Carbohydrate Polymers 87 (2012) 551–556

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Analysis of Herba Asari polysaccharides and their immunological activity

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ARTICLE INFO

Article history:
Received 16 February 2011
Received in revised form 4 August 2011
Accepted 9 August 2011
Available online 22 August 2011

Keywords:
Herba Asari
Polysaccharide
Lymphocyte proliferation

ABSTRACT

A water-soluble polysaccharide (HA) was extracted from the Herba Asari root. HA was separated into a starch-like glucan fraction (HA1) and a pectin fraction (HA2) using DEAE-cellulose. HA2 was further fractionated into three pectic polysaccharides, HA2-a, HA2-b and HA2-c, using ion-exchange chromatography. NMR and sugar composition analyses demonstrated that HA2-a is an arabinogalactan (AG) and HA2-b and HA2-c are xylogalacturonans (XGA) with AG domains. Lymphocyte proliferation assays showed that both the neutral polysaccharide and acidic polysaccharide were potent B and T cell stimulators that may have two different modes of action.

1. Introduction

Many polysaccharides extracted from herbal medicines possess anti-tumor and immune-stimulating properties (Inngjerdingen et al., 2008; Ma, Wang, Zhang, Zhang, & Ding, 2010; Wang, Liu, & Fang, 2005). These polysaccharides, which are usually low in toxicity and cause few side effects, may be potential candidates for immunomodulatory or antitumor therapeutics and adjuvants (Schepetkin & Quinn, 2006). Herba Asari (Xixin, Manchurian Wildginger, *Asarum* spp.) was first reported in the monograph *Shennong Compendium of Materia Medica* (Shennong Bencaojing) compiled during the Eastern Han dynasty (25–220 AD) (Gao, 2004). Currently, it is employed in the clinic to treat cough, dyspnea, headache, rheumatic arthralgia, sinusitis and toothache (Drew et al., 2002). In recent years, aristolochic acid, volatile oils and flavonoid glycosides in the Herba Asari have been studied (Zha et al., 2008), but there are no similar reports on polysaccharides. In this paper we report the fractionation, compositional analysis, structural characterization and immunological activity of the polysaccharides from the Herba Asari root.

2. Experimental

2.1. Materials

The dried roots of Herba Asari were cultivated and collected from Tonghua in the Jilin province of China. DEAE-cellulose, Sepharose CL-6B, α-amylase (E.C.3.2.1.1) from *Bacillus* sp., concanavalin A (ConA) and lipopolysaccharide (LPS) were from Sigma. All other chemicals were of analytical grade and made in China.

2.2. General methods

Total carbohydrate content was determined by the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Standards were prepared from monosaccharides that constitute the polysaccharide to be tested. Uronic acid content was determined by the m-hydroxydiphenyl method using galacturonic acid as the standard (Blumenkrantz & Asboe-Hansen, 1973). Gel permeation and anion exchange chromatography were monitored by assaying the total sugar and uronic acid contents. Protein content was determined by the Bradford assay (Sedmark & Grossberg, 1979), using bovine serum albumin as the standard.

2.3. Extraction and fractionation

Herba Asari roots were extracted with boiling water three times for 6 h each and subjected to precipitation by the addition of 95% ethanol (4 volumes). The precipitate was re-dissolved in distilled water, deproteinised using the Sevag method, and exhaustively

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doi:10.1016/j.carbpol.2011.08.019

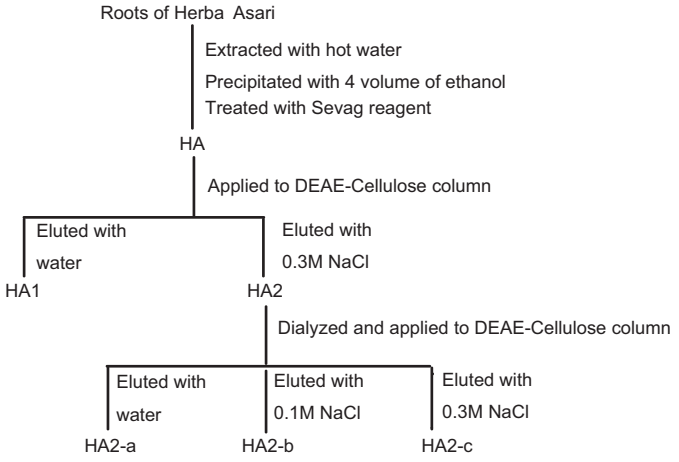

Fig. 1. Fractionation procedure of the polysaccharides from Herba Asari.

dialyzed against distilled water. The ethanol precipitation step was repeated and the residue was dried by solvent exchange (95% ethanol, acetone, and ether) to give the deproteinised polysaccharide fraction HA. HA was re-dissolved in distilled water and loaded onto a column of DEAE-cellulose (3.8 cm × 27 cm), eluting stepwise with H_2O and 0.3 M NaCl to give fractions HA1 and HA2, respectively. HA1 is a neutral polysaccharide and HA2 is an acidic polysaccharide. HA2 was further fractionated using a DEAE-cellulose column and eluted stepwise with H_2O, 0.1 and 0.3 M NaCl to give fractions HA2-a, HA2-b and HA2-c, respectively. The fractionation procedure is shown in Fig. 1.

2.4. Sugar composition analysis

Sugar composition analysis was performed as described by Zhang et al. (2009). Polysaccharide samples (2 mg) were hydrolyzed first with anhydrous methanol containing 1 M HCl for 16 h at 80 °C and then with 2 M TFA for 1 h at 120 °C. The resulting hydrolysates were derivatized with 1-phenyl-3-methyl-5-pyrazolone (PMP) and analyzed on a DIKMA Inertsil ODS-3 column (4.6 mm × 150 mm) connected to a Shimadzu HPLC system (LC-10ATvp pump and SPD-10AVD UV-VIS detector). The PMP derivative (20 μL) was injected, eluted with 82.0% PBS (0.1 M, pH 7.0)/18.0% acetonitrile (v/v) at a flow rate of 1.0 mL/min and monitored by UV absorbance at 245 nm. Sugar compositions of the purified polysaccharides were identified by comparing retention times with those of standard sugars and the contents were calculated from the peak areas using response factors.

2.5. Homogeneity and molecular weight determination

The homogeneity and molecular weight of each fraction were determined by Sepharose CL-6B chromatography. Each sample (5–10 mg) was dissolved in 0.15 M NaCl (1 mL), loaded onto a Sepharose CL-6B column (1.5 cm × 90 cm) and eluted with 0.15 M NaCl at a flow rate of 0.15 mL/min. The eluate was collected at 3 mL per tube and assayed for total sugar and uronic acid contents. Standard dextrans with molecular weight of 12, 50, 150, 470 and 670 kDa were used for column calibration. The elution volumes of standard dextrans were plotted against the logarithms of their respective molecular weights. The elution volumes of samples were then plotted in the same graph, and the molecular weights of polysaccharides were calculated.

2.6. NMR spectra analysis

The ^{13}C NMR spectra were obtained on a Bruker AV600 spectrometer at 150 MHz. The sample (20 mg) was dissolved in D_2O (1 mL, 99.8%) with overnight stirring at room temperature. Acetone was used as an internal standard. The spectra were recorded at 25 °C over 57,000 scans.

2.7. In vivo lymphocyte proliferation assay

Male ICR mice (18–22 g) were purchased from the Pharmacology Experimental Center of Jilin University (China) and acclimatized before use. All mice were housed under standard conditions at room temperature, with a 12 h light–12 h dark cycle and access to standard rodent chow and water. All efforts were made to reduce the number of animals used and to minimize their suffering.

The mice were classified into test and control groups (6 mice/group). The polysaccharide samples HA, HA1 and HA2 were dissolved in physiological saline and administered intraperitoneally (i.p.) into mice at different dosages (10, 50 and 100 mg/kg; 0.2 mL) daily for 10 days. Control mice were given physiological saline instead of the polysaccharide solution. Spleens were collected from the immunized mice under aseptic conditions. Spleen single cell suspensions were pooled in D-Hanks solution (Gibco) filtered through a fine steel mesh. The erythrocytes were lysed with ammonium chloride (0.8%, w/v). After centrifugation (2000 rpm for 10 min), the cells were washed three times with D-Hanks solution and resuspended in complete medium [RPMI 1640 (Gibco) supplemented with 25 mM HEPES (Sigma), 10% heat-inactivated fetal calf serum (Gibco), 1×10^5 U/L penicillin G (Hyclone) and 100 mM streptomycin (Hyclone)]. Splenocyte activity was measured above 95% as assessed by the trypan blue dye exclusion method.

The splenocytes (100 μL/well, 5×10^6/mL) were seeded in a 96-well plate in the presence of medium, ConA (5.0 μg/mL) or LPS (10.0 μg/mL) to a final volume of 200 μL. The plates were incubated at 37 °C in a humidified atmosphere with 5% CO_2. After 44 h, MTT (20 μL, 5 mg/mL) was added to each well and the plate was incubated for another 4 h. After aspirating the supernatant from the wells, DMSO (150 μL) was added to dissolve formazan crystals. The absorbance at 570 nm was measured using a microplate reader (Han et al., 1998).

2.8. Statistical analysis

Results were expressed as the mean ± S.D. of the indicated number of experiments. The data were analyzed for significance using the Student's *t* test. *P*-values of <0.05 and <0.01 were considered statistically significant.

3. Results and discussion

3.1. Polysaccharides composition and structural characterization

A water-soluble polysaccharide (HA) was extracted from the Herba Asari root with boiling water and deproteinised using the Sevag method to a yield of 9.5% (w/w). HA contained 88.7% total sugar, 17.4% uronic acid and less than 1% protein. Sugar component of HA consisted mainly of Glc and GalA (Table 1). HA was fractionated using a DEAE-Cellulose column eluted with water and 0.3 M NaCl to give two respective fractions: HA1 (24%) and HA2 (20%). The total recovery of HA1 and HA2 was 44% because the impurities (including pigments and salts) and insoluble materials (including residues of Herba Asari roots and starch granules) in crude polysaccharide fraction HA were removed by centrifugation before loading the sample on a DEAE-cellulose column. Iodine and phenol–sulfuric acid test showed that the insoluble material contained about 85%

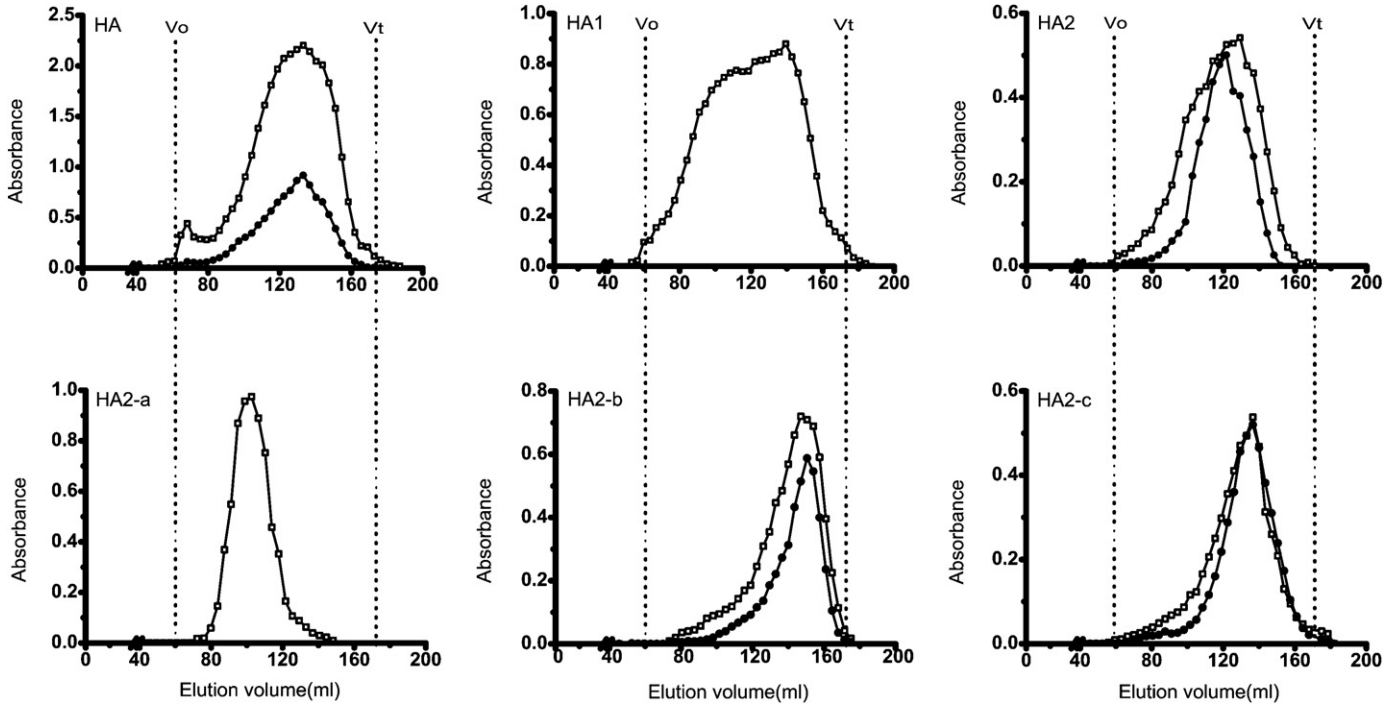

Fig. 2. Molecular weight distributions of the polysaccharide fractions on Sepharose CL-6B (total sugar, □; uronic acid, ●).

starch. As seen in Table 1, HA1 is a neutral fraction mainly composed of glucose (95.8%). A positive iodine test indicated that HA1 is a starch-like glucan. HA2 is a pectic fraction, composed of GalA, Ara, Xyl, Gal and GlcA in a ratio of 18.7:2.4:1.8:1.1:1.0; no Rha was detected in HA2 by HPLC analysis. HA2 was further separated using a DEAE-cellulose column with water and 0.1 and 0.3 M NaCl with stepwise elution to give HA2-a (2%), HA2-b (13%) and HA2-c (58.5%), respectively. Based on the monosaccharide composition, HA2-a is an arabinogalactan (AG), and HA2-b and HA2-c are xylogalacturonan (XGA)-type pectins with AG domains. HA2-b contains more Xyl than HA2-c, indicating that HA2-b has more branched Xyl residues in the HG backbone.

The molecular weight distribution of each Herba Asari polysaccharide fraction from the Sepharose CL-6B column is shown in Fig. 2. HA shows a wide molecular weight distribution. After separation using DEAE-cellulose, the distribution of HA1 still shows a wide peak, while HA2 appears as a relatively narrow peak. However, the HA2-a, HA2-b and HA2-c fractions offer relatively narrow, symmetrical peaks, indicating a homogeneous molecular weight distribution. Using a calibration curve of dextran standards, the molecular weights were estimated to be about 158,000, 12,000 and 21,200 for HA2-a, HA2-b and HA2-c, respectively.

The NMR spectrum of HA1 is shown in Fig. 3. As seen, six signals of glucose appear at 98.52 ppm (C-1), 70.45 ppm (C-2), 72.24 ppm (C-3), 75.73 ppm (C-4), 70.10 ppm (C-5), 59.38 ppm (C-6), which was consistent with those of the 4-linked α-D-Glc*p* (Ni et al., 2009). This result supported that HA1 was a starch-like glucan.

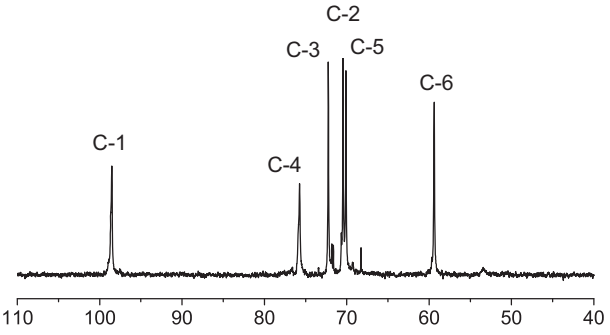

Fig. 3. NMR spectrum of HA1 from Herba Asari.

The NMR spectra of HA2-a, HA2-b and HA2-c are shown in Fig. 4, and the major chemical shifts are listed in Table 2. HA2-a is an arabinogalactan. Although not all NMR signals can be assigned because of overlap of the peaks, some characteristic peaks of Gal and Ara residues are clearly identifiable based on data in the literature (Classen, 2007; Thude & Classen, 2005). In the anomeric

Table 1
Yield and monosaccharide composition of collected fractions.

Fraction	Yield (%)	Monosaccharide composition (%)							
		Man	GlcA	Rha	GalA	Glc	Gal	Xyl	Ara
HA	9.5[a]	5.7	1.2	0.5	15.3	67.6	3.9	3.8	7.2
HA1	24.0[b]	–	–	–	–	95.8	3.4	–	0.8
HA2	20.3[b]	–	4.0	–	71.7	–	5.6	8.7	11.4
HA2-a	2.0[b]	–	–	–	–	–	35.7	–	63.3
HA2-b	13.4[b]	–	1.5	–	66.4	2.2	4.9	18.0	8.5
HA2-c	58.5[b]	–	2.3	–	74.7	–	4.4	7.1	9.7

[a] Yield as % of Herba Asari root dry weight.
[b] Yield as % of fraction applied to column.

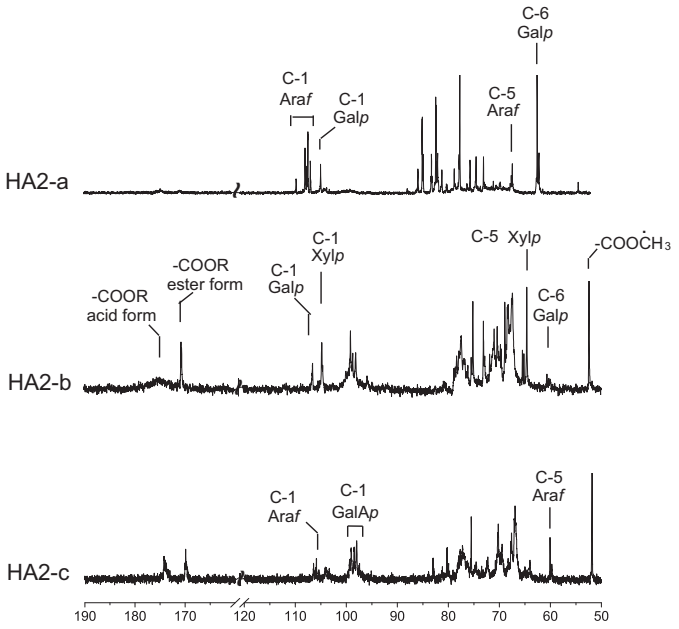

Fig. 4. NMR spectra of pectin from Herba Asari.

carbon region, a cluster of signals at about 103.8 ppm is assigned to C-1 of 1,3-, 1,6- and 1,3,6-linked β-D-Gal*p*. We attribute resonance at 60.61 ppm to C-6 of 1,3-linked β-D-Gal*p* and at 68.60 ppm to 1,6- and 1,3,6-linked β-D-Gal*p* (Thude & Classen, 2005). These Gal linkages are usually present in type II arabinogalactan (AG-II), which has backbone Gal residues and Ara residues in side chains or linked to non-reducing terminals (Golovchenko, Ovodova, Shashkov, & Ovodov, 2002; Habibi, Heyraud, Mahrouz, & Vignon, 2004).

Table 2
Major chemical shifts of carbon atom signals (δ, ppm) in the ^{13}C NMR spectra of HA2-a, HA2-b and HA2-c fractions.

Fraction	Residue	Carbon	ppm
HA2-a	1,3-, 1,6-, 1,3,6-β-D-Gal*p*	C-1	103.80
	1,3-β-D-Gal*p*	C-6	60.61
	1,6-, 1,3,6-β-D-Gal*p*	C-6	68.60
	t-α-L-Ara*f*-(3-	C-1	108.75
	t-α-L-Ara*f*-(5-	C-1	106.59
	1,5-α-L-Ara*f*	C-1	106.96
	1,3-α-L-Ara*f*	C-1	106.38
	1,3,5-α-L-Ara*f*	C-1	105.91
HA2-b	1,4-α-D-Gal*p*A	C-1	97.96
	1,3-α-D-Gal*p*A	C-1	98.50
	1,3,4–α-D-Gal*p*A	C-1	99.09
	t-β-D-Gal*p*A	C-1	97.47
	α-D-Gal*p*A(free)	C-6	174.17
	α-D-Gal*p*A(ester)	C-6	170.02
	α-D-Gal*p*A(ester)	OCH_3	51.84
	t-β-D-Xyl*p*	C-1	104.04
	t-β-D-Xyl*p*	C-5	64.01
	1,4-β-D-Gal*p*	C-1	105.87
	t-α-L-Ara*f*	C-1	106.43
HA2-c	1,4-α-D-Gal*p*A	C-1	97.97
	1,3-α-D-Gal*p*A	C-1	98.46
	1,3,4–α-D-Gal*p*A	C-1	99.05
	t-β-D-Gal*p*A	C-1	97.40
	α-D-Gal*p*A(free)	C-6	174.20
	α-D-Gal*p*A(ester)	C-6	170.02
	α-D-Gal*p*A(ester)	OCH_3	51.82
	t-β-D-Xyl*p*	C-1	104.08
	t-ββ-D-Xyl*p*	C-5	106.42
	1,4-β-D-Gal*p*	C-1	105.86
	t-α-L-Ara*f*	C-1	106.42

The HA2-a NMR signals for Ara are more complex than those for Gal. As seen in Fig. 4, five anomeric carbon signals of α-L-Ara*f* appear between 108.7 and 105.9 ppm, indicating that Ara residues are present in five linkage forms, including terminal α-L-Ara*f* linked to C-3 of Gal*p* (108.75 ppm) or C-5 of Ara*f* (106.59 ppm), 1,5-linked α-L-Ara*f* (106.96 ppm), 1,3-linked α-L-Ara*f* (106.38 ppm) and 1,3,5-linked α-L-Ara*f* (105.91 ppm) (Redgwell, Curti, Fischer, Nicolas, & Fay, 2002; Thude & Classen, 2005). The signals at 60.24 and 65.77 ppm are assigned to the C-5 of Ara*f* residues. These linkage forms of Ara*f* are often observed in type II arabinogalactans (Thude & Classen, 2005).

The ^{13}C NMR spectra of HA2-b and HA2-c are similar to each other and to the chemical shifts of xylogalacturonans in the cell walls of apples (Schols, Bakx, Schipper, & Voragen, 1995) and watermelons (Mort, Zheng, Qiu, Nimtz, & Bell-Eunice, 2008). Typical signals for the C-6 carboxyl group of GalA are observed at 174.20 and 170.02 ppm, indicating the presence of free and esterified carboxyl groups of α-D-Gal*p*A (Catoire, Goldberg, & Pierron, 1998; Sun, Cui, Tang, & Gu, 2010). The signal at 51.84 ppm is assigned to the *O*-methyl of the carboxylic acid methyl ester. Based on the integration of the signals for the ester carbonyl and carboxyl carbons, the degree of methyl-esterification is estimated to be 30% and 50% for HA2-b and HA2-c, respectively. In the anomeric region, the signals at 99.09, 98.50, 97.96 and 97.47 ppm are assigned to 3,4-linked α-D-Gal*p*A, 3-linked α-D-Gal*p*A, 4-linked α-D-Gal*p*A and the reducing terminal of β-D-Gal*p*A, respectively. The anomeric carbon signal at 104.08 ppm and the corresponding C-5 signal at 64.01 ppm are attributable to terminal β-D-Xyl*p* (Huisman et al., 2001; Mort et al., 2008). These results imply that both HA2-b and HA2-c contain xylogalacturonan structures in which 4-linked α-D-Gal*p*A acids and esters appear in the backbone and terminal β-D-Xyl*p* residues are linked to the *O*-3 of 4-linked α-D-Gal*p*A. In addition, NMR signals indicated that 3-linked α-D-Gal*p*A residues are present in HA2-b and HA2-c, which is consistent with the previous observation that xylogalacturonan structures contain 3-linked α-D-Gal*p*A (Mort et al., 2008). The C-1 signals of Xyl in HA2-b are stronger than in HA2-c, in accordance with our finding that HA2-b contains more Xyl. Six Gal residue signals appear at 105.87 ppm (C-1), 70.36 ppm (C-2), 72.27 ppm (C-3), 76.79 ppm (C-4), 73.47 ppm (C-5) and 59.71 ppm (C-6), typical of a 4-linked β-D-Gal*p* (Cipriani et al., 2009). The C-1 signal at 106.43 ppm and the matching signal at 60.07 ppm for the C-5 of Ara indicate the presence of terminal α-L-Ara*f*. Therefore, we speculate that type I arabinogalactan might be present in both HA2-b and HA2-c.

Pectins are a family of complex polysaccharides that usually contain arabinogalactan (AG), homogalacturonan (HG), rhamnogalacturonan I (RG-I), xylogalacturonan (XGA) and rhamnogalacturonan II (RG-II) domains (Ridley, O'Neill, & Mohnen, 2001; Schols & Voragen, 1996). AG-I is composed of a β-1,4-Gal*p* backbone with α-L-Ara*f* attached to the O-3 or O-6 of Gal residues, while AG-II has a β-1,3-Gal*p* backbone with β-1,6-Gal*p* side chains branched at the C-6 of the β-1,3-Gal*p* backbone; the Gal residues of the side chains can be substituted with α-L-Ara*f* (Voragen, Coenen, Verhoef, & Schols, 2009). HG is a linear chain of 1,4-linked α-D-GalA residues whose carboxyl groups are often partially methyl-esterified (Ridley et al., 2001). RG-I has a backbone of alternating α-L-Rha and α-D-GalA residues to which Ara- and Gal-rich side chains are attached via the C-4 of Rha residues (McNeil, Darvill, & Albersheim, 1980). XGA is a polymer with an HG backbone that incorporates varying amounts of β-D-xylose substitutions at the C-3 of GalA residues (Caffall & Mohnen, 2009). RG-II is an HG backbone composed of nine to ten α-1,4-linked GalA and four different oligosaccharide chains attached to a uronic acid backbone via 2- or 3-O of GalA (Ridley et al., 2001). In this paper, we isolated Herba Asari polysaccharide and fractionated the polysaccharide into AG- and XGA-type pectin. Little Rha

Table 3
Effect of Herba Asari polysaccharides on ConA- or LPS-induced lymphocyte proliferation in mice.

	Dose (mg/kg)	Lymphocyte	
		T cell (*A*570)	B cell (*A*570)
Control	NaCl	0.303 ± 0.01	0.300 ± 0.04
HA	10	0.310 ± 0.01	0.310 ± 0.06
	50	0.334 ± 0.01*	0.520 ± 0.03*
	100	0.340 ± 0.01*	0.590 ± 0.07**
HA1	10	0.614 ± 0.03**	0.419 ± 0.01*
	50	0.662 ± 0.03**	0.693 ± 0.01**
	100	0.440 ± 0.01*	0.493 ± 0.01*
HA2	10	0.428 ± 0.03*	0.350 ± 0.03*
	50	0.717 ± 0.08**	0.564 ± 0.05*
	100	0.804 ± 0.08**	0.996 ± 0.04**

Each value represents the mean ± S.D. for each group of six mice.
* $P < 0.05$, significantly different from the control.
** $P < 0.01$, significantly different from the control.

was detected; we conclude that compared to the polysaccharides isolated from ginseng roots by our group (Zhang et al., 2009), the Herba Asari polysaccharides have more XGA domains and fewer RG-I domains. Some pectins contain trace amounts of RG-II domains (Hilz, Williams, Doco, Schols, & Voragen, 2006), but none were detected in our Herba Asari and ginseng polysaccharides.

3.2. In vivo lymphocyte proliferation activity

The polysaccharides from Herba Asari were tested for ConA- and LPS-induced lymphocyte proliferation in vivo. The results are listed in Table 3. HA significantly enhanced both T and B lymphocyte proliferation in the relatively high 50 and 100 mg/kg doses. After separation, the neutral HA1 fraction significantly enhanced both T and B lymphocyte proliferation at a relatively low dose. A bell-shaped dose response was recorded; increases in T/B cell proliferation peaked at 50 mg/kg. In the acidic HA2 fraction, both T and B lymphocyte proliferation was enhanced in a dose-dependent manner.

The immunological activity of a polysaccharide depends on its structure (Raveendran Nair et al., 2004). In this paper, starch-like glucan HA1 enhances T and B lymphocyte proliferation in a bell-shaped dose response, similar to our previous results for the starch-like ginseng glucan (Ni et al., 2010). Several starch-like glucans are also reported to have similar immunological activity (Cao et al., 2006; Dong, Yao, Fang, & Ding, 2007; Liu et al., 2008). Unlike HA1, the pectic fraction HA2 is immunologically active in a dose-dependent manner, consistent with our ginseng pectin results (Zhang et al., 2009) and previous literature (Inngjerdingen et al., 2007; Nergard et al., 2005). Pectin has a complex structure that includes AG, RG-I, RG-II, XGA and HG domains. Based on its sugar composition, HA2 is composed mainly of AG and XGA domains, while RG-I and RG-II domains were not detected in HA2. AG domains are more structurally diverse, and probably more diverse in their activity. Recent investigation has shown that AG domains of pectin play an important role in immune-modulating activities (Inngjerdingen et al., 2007). A study of the structure–activity relationship of pectin indicated that its immunological activities were highly related to the AG domains (Diallo, Paulsen, Liljebäck, & Michaelsen, 2001; Kiyoharaa & Yamada, 1989). The enhancement of T and B cell proliferation by HA2 might similarly be related to its AG domains. We deduced that both the starch-like glucan HA1 and the pectic polysaccharide HA2 contribute to the stimulation of lymphocytes; however, the stimulatory mechanisms may be different. Lymphocytes are important immune cells that play a pivotal role in immune response by producing many kinds of cytokines after differentiation and activation. The immunomodulatory activity of Herba Asari polysaccharides may derive from its ability to stimulate the proliferation of lymphocytes, which results in the release of more cytokines.

4. Conclusion

In the present work, a starch-like glucan HA1 and three pectic polysaccharides HA2-a, HA2-b and HA2-c were purified from the Herba Asari roots via hot water extraction, ethanol precipitation and anion-exchange chromatography. Sugar composition and NMR results indicated that HA2-a is an arabinogalactan; HA2-b and HA2-c are xylogalacturonans with arabinogalactan domains. Both the neutral and acidic polysaccharides may be potent B and T cell stimulators with different modes of action by lymphocyte proliferation assays.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No: 30973857) and the Basic Research Found of Chinese University (Studies on Fine Structures of Ginseng Pectins, No: 10SSXT146).

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