Salvia officinalis* L. possessing immunomodulatory activity

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Abstract

A water-soluble polysaccharide complex (A) composed of galactose (17.9%), 3-*O*-methyl-galactose (3.0%), glucose (15.5%), mannose (8.3%), arabinose (30.4%), xylose (7.6%), fucose (2.6%), rhamnose (6.7%), and uronic acids (8.0%) has been isolated from the aerial parts of sage (*Salvia officinalis* L.) by cold water extraction. It showed a broad molecular-mass distribution pattern ($M_w \sim 2000$–$93\,000$) with a predominance of polymers with $M_w < 10\,000$. Ion-exchange chromatography of A afforded six polymeric fractions (A_1–A_6) in which arabinogalactans associated with galacturonan and/or rhamnogalacturonan backbones prevail. Sage polysaccharides were examined for their immunomodulatory activity in the comitogenic thymocyte test which is interpreted as being an in vitro correlate of adjuvant activity. The acidic polysaccharide fractions A_2, A_3 and A_4 exhibited the highest mitogenic and comitogenic activities of all fractions tested, and relatively high SI_{comit}/SI_{mit} ratios ~3 indicate potential adjuvant properties of these polysaccharides.

Keywords: Sage; *Salvia officinalis*; Arabinogalactans; Rhamnogalacturonans; FT-IR and NMR spectroscopies; Immunostimulatory activity

1. Introduction

Sage (*Salvia officinalis* L.) is a known medicinal plant widely cultivated for use in traditional medicine. Various extracts and decoctions of this herb are employed for treatment of many kinds of ailments (inflammations of the oral cavity, digestive and intestinal tracts, in gastritis and tonsillitis). Recently, the curing effects of sage have been ascribed to the low molecular-mass compounds contained in the herb in relatively large amounts (phenolic acids, phenolic glycosides, diterpenoids, flavonoids), many of which possess a variety of biological activities including those that are antioxidant, antiplatelet, antitumor and antiviral (Lu and Foo, 2001, 2002). In a search for biological active polysaccharides of plant origin, we have described mitogenic and comitogenic activities in crude polysaccharides isolated from sage (Capek et al., 2003). Presently, we are reporting on the isolation and characterization of ion-exchange fractions of water-extractable polysaccharide complex from sage possessing mitogenic and co-mitogenic activities.

2. Results and discusion

2.1. Extraction and characterization of water-soluble polysaccharides

Aerial parts of the plant were repeatedly treated with methanol–chloroform and the drug residue was extracted with cold water. A crude polysaccharide complex (A) was obtained by precipitation of the water extract with ethanol, was dialyzed and freeze-dried. About 4% of the polysaccharide material (A) was solubilized by water extraction from the drug. Polysaccharide complex A showed a broad molecular-mass distribution pattern ($M_w \sim 2000$–$93\,000$) with a predominance of polymers of $M_w < 10\,000$ (Fig. 1). It was composed (Table 1) of arabinose (30.4%), galactose

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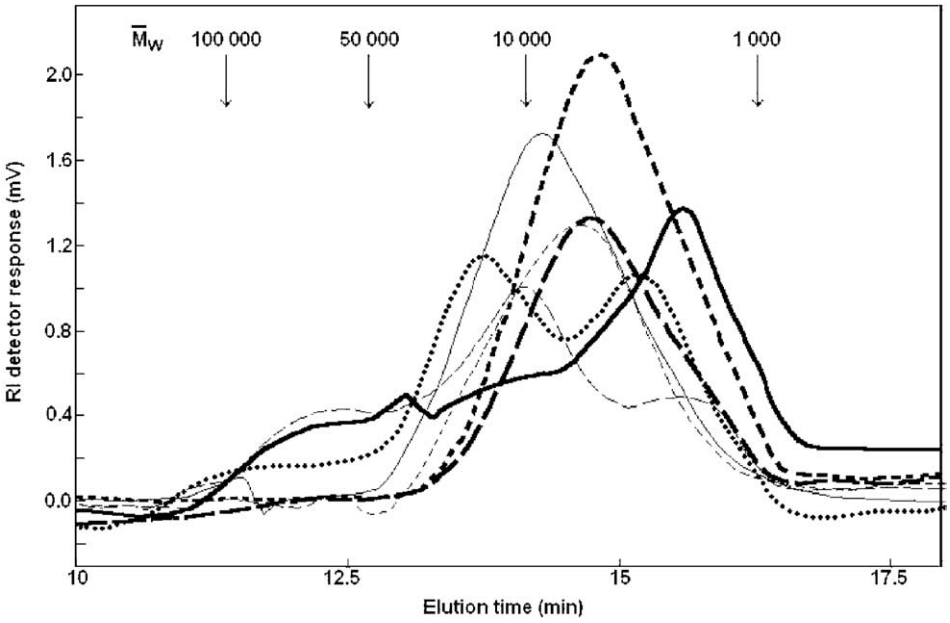

Fig. 1. Molecular-mass distribution of DEAE–sephacel fractions of the crude polysaccharide A. A (–), A_1 (–), A_2 (- - -), A_3 (– – –), A_4 (· · ·), A_5 (– – –), and A_6 (– –).

(17.9%), 3-*O*-methylgalactose (3.0%), glucose (15.5%), mannose (8.3%), xylose (7.6%), fucose (2.6%), rhamnose (6.7%), and uronic acids (8.0%). Besides that, it contained methoxyl groups (1.7%), protein (9.4%), and inorganic material (9.8%). The high content of arabinose and galactose residues (over 50%) as well as the presence of rhamnose and uronic acids (~15%) indicate the presence of arabinogalactans associated with a galacturonan and/or rhamnogalacturonan core. FT-IR spectrum of A (Fig. 2) showed bands at 1072 cm^{-1} (galactans), 1036 cm^{-1} (arabinans), 1614 cm^{-1} ($\nu_{as}COO^-$) and 1414 cm^{-1} (ν_sCOO^-) characteristic for the carboxylic group of uronic acid (Gilli et al., 1994).

The water-soluble polysaccharide complex (A) is important from the point of view of therapeutic applications. To obtain any indication of the presence of the polysaccharides constituting this complex, it was chromatographied on DEAE–sephacel into six fractions by step-wise elution with water (A_1), sodium chloride solutions of increasing concentrations (A_1–A_5) and sodium hydroxide solution (A_6). The individual polysaccharide fractions differed in molecular-mass distribution (Fig. 1), in the composition of neutral sugars, and in uronic acid content (Table 1). Uronic acids were found in all fractions, their content varying from 4.7% to 23.8%; it was increasing in the non-retained fraction A_1 to retained ones A_2, A_3, and A_4 (0.5 M sodium chloride) and then decreasing in 1 M eluents.

The non-retained fraction A_1 was homogeneous on free-boundary electrophoresis and on HPLC showed one symmetrical peak (Fig. 1) with $M_w \sim 8000$. Compositional analysis of A_1 showed the predominance of

Table 1
Sugar composition of DEAE–sephacel fractions of the water-soluble polysaccharides (A) from sage

Fraction	eluent	Monosaccharide composition (mol%)								
		Rha	Fuc	Ara	Xyl	Man	Glc	Gal	3-Gal[a]	UA
A	–	6.7	2.6	30.4	7.6	8.3	15.5	17.9	3.0	8.0
A_1	H_2O	1.2	0.4	35.0	2.9	10.1	13.0	22.1	10.6	4.7
A_2	0.1 M NaCl	4.8	–	34.4	1.5	4.2	6.0	23.4	17.1	8.6
A_3	0.25 M NaCl	11.8	0.7	32.5	2.0	5.6	6.7	21.8	–	18.9
A_4	0.5 M NaCl	18.7	2.0	25.5	1.7	3.3	6.6	18.3	–	23.8
A_5	1 M NaCl	8.9	2.0	34.3	2.5	8.0	14.3	23.8	–	6.2
A_6	1 M NaOH	6.8	2.4	37.7	2.3	8.6	16.2	21.2	–	4.8

[a] 3-*O*-Methyl-galactose.

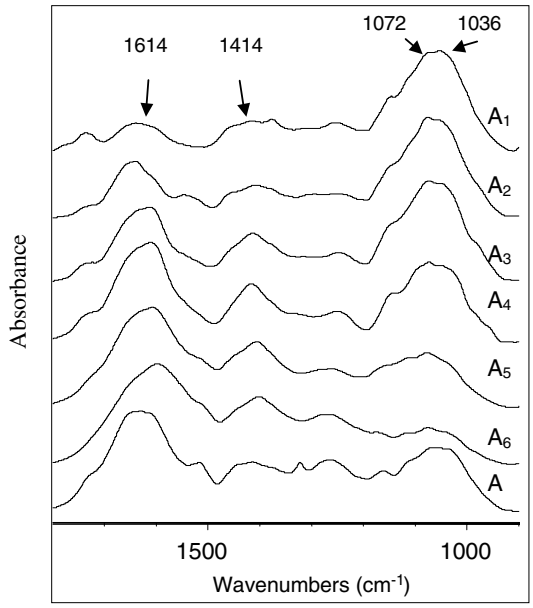

Fig. 2. FT-IR spectra of DEAE–sephacel fractions (A_1–A_6) of the crude polysaccharide complex A.

galactose, glucose and arabinose residues, and the other sugars as mannose, xylose, rhamnose, and uronic acids were present in smaller proportions (Table 1). ^{13}C NMR spectrum of A_1 (Fig. 3) was well resolved with predominant resonances in the anomeric region at 110.1, 108.8, 108.3 and 104.2 ppm due to arabinose and galactose residues, respectively, common constituents of plant arabinogalactans (Capek and Kardoš, 1995; Samuelson et al., 1998). The relatively intensive signal at 57.0 ppm is due to etherification of some galactose residues in arabinogalactan (Capek et al., 2002). Besides, a high-field resonance at 21.5 ppm (CH_3) and one in the lowest magnetic field at 174.7 ppm indicate acetylation of some arabinogalactan molecules (Agrawal, 1992). The FT-IR spectrum of A_1 (Fig. 2) had maxima at 1073 and 1037 cm^{-1} belonging to two components of the arabinogalactan moiety, i.e., galactan and arabinan, respectively (Kačuráková et al., 2000). Sugar linkage analysis revealed a great number of derivatives and indicated a wide range of glycosidic linkages in A_1 (Table 2). The dominant sugar components of A_1 – galactose and arabinose—were found to be involved mainly in 1,6 and 1,3,6; and 1,5; 1,3 and 1,2 linkages, respectively. Such linkages are common for arabino-3,6-galactan type II widely distributed in plant kingdom. The main derivatives of mannose and glucose residues indicated 1,4-linked position of these sugars in polymeric chains.

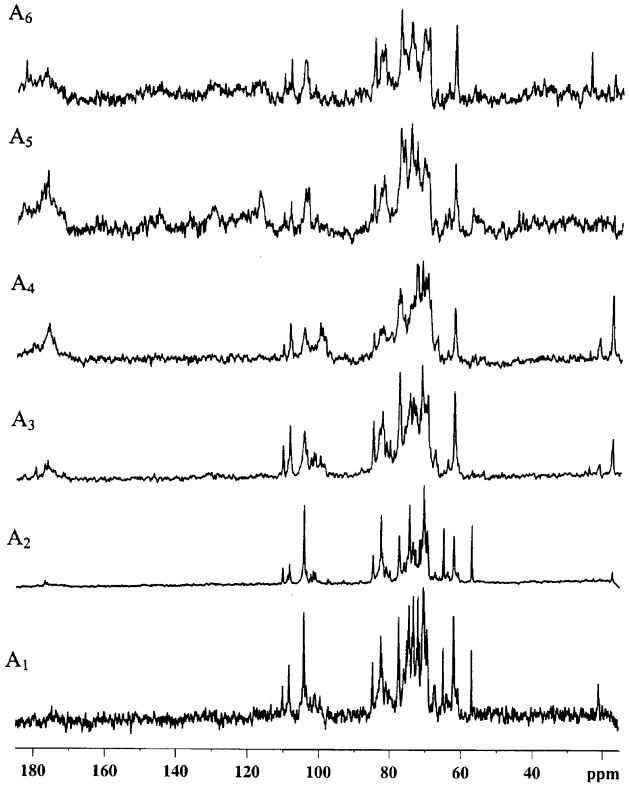

Fig. 3. ^{13}C NMR spectra of polysaccharide fractions A_1–A_6.

The retained fraction A_2, eluted with 0.1 M sodium chloride solution, showed a similar molecular-mass pattern to that of fraction A_1. On HPLC it showed a dominant peak with $M_w \sim 11\,000$, shifted to the lower values (Fig. 1) in comparison with that of A_1, and one smaller peak of $M_w \sim 60\,000$. Its heterogeneity was shown as well by free-boundary electrophoresis, where two peaks were observed. Monosaccharide analysis of A_2 was similar to that of fraction A_1, differences were found only in the content of sugar constituents (Table 1). The relationship of A_2 and A_1 was revealed as well by ^{13}C NMR spectroscopy (Fig. 3). The anomeric region of the spectrum showed the signals at 110.1, 108.8, and 108.3 ppm for arabinose, at 104.2 and 103.5 ppm for galactose residues indicating the arabinogalactan type of polysaccharide in A_2 as the predominant component (Capek and Kardoš, 1995; Samuelson et al., 1998). The other anomeric signals in the region at 102.3–101.0 ppm are due to the less abundant sugar components, i.e., glucose, mannose, rhamnose, and uronic acid residues. Some galactose units of the arabinogalactan moiety are etherified by methyl groups (57.0 ppm) and no signals evidencing the presence of *O*-acetyl groups were found in A_2 (Capek et al., 2002). The FT-IR spectrum of A_2 (Fig. 2) exhibits similar maxima as infraction A_1. Differences were found in the relative absorption intensities of IR maxima, i.e., less intensive arabinose and more intensive galactose related maxima in A_2 in comparison with those of fraction A_1 (Kačuráková et al., 2000). Results of methylation analysis of A_2 showed a similar linkage pattern compare to that of A_1, i.e., the presence of arabino-3,6-galactan as the dominant polysaccharide component. However, the glucose and mannose residues

Table 2
Methylation analysis data of polysaccharide fractions A_1–A_4

Sugar derivatives	Mol%				Linkages
	A_1	A_2	A_3	A_4	
2,3,4-Me_3-Rha*p*[a]	0.7	4.0	6.3	6.2	Terminal
3,4-Me_2-Rha*p*	0.4	0.2	1.3	–	1,2-
2,4-Me_2-Rha*p*	–	0.2	1.2	3.2	1,3-
2,3-Me_2-Rha*p*	–	–	–	2.9	1,4-
2-Me-Rha*p*	0.3	–	–	–	1,3,4-
3-Me-Rha*p*	Trace	Trace	4.6	8.4	1,2,4-
2,3,5-Me_3-Ara*f*	11.4	12.2	14.3	10.7	Terminal
2,3,4-Me_3-Ara*p*	4.2	1.7	2.7	3.0	Terminal
3,5-Me_2-Ara*f*	–	1.4	2.1	2.8	1,2-
2,5-Me_2-Ara*f*	8.0	7.1	6.6	4.6	1,3-
2,3-Me_2-Ara	9.5	8.6	11.5	6.5	1,5- or 1,4-
2-Me-Ara	–	2.1	–	–	1,3,5- or 1,3,4-
3-Me-Ara	–	2.2	–	6.6	1,2,5- or 1,2,4-
Ara	2.6	1.0	2.1	–	1,2,3,5- or 1,2,3,4-
2,3,4-Me_3-Fuc*p*	Trace	Trace	0.3	–	Terminal
2,4-Me_2-Fuc*p*	–	–	0.2	1.8	1,3-
2,3,4-Me_3-Xyl*p*	1.9	0.5	1.6	1.4	Terminal
2,3-Me_2-Xyl*p*	–	1.2	1.2	0.7	1,4-
3,4-Me_2-Xyl*p*	2.1	0.2	–	0.1	1,2-
2,3,4,6-Me_4-Man*p*	0.8	0.4	0.4	0.5	Terminal
3,4,6-Me_3-Man*p*	Trace	0.2	1.2	0.9	1,2-
2,4,6-Me_3-Man*p*	–	2.2	4.9	4.3	1,3-
2,3,6-Me_3-Man*p*	7.9	1.7	0.2	–	1,4-
2,3,4-Me_3-Man*p*	Trace	–	–	–	1,6-
2,3-Me_2-Man*p*	2.9	0.9	–	–	1,4,6-
Man*p*	–	–	0.9	–	1,2,3,4,6-
2,3,4,6-Me_4-Glc*p*	1.2	0.9	1.8	1.6	Terminal
3,4,6-Me_3-Glc*p*	–	0.1	0.2	–	1,2-
2,3,6-Me_3-Glc*p*	8.1	1.0	1.4	7.4	1,4-
2,6-Me_2-Glc*p*	0.1	–	–	–	1,3,4-
2,3-Me_2-Glc*p*	4.8	–	–	0.3	1,4,6-
2,4-Me_2-Glc*p*	–	3.9	4.8	–	1,3,6-
2,3,5,6-Me_4-Gal*f*	0.4	0.9	2.0	–	Terminal
2,3,4,6-Me_4-Gal*p*	5.3	3.2	4.2	4.6	Terminal
3,4,6-Me_3-Gal*p*	–	0.5	–	–	1,2-
2,4,6-Me_3-Gal*p*	–	–	–	2.9	1,3-
2,3,6-Me_3-Gal*p*	0.5	0.2	1.3	2.8	1,4-
2,3,4-Me_3-Gal*p*	16.6	30.1	5.7	5.1	1,6-
2,6-Me_2-Gal*p*	0.1	–	–	–	1,3,4-
2,3-Me_2-Gal*p*	0.4	–	–	0.1	1,4,6-
2,4-Me_2-Gal*p*	7.3	11.2	15.0	10.6	1,3,6-
3,4-Me_2-Gal*p*	2.5	–	–	–	1,2,6-

[a] 2,3,4-Me_3-Rha = 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylrhamnitol, etc.

were found to occupy predominantly 1,3,6- and 1,3-linkages (Table 2), respectively.

The polysaccharide material eluted with 0.25 M sodium chloride solution (A_3) was heterogeneous on free-boundary electrophoresis, and on HPLC showed a wide range of M_w with three maxima at ~2000, 9000 (a dominat peak), and 66 000 (Fig. 1). The sugar composition revealed the predominance of arabinose, galactose, and uronic acid residues. The other sugars, i.e., fucose, xylose, mannose, and glucose were found in lesser amounts (Table 1). A relatively high content of uronic acids (~19%) and the presence of rhamnose residues (~12%) indicate in addition to the arabinogalactan moiety (band maxima in IR spectrum at 1073 and 1037 cm^{-1}) a rhamnogalacturonan type of polymer as well (bands at 1734, 1612 and 1414 cm^{-1} due to COO^- and at 983, 917, 900 and 820 cm^{-1} for the anomeric region; Fig. 2) in A_3 (Kačuráková et al., 2000). The main structure reporter signals in the ^{13}C NMR spectra of A_3 supported the results of compositional analysis.

The occurrence of the anomeric signals at 110.2, 108.8, and 108.3 ppm are due to arabinose residues, at 104.2 and 103.5 ppm arise from galactose units, and resonances in the region 102.3–99.7 ppm could be assigned to mannose, rhamnose, glucose, and uronic acid residues. In addition to the above-mentioned signals, the resonances in the lowest field at 176.1–174.0 ppm indicate the presence of uronic acids and *O*-acetyl groups, two high-field signals at 21.2 and 17.5 ppm derive from acetyl groups and rhamnose residues, respectively (Agrawal, 1992; Capek and Kardoš, 1995; Vignon and Garcia-Jaldon, 1996; Capek et al., 1997; Samuelson et al., 1998). Sugar linkage analysis of A_3 indicated 3,6-linked type of arabinogalactan similarly as in fractions A_1 and A_2. However, in A_1 and A_2 was found the ratio 1,3,6- and 1,6-linked galactose residues 1:2.3 and 1:2.7, respectively, while in A_3 2.6:1. The higher content of 3,6-linked galactose units indicates more branched type of arabinogalactan moiety in A_3. Similarly as in A_2, glucose and mannose residues were found in A_3 mainly in 1,3,6- and 1,3-linked positions, respectively. The higher content of rhamnose derivatives (~12%) indicates the presence of rhamnogalacturonan type of polymer in this fraction.

The polysaccharide fraction eluted with 0.5 M sodium chloride solution (A_4) showed molecular heterogeneity on free-boundary electrophoresis and HPLC. Two main peaks with $M_w \sim 3000$ and 13 000, and one small of higher $M_w \sim 120\,000$ were found in this fraction (Fig. 1). On hydrolysis it yielded rhamnose, arabinose, galactose, and uronic acid residues as the predominant sugar components and the other sugars, i.e., glucose, mannose, fucose, and xylose were detected only in low amounts (Table 1). It contained the highest proportions of uronic acids (~24%) and rhamnose residues (~19%) of all ion-exchange fractions. FT-IR and ^{13}C NMR patterns of A_4 (Figs. 2 and 3) showed similarity with the spectra of fraction A_3. In the anomeric region of the ^{13}C NMR spectrum the signals at 110.2, 108.8, and 108.3 ppm are due to arabinose residues, at 104.2 and 103.5 ppm to galactose units typical for arabinogalactan moiety (Capek and Kardoš, 1995; Samuelson et al., 1998), resonances at 99.9–98.4 ppm being due to galacturonic acid and rhamnose units derived from rhamnogalacturonan types of polysaccharides. The split signals in the low field at 175.9, 175.8, and 174.0 ppm derive from the resonances of carboxyl groups of uronic acids and acetyl groups, respectively. The two signals in the high field at 21.2 and 17.5 ppm are due to acetyl groups and rhamnose residues, respectively (Agrawal, 1992; Vignon and Garcia-Jaldon, 1996; Capek et al., 1997). Sugar linkage analysis of A_4 showed the similar arabino-3,6-galactan linkage pattern as those of A_3. The highest content of rhamnose (~21%) derivatives indicates as well the presence of rhamnogalacturonans in this fraction (Table 2).

The ion-exchange fractions eluted with 1 M sodium chloride (A_5) and sodium hydroxide (A_6) solutions showed similar molecular-mass patterns with one symmetrical peak of $M_w \sim 4$–5000 (Fig. 1). Both fractions were rich mainly in arabinose, galactose, and glucose (Table 1). The higher content of rhamnose residues in comparison with uronic acids was noticeable in A_5 and A_6. ^{13}C NMR spectra of A_5 and A_6 fractions displayed similar pattern (Fig. 3). Both dark brown materials were contained in the lower magnetic field signals characteristic for colour substances and phenolic compounds (in IR spectra the band at 1512 cm^{-1}) (Himmelsbach et al., 1994). In the anomeric regions there were found signals characteristic for arabinose residues (110.2, 108.8, and 108.3 ppm) as well as for galactose units (104.2 and 103.5 ppm) confirming the presence of arabinogalactans as the predominant polysaccharide components in these fractions (Capek and Kardoš, 1995; Samuelson et al., 1998).

2.2. Mitogenic and comitogenic activities of ion-exchange fractions of A

The immunomodulatory activities of the water-soluble polysaccharide complex A and its ion-exchange fractions A_1–A_6 were examined in the in vitro comitogenic thymocyte test. Originally, this test was performed with guinea-pig thymocytes which are refractory to lipopolysaccharide (LPS) in this test (Iribe and Koga, 1984). We have found that several immunostimulatory compounds which are not noticeably comitogenic for guinea-pig thymocytes exhibited the comitogenic effect with mouse or rat thymocytes. Also plant polysaccharides exhibited a more marked response with rat than guinea-pig thymocytes (Ebringerová et al., 1995) and therefore, the rat system has been used to screen all further plant polysaccharides studied. However, in this system, immunomodulatory-active polysaccharides were also directly mitogenic, and the same has applied to LPS. For this reason, to avoid the stimulatory effect of eventual endotoxin contamination of the polysaccharide preparations, all the polysaccharides have been tested in the presence of polymyxin B (PMB) which inhibits stoichiometrically the biological effects of LPS including its mitogenicity (Jacobs and Morrison, 1977). In our hands, the dose of 20 μg PMB ml^{-1} culture which by itself had no effect on cell proliferation, inhibited the proliferative response of rat thymocytes to 5 μg LPS ml^{-1} (LPS *E. coli* 055, Difco) completely, to 50 μg LPS ml^{-1} by about 50%. According to the results of the Limulus amoebocyte lysate test, at the highest used concentration of the tested preparations the content of contaminating endotoxin was from 0.075 ng to 1.6 μg ml^{-1} culture.

The immunomodulatory effect of the polysaccharides tested is illustrated in the form of a log dependence of

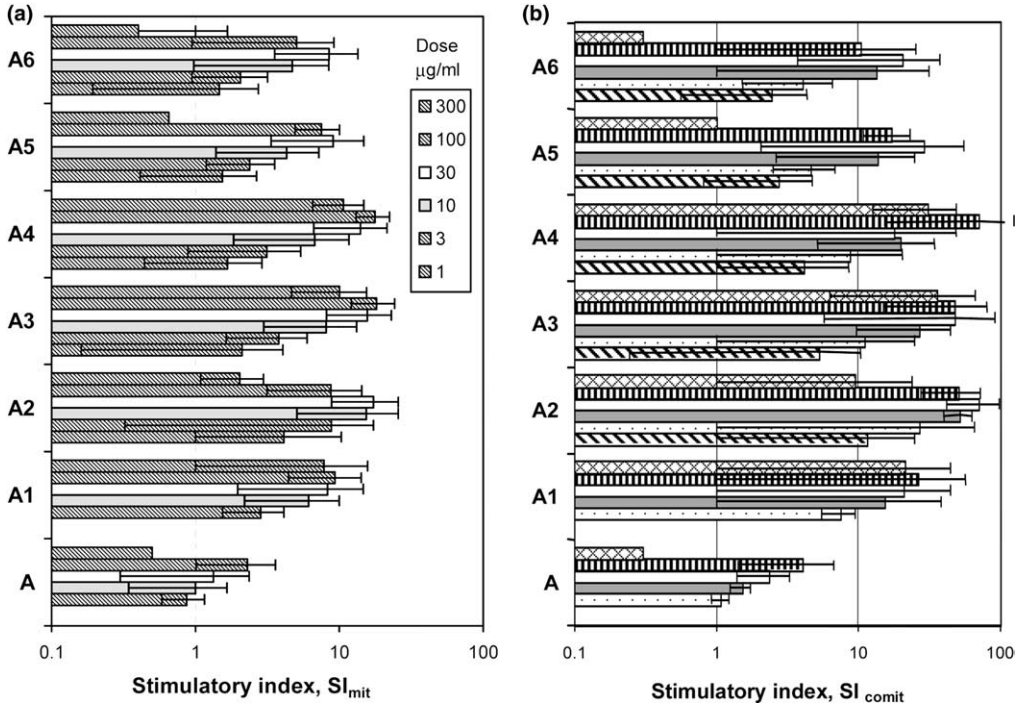

Fig. 4. Mitogenic (a) and comitogenic (b) activities of the crude polysaccharide complex A and its DEAE–sephacel fractions A_1–A_6. The columns represent arithmetical means of stimulation indices (SI) from 3 to 4 repeated experiments including 95% confidence interval limits.

the stimulatory indices on the applied dose (Fig. 4(a) and (b)). The proliferative responses at doses from 3 to 300 μg ml^{-1} for A and A_1 and 1–300 μg ml^{-1} for A_2–A_6 are illustrated. In the dose range used, the proliferative responses have risen dose-dependently to a certain maximum; with any further increase of the dose above the optimum, the response fell off more or less markedly. The inhibiting dose was not the same for all fractions. For polysaccharide complex A, A_5 and A_6 marked inhibition appeared at 300 μg ml^{-1}. For fractions A_1–A_4, the decrease of the mitogenic response even at the dose of 300 μg ml^{-1} was insignificant. In repeated testing of the same sample, the inhibition appeared always at the same dose, indicating that in question were not fluctuations in the sensitivity of the biological system implemented. An inhibition effect has already been noticed in evaluating the mitogenic activity of polysaccharides isolated from other herbal plants (Capek et al., 2003; Hromádková et al., 2002; Ebringerová et al., 2003; Yamada et al., 1990), being manifested by a fall or levelling off of the proliferative response at higher doses (>500 μg ml^{-1}). Its cause has not been discussed and is still unknown. It might result in some cases from the effect of contaminating non-carbohydrate impurities.

The highest values of mitogenic and comitogenic activities were observed in the acidic fractions A_2, A_3 and A_4. From the point of view of efficacy, fractions A_3 and A_4 can be considered as a single entity; the responses to all doses tested, the maximum at 100 μg ml^{-1}, and only an insignificant decrease of responses at 300 μg ml^{-1}, all being quite comparable in both fractions. Fraction A_2 differed considerably from the two ones mentioned above. Its maximum responses, the values of which were comparable to that observed with fractions A_3 and A_4, were obtained at 30 μg ml^{-1}, i.e., at a three times lesser dose. Moreover, the response began to fall off already at the dose of 100 μg ml^{-1}. In comparison with A_3 and A_4 (in the statistical evaluation of results, treated as one preparation), a significantly higher comitogenic response in A_2 at all doses up to 30 μg ml^{-1} has been demonstrated in the region of the rising dose–response curves. However, in view of the dispersion of the SI values obtained from individual repeat experiments with A_2, the increase in mitogenic activity was not statistically significant. Nevertheless, fraction A_2 could be considered the most effective of all three acidic fractions tested. In these fractions, the SI_{comit}/SI_{mit} ratios of about 3 is suggestive of possible adjuvant properties.

The acidic fractions A_2–A_4 of the polysaccharide complex A in their immunomodulatory activities surpassed fraction D described in our previous paper (Capek et al., 2003) as the most potent polysaccharide isolated from sage. Fraction A_2 was significantly more potent in the whole range of doses from 1 to 100 μg ml^{-1}, while fractions A_3 and A_4 were signficantly more potent only in the dose range from 30 to 300 μg ml^{-1}.

The activities of A_5 and A_6 were comparable mutually as well as with those of the neutral fraction A_1 within the dose range from 3 to 100 μg ml^{-1}. However, at 300 μg ml^{-1} fractions A_5 and A_6 elicited a marked

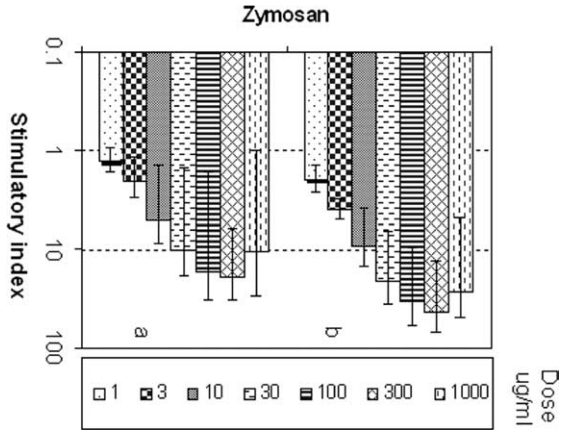

Fig. 5. Mitogenic (a) and comitogenic (b) activities of the zymosan.

inhibitory effect. In all these three fractions, the SI_{comit} / SI_{mit} ratios were low (<3).

The polysaccharide complex A elicited maximum response at 100 μg ml^{-1}. At that dose, the responses to fractions A_1–A_6 were significantly higher than in the case of A. All the ion-exchange fractions (A_1–A_6) were more potent in the whole range of doses than the parent one. Besides, the maximum response in fraction A at dose 100 μg ml^{-1} was comparable with that observed in all fractions A_1–A_6 at doses 1–3 μg ml^{-1} (Fig. 4(a) and (b)).

The immunostimulatory activities of tested preparations have been compared with that of zymosan (yeast β-glucan) as has been usually done in fungal and plant polysaccharides hitherto examined by us in the comitogenic tests (Fig. 5). With the mitogenic response to zymosan, there were roughly comparable the thymocyte responses to the most active fractions A_2–A_3 with the difference that in zymosan the response increases dosewise up to 300 μg ml^{-1}, whereas in the fractions examined the response more or less markedly decreases at that concentration; the maximums have been attained in the range of 30–100 μg ml^{-1}. The comitogenic responses of the most active fractions were higher than that of zymozan, because in zymosan the ratio $SI_{comit}/SI_{mit} = 2$, while in the fractions it is about 3.

2.3. Conclusion

The present study revealed the capacity of all ion-exchange fractions of the water-soluble polysaccharide complex A to induce the proliferation of rat thymocytes in the order $A_2 > A_3 = A_4 > A_1 = A_5 = A_6 > A$. Besides, fractions A_2, A_3 and A_4 possessed a significant comitogenic effect, and the SI_{comit} /SI_{mit} ratios ~3 indicate the potential adjuvant properties of these acidic polysaccharides. A separation of the polysaccharide complex A according to uronic acid content and molecular masses into six polymeric fractions (A_1–A_6) led to a significant increase of their immunomodulatory activities in comparison with the parent material. Compositional and methylation analyses, as well as ^{13}C NMR measurements indicated an arabino-3,6-galactan as the major component in A_1, arabinogalactans associated with rhamnogalacturonan chains in A_2, A_3, and A_4, and hexosan-type polysaccharides in A_5 and A_6fractions. Our previous biological study on the crude sage carbohydrate fractions (Capek et al., 2003) revealed their immunological activities in the order D > B > A. The main polysaccharide component of D comprised a glucuronoxylan, that of B a pectin material, and that of A an arabinogalactan associated with a rhamnogalacturonan core. However, the acidic fractions A_2–A_4 of the polysaccharide complex A surpassed in immunomodulatory activities fraction D described as the most potent polysaccharide isolated from sage (Capek et al., 2003). The results suggested that a ramified RG-I core with arabinan- or arabinogalactan-rich side chains (A_2–A_4) and glucuronoxylan-related polymers (D) are the most potent polysaccharide constituents of sage. Although both types of polysaccharides possess similar biological activities, they differ greatly in the primary structure of their backbones as well as in the size of their molecules. Besides, in the fraction A_2 was determined the highest content of 3-*O*-methylgalactose residues which could be an important structural factor for its immunomodulatory activity. The arabinan or arabinogalactan chains attached to a galacturonan and/or rhamnogalacturonan core have been isolated from various medicinal plants and were reported to exhibit anti-complementary and mitogenic activities (Yamada et al., 1990; Samuelson et al., 1996; Kiyohara et al., 1996; Zhao et al., 1991; Hirano et al., 1994; Zhang et al., 1996). Similar immunological activities have been reported in the case of glucuronoxylan-related polymers isolated from various herbal plants (Ebringerová et al., 2003; Samuelson et al., 1996; Ebringerová et al., 2002). It seems that much efforts should be made to elucidate the possible structure/activity relationship of plant polysaccharides.

3. Experimental

3.1. Material

The aerial parts of *Salvia officinalis* L. were purchased from Slovakofarma, Medicinal Plants, Malacky, Slovakia. RPMI-1640 medium and fetal calf serum were supplied by Sigma (Sigma–Aldrich Chemie GmbH, Germany). Phytohaemagglutinin (PHA) and polymyxin B (Aerosporin) were supplied by Wellcome Diagnostics, UK. ^3H-Thymidine (specific activity 960 GBq $mmol^{-1}$) was purchased from Lacomed, Ltd. (Řež near Prague, Czech Republic). Zymosan (yeast β-glucan) was purchased from Likospol Ltd. (Bratislava, Slovakia).

3.2. General methods

Solutions were concentrated under diminished pressure below 40 °C. Free-boundary electrophoreses of 1% solutions of the polysaccharides were effected with a Zeiss 35 apparatus, using 0.05 M sodium tetraborate buffer (pH 9.2) at 150 V/cm and 6 mA for 30 min. Polysaccharides were hydrolyzed with 2 M trifluoroacetic acid for 1 h at 120 °C. Quantitative determination of the neutral sugars was carried out in the form of their trifluoroacetates (Shapira, 1969) by gas chromatography on a Hewlett–Packard Model 5890 Series II chromatograph equipped with a PAS-1701 column (0.32 mm × 25 m), the temperature program of 110–125 (2 °C/min)–165 °C (20 °C/min) and flow rate of hydrogen 20 ml min^{-1}. The uronic acid content was determined with 3-hydroxybiphenyl reagent (Blumenkrantz and Asboe-Hansen, 1973). Protein was calculated from the nitrogen content (%N × 6.25). Gas chromatography–mass spectrometry of partially methylated alditol acetates (Jansson et al., 1976) was effected on a FINNIGAN MAT SSQ 710 spectrometer equipped with an SP 2330 column (0.25 mm × 30 m) at 80–240 °C (6 °C/min), 70 eV, 200 A, and ion-source temperature 150 °C.

3.3. Extraction of polysaccharides

The air-dried and crushed aerial parts of *Salvia officinalis* L. (480 g) were exhaustively extracted with methanol–chloroform (10:1 v/v) to remove the main extractive compounds. The drug residue (440 g) was extracted twice in distilled water (10 l) for 24 h at laboratory temperature in the presence of 0.02% solution of sodium azide. The aqueous extracts were combined, concentrated and poured into 96% ethanol containing 1% (v/v) of acetic acid. The precipitate was dissolved in water, dialyzed and freeze-dried (A; 17.2 g).

3.4. Ion-exchange chromatography

The water-extractable polysaccharide complex A (5 g) was dissolved in distilled water (500 ml) and the solution was centrifuged to remove the insoluble portion. The soluble portion was applied to a column (8 × 20 cm) of DEAE–sephacel and eluted successively with water (A_1), 0.1 M (A_2), 0.25 M (A_3), 0.5 M (A_4) and 1 M (A_5) NaCl solutions, and finally with 1 M NaOH (A_6). Fractions of 10 ml were collected and analyzed for the carbohydrate content by phenol–sulfuric acid assay (Dubois et al., 1956). The subfractions eluted with NaCl solutions were concentrated, dialyzed and freeze-dried. The strongly coloured subfraction eluted with 1 M NaOH solution was neutralized, concentrated, dialyzed and freeze-dried. Yields of the individual subfractions were the following: 373 mg of A_1, 242 mg of A_2, 354 mg of A_3, 326 mg of A_4, 510 mg of A_5, and 1800 mg of A_6.

3.5. Determination of molecular mass

The apparatus used (Shimadzu, Wien, Austria) comprised an LC-10AD high-pressure pump, a GT-104 membrane degasser, an Reodyne 77251 injector equipped with a 0.02 ml sample loop, an RID-6A differential refractometer, and a SPD-10AV UV–Vis detector. Chromatographic experiments were performed using a tandem of two HEMA-BIO 100 columns followed by a HEMA-BIO 40 column (Tessek, Prague, Czech Republic) of dimensions 8 × 250 mm and particle size 10 μm. As a mobile phase, 0.02 M phosphate buffer pH 7.2 containing 0.1 M NaCl was used at flow rate 0.8 ml min^{-1}. Samples and calibration dextrans (within the range of M_w from 1000 to 100 000) were injected at 1 mg ml^{-1} concentration. Output signals from the differential refractometer as well as from the UV detector (280 nm) were processed on-line using a Class-VP-chromatography software package.

3.6. NMR and FT-IR spectroscopies

The samples were dissolved in 0.5 ml D_2O (99.99 atom%) in 5-mm tubes. ^1H and ^{13}C NMR spectra of carbohydrates were recorded at 25 °C, on a Bruker DPX AVANCE 300 spectrometer operating at a 300 MHz for ^1H and 75.46 MHz for ^{13}C. Acetone was used as an internal standard (δ 2.225 ppm for ^1H and 31.07 ppm for ^{13}C). Infrared spectra were obtained on a NICOLET Magna 750 spectrometer with DTGS detector and OMNIC 3.2 software. 128 scans were recorded with 4 cm^{-1} resolution. The samples were pressed into KBr pellets with a sample/KBr ratio of about 2/200 mg. In order to obtain more exact band positions, Fourier self-deconvolution was applied using Omnic 3.2 software (bandwidth 50 cm^{-1}, enhancement factor 2.6).

3.7. Methylation analysis

The dry samples of polysaccharides (~5 mg) were solubilized in dry Me_2SO (1 ml) and methylated in the presence of methylsulfinylmethanide with CH_3I (Hakomori, 1964). The methylated products were purified using a Sep-Pak C_{18} cartridge (Waters Assoc.), hydrolyzed with 90% formic acid at 100 °C for 1 h and with 2 M trifluoroacetic acid at 120 °C for 1 h, reduced with sodium borodeuteride, acetylated and analyzed by GLC–MS.

3.8. Mitogenic and comitogenic tests

Immunostimulatory activities of samples were screened according to Iribe method (Iribe and Koga, 1984) with slight modifications. Wistar rat thymocytes in RPMI-1640 medium supplemented with 5% fetal calf serum were cultivated at a concentration of 1.5×10^6/0.2 ml per well. Test compounds were added at final con-

centrations from 1 to 300 μg ml^{-1}. In the comitogenic test, PHA at a final concentration of 25 μg ml^{-1} was added. Possible contamination of the tested preparations with endotoxin was checked by cultivation in the presence of polymyxin B (20 μg ml^{-1} culture). In each experiment, the geometrical means of counts per minute (cpm) for each set of 4 replicas were used for calculating the stimulation indices (SI). The direct mitogenic effect of the compounds tested was expressed as SI_{mit} = mean cpm test compound/mean cpm control, and the comitogenic effect was expressed as SI_{comit} = mean cpm (PHA + test compound)/mean cpm PHA. Each substance was tested minimally in at least three independent experiments.

Statistical analysis. At the level of individual experiments, each geometrical mean of cpm of stimulated cultures was compared with that of a respective control (i.e., cells without any stimulant for the mitogenic effect; cells + PHA for the comitogenic effect) by Student's t test as the preliminary estimation of stimulatory ability of the tested compounds. Results of individual experiments are not presented graphically. At the level of repeated experiments, arithmetical means of SI values with their respective values of variability (SD) were calculated. Differences between the effects of the tested compounds were evaluated by analysis of variance (ANOVA), in cases of significant differences among dispersions of compared results by a non-parametric technique of the Kruskal–Wallis test. All the calculations were done by program EP16, which is freely available. The graphical expression of the results relates to mean SI values from repeated experiments; in order to also present the variability, there have been added 95% confidence interval limits: $[SI - (t \times SE); SI + (t \times SE)]$, wherein the t value is Student's quantile for 95% probability and $(n - 1)$ degrees of freedom (n is number of repeated experiments), and SE is the standard error of the mean. By this method, the comparison of effects for different compounds in different dilutions is very simple – if the compared intervals overlap, the activities are similar; if not, the compared activities are significantly different.

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