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Structure elucidation of an immunoenhancing pectic polysaccharide isolated from aqueous extract of pods of green bean (*Phaseolus vulgaris* L.)

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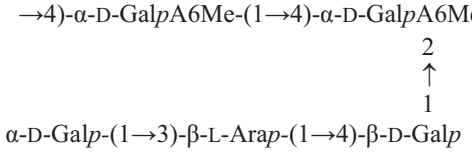
ABSTRACT

A water-soluble pectic polysaccharide (PS-I) isolated from the hot water extract of the pods of green bean (*Phaseolus vulgaris* L.) by successive purification through dialysis, centrifugation and Sepharose-6B gel filtration was found to consist of methyl ester of D-galacturonic acid, D-galactose, and L-arabinose in a molar ratio of nearly 2:2:1. This molecule showed splenocyte, thymocyte activation as well as antioxidant properties. Structural studies of PS-I was performed using acid hydrolysis, methylation analysis, periodate oxidation studies along with NMR analysis (^{1}H, ^{13}C, TOCSY, DQF-COSY, NOESY, ROESY, HMQC, and HMBC) and the repeating unit of PS-I was established as:



1. Introduction

Pectic polysaccharides (pectins), an important structural component of higher plant cells, in particular fruits and vegetables (Ridley, O'Neil, & Mohnen, 2001; Voragen & Pilnik, 1989), are complex heterogeneous polysaccharides containing galacturonic acid or its ester in the main chain. These are used in food industry as gelling and thickening agents and exhibit different pharmaceutical activities (Voragen, Pilnik, Thibault, Axelos, & Renard, 1995) as well as play an important role in the regulation of some physiological process to prevent hyperlipidemia and bowel cancer (Lim, Yamada, & Nonaka, 1998; Willats, McCartney, Mackie, & Knox, 2001). Ginseng pectin inhibit adhesion of bacteria to host cells (Lee, Shim, Chung, Lim, & Kim, 2009; Lee et al., 2006), protect animals from the lethal effects of ionizing radiation (Kim et al., 2007; Song et al., 2003), reduce blood sugar in normal and hyperglycemic mice (Konno, Sugiyama, Kano, Takahashi, & Hikino, 1984; Suzuki & Hiking, 1989) and also inhibit tumor growth and metastasis (Kim, Kang, & Kim, 1990; Shin et al., 2004). A Pectic polysaccharide, isolated from sweet pepper using a stimulated gastric medium was found to decrease tumor necrosis factor TNF-alpha release and increase production of interleukin-10 in lipopolysaccharide-stimulated whole blood (Popov et al., 2011). In vitro bioactive test, the pectic polysaccharide from the stems of Dendrobium nobile Lindl showed remarkable immunostimulant activities on T- and B-lymphocytes (Wang, Luo, & Zha, 2010).

In present study, we have carried out the structural characterization and study of splenocyte and thymocyte activation as well as antioxidant property of a pectic polysaccharide (PS-I) isolated from the pods of green beans (*Phaseolus vulgaris* L.) and reported herein for the first time.

2. Materials and methods

2.1. Isolation, fractionation, and purification of the crude pectic polysaccharide

The green beans (1.0 kg), collected from the local market were washed with water, crushed and boiled in distilled water for 12 h at 100 °C. After filtration through linen cloth, the filtrate was centrifuged at 10,000 rpm (using a Heraeus Biofuge stratos centrifuge) for 45 min at 4 °C and supernatant was collected and precipitated in ethanol (1:5, v/v). It was kept overnight at 4 °C and again centrifuged as above. The precipitated material was washed four times

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with ethanol and then freeze dried followed by dialysis through cellulose membrane (Sigma–Aldrich, retaining >M_w 12,400) against distilled water for 48 h. The aqueous solution was then collected from the dialysis bag and freeze-dried, to yield crude polysaccharide (560 mg).

The crude polysaccharide (30 mg) was purified by gel permeation chromatography on column (90 cm × 2.1 cm) of Sepharose 6B in water as eluant (0.4 mL/min) using Redifrac fraction collector. Ninety-five test tubes (2 mL each) were collected and monitored spectrophotometrically at 490 nm with phenol-sulfuric acid reagent (York, Darvill, McNeil, Stevenson, & Albersheim, 1985) using Shimadzu UV-VIS spectrophotometer, model-1601. Two fractions (test tubes 15–22 and test tubes 31–42) were collected and freeze-dried, to yield Fr-I (PS-I); 8 mg, and Fr-II (PS-II); 8.5 mg. The purification process was carried out in several lots and collected, yield PS-I ~68 mg and PS-II ~73.5 mg. PS-I was again separately purified by gel permeation chromatography and a homogeneous peak was obtained.

2.2. Monosaccharide analysis

2.2.1. Alditol acetate analysis

PS-I (3.0 mg) was hydrolyzed with 2 M CF_3COOH (2 mL) in a round-bottom flask at 100 °C for 18 h in a boiling water bath. The excess acid was completely removed by co-distillation with water. Then, the hydrolyzed product was divided into two parts. One part was examined by paper chromatography (Hoffman, Lindberg, & Svensson, 1972) in solvent systems *X* and *Y*. Another part was reduced with $NaBH_4$ (9 mg), followed by acidification with dilute CH_3COOH, and then co-distilled with pure CH_3OH to remove excess boric acid. The reduced sugars (alditol) were acetylated with 1:1 pyridine–acetic anhydride in a boiling water bath for 1 h to give the alditol acetates, which were analyzed by GLC.

2.2.2. Preparation of carboxyl reduced pectic polysaccharide

PS-I (10.0 mg) was dissolved in 1 M imidazole-hydrochloric acid buffer, pH 7.0 (200 μL/mg) and cooled on ice. Sodium borohydride (40 mg) was then added and reacted on ice for at least 1 h. The excess borohydride was destroyed by adding glacial acetic acid (100 μL/40 mg borohydride) slowly to the cooled sample. An equal volume of redistilled water was then added, and the reduced PS-I was precipitated by adding 3–4 volume of 95% (v/v) ethanol (2 mL). The sample was reprecipitated two more times with 95% ethanol and freeze-dried. The carboxyl-reduced PS-I (Maness, Ryan, & Mort, 1990) was hydrolysed with 2 M CF_3COOH for 18 h at 100 °C, and after usual treatment, the sugars were analyzed by GLC.

2.3. Methylation analysis

PS-I (6 mg) was methylated using Ciucanu and Kerek (1984) method. The methylated products were isolated by partition between $CHCl_3$ and H_2O (5:2, v/v). The organic layer containing products was washed with 5 mL water for three times and dried. The methylated product (2 mg) was then hydrolyzed with 90% formic acid (1 mL) at 100 °C for 1 h, reduced with sodium borohydride, acetylated with (1:1) acetic anhydride–pyridine, and analyzed by GLC and GLC–MS. Another portion of the methylated product (2.0 mg) was dissolved in dry THF (2 mL) and refluxed with lithium aluminium hydride (LAH) (Abdel-Akher & Smith, 1950) (40 mg) for 5 h and kept overnight at room temperature. The excess of the reductant was decomposed by drop wise addition of ethyl acetate and aqueous THF. The inorganic materials were filtered off. The filtrate was evaporated to dryness giving the permethylated carboxyl-reduced product. The product was hydrolyzed with formic acid as before and the alditol acetates of the methylated, carboxyl-reduced sugars were prepared, analyzed by GLC and GLC–MS.

2.4. Periodate oxidation

PS-I (5 mg) was oxidized with 0.1 M sodium metaperiodate (2 mL) at 27 °C in the dark during 48 h. The excess periodate was destroyed by adding 1,2-ethanediol, and the solution was dialyzed against distilled water. The dialyzed material was reduced with $NaBH_4$ for 15 h and neutralized with acetic acid. The resulting material was obtained by co-distillation with methanol. The periodate-oxidized material (Goldstein, Hay, Lewis, & Smith, 1965; Hay, Lewis, & Smith, 1965) was divided into three portions. One portion was hydrolyzed with 2 M CF_3COOH for 18 h, and alditol acetate was prepared as usual. Another portion was methylated by the method of Ciucanu and Kerek (1984), followed by preparation of alditol acetates which were analyzed by GLC and GLC–MS. Another portion was reduced by LAH and then kept with 0.5 M CF_3COOH for 48 h at room temperature. The acid was removed and the hydrolyzate was analyzed by GLC (as alditol acetates).

2.5. General methods

Optical rotation was measured on a Jasco Polarimeter model P-1020 at 25 °C. All the GLC–MS experiments were carried out in a Hewlett-Packard 5970 MSD instrument using HP-5 fused silica capillary column. The program was isothermal at 150 °C; hold time 2 min, with a temperature gradient of 4 °C/min up to a final temperature of 200 °C. Paper partition chromatographic studies (Hoffman et al., 1972) were performed on Whatmann No. 1 and 3 MM sheets. Solvent systems used were: (*X*) BuOH–HOAc–H_2O (4:1:5, v/v/v, upper phase) and (*Y*) EtOAc–pyridine–H2O (8:2:1, v/v/v). The spray reagent used was alkaline silver nitrate solution. The molecular weight (Hara, Kiho, Tanaka, & Ukai, 1982) of PS-I was determined by a gel-chromatographic technique. Standard dextrans T-200, T-70, and T-40 were passed through a Sepharose-6B column and then the elution volumes were plotted against the logarithms of their respective molecular weights. The elution volume of PS-I was then plotted on the same graph, and the average molecular weight of PS-I was determined.

2.6. Absolute configuration of monosaccharide

The method used was based on Gerwig, Kamarling, & Vliegenthart (1978). PS-I (1.0 mg) was hydrolyzed with CF_3COOH, and then the acid was removed. A solution of 250 μL of 0.625 M HCl in R-(+)-2-butanol was added and heated at 80 °C for 16 h. Then the reactants were evaporated and TMS-derivatives were prepared with N,O-bis (trimethylsilyl) trifluroacetamide (BSTFA). The products were analyzed by GLC using a capillary column SPB-1 (30 m × 0.26 mm), a temperature program (3 °C/min) from 150 to 210 °C. The 2,3,4,6-tetra-*O*-TMS-(+)-2-butylglycosides obtained were identified by comparison with those prepared from the D and L enantiomers of different monosaccharides.

2.7. NMR studies

PS-I was kept over P_2O_5 in vacuum for several days and then exchanged with deuterium (Dueñas Chasco et al., 1997) by lyophilizing with D_2O (99.96% atom ^2H, Aldrich) for four times. With a Bruker Avance DPX-500 spectrometer, ^1H, TOCSY, DQF-COSY, NOESY and HMBC NMR spectra were recorded in D_2O at 27 °C. The ^1H NMR spectrum was recorded by suppressing the HOD signal (fixed at δ 4.73 ppm) using the WEFT pulse sequence (Hård, Zadelhoff, Moonen, Kamerling, & Vliegenthart, 1992). The TOCSY experiment was recorded at mixing time of 150 ms, and complete

assignment required several TOCSY experiments having mixing times ranging from 60 to 300 ms. The NOESY mixing delay was 200 ms. The delay time in the HMBC experiment was 80 ms.

2.8. Splenocyte and thymocyte proliferation assay

A single cell suspension of spleen and thymus were prepared from the normal mice under aseptic conditions by frosted slides in Phosphate Buffer Saline (PBS). The suspension was centrifuged to obtain cell pellet. The contaminating RBC was removed by hemolytic Gey's solution. After two washes in PBS the cells were resuspended in complete RPMI (Rose well Park Memorial Institute) medium. Cell concentration was adjusted to 1×10^5 cells/mL and viability of the suspended cells (as tested by trypan blue dye exclusion) was always over 90%. The cells (180 μL) were plated in 96-well flat-bottom plates and incubated with 20 μL of various concentrations (10–200 μg/mL) of the PS-I with lipopolysaccharide (LPS, which is positive control) of 4 μg/mL. Cultures were set-up for 72 h at 37 °C in a humidified atmosphere of 5% CO_2. Proliferation was checked by MTT assay method (Ohno et al., 1993). Data are reported as the mean ± standard deviation of six different observations and compared against PBS control (Maiti et al., 2008; Sarangi, Ghosh, Bhutia, Mallick, & Maiti, 2006).

2.9. DPPH radical scavenging activity

The antioxidant activity of PS-I was measured on the basis of the scavenging activity of the stable 1,1-diphenyl-2-picryl hydrazyl (DPPH) free radical test according to the method described by Yen, Yang, & Mau (2008). Samples were dissolved in distilled water at 0.2, 0.5, 1, 2, 4, and 8 mg/mL. One milliliter test samples were mixed with 2 mL of freshly prepared DPPH (0.1 mM) in 50% ethanol. After shaking vigorously, the mixture was incubated at 25 °C for 30 min in the dark, and then the absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. Ascorbic acid was used as a standard antioxidant material. The scavenging activity of the DPPH radicals was calculated as:

$$Q = \frac{A_0 - A_c}{A_0} \times 100\%$$

where Q is the percentage reduction of the DPPH, A_0 the initial absorbance, A_c is the absorbance after added sample concentration c.

3. 3. Results and discussion

3.1. Chemical analysis of the pectic polysaccharide

The molecular weights (Hara et al., 1982) of both the polysaccharide, PS-I and PS-II were estimated as $\sim 1.8 \times 10^2$ kDa and $\sim 2.0 \times 10^2$ kDa respectively. We are reporting herein the structural characterization of PS-I only. PS-II polysaccharide contains similar sugar units like PS-I but different in molar ratio. It will be communicated later.

The pure pectic polysaccharide (PS-I) showed a specific rotation of $[\alpha]_D^{25}$ +112.0 (*c* 0.86, water). The GLC analysis of the alditol acetate of hydrolyzed PS-I showed the presence of galactose, and arabinose in a molar ratio of nearly 2:1 but the carboxyl-reduced (Maness et al., 1990) PS-I on hydrolysis followed by GC and GC–MS analysis showed the presence of galactose, and arabinose in a molar ratio of nearly 4:1. These results confirmed molar ratio of galacturonic acid, galactose, and arabinose present in PS-I was 2:2:1. The absolute configuration of sugar units present in PS-I was determined by the method of Gerwig et al. (1978) and it was found that galactose and galacturonic acid had D configuration, whereas, arabinose had L configuration. The alditol acetates of the methylated PS-I (Ciucanu & Kerek, 1984) were analyzed by GC and GC–MS and showed the presence of 2,3,4,6-Me_4-Gal; 2,3,6-Me_3-Gal; and 2,4-Me_2-Ara in a molar ratio of nearly 1:1:1. This result indicated the presence of terminal galactopyranosyl, (1 → 4)-linked galactopyranosyl, and (1 → 3)-linked arabinopyranosyl moieties. The methylated carboxyl-reduced (Abdel-Akher & Smith, 1950) PS-I showed the presence of the above peaks along with two new peaks corresponding to 2,3-Me_2-Gal and 3-Me-Gal in a molar ratio of nearly 1:1:1:1:1. This result indicated the presence of (1 → 4)-linked and (1 → 2,4)-linked Gal*p*A in PS-I.

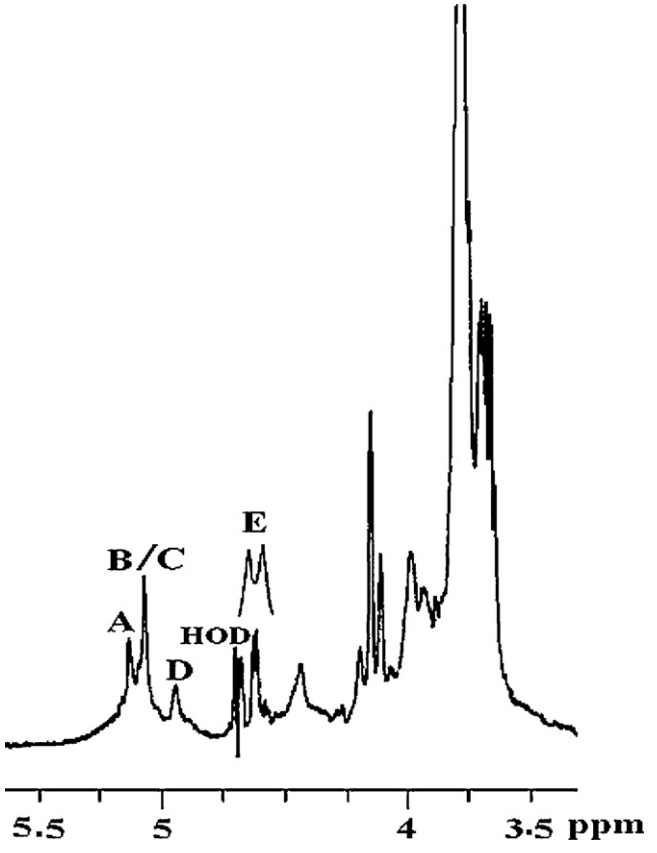


Fig. 1. ^{1}H NMR spectrum (500 MHz, D_2O, 27 °C) of PS-I, isolated from pods of green bean (*Phaseolus vulgaris* L.).

The periodate-oxidized (Goldstein et al., 1965; Hay et al., 1965) material upon hydrolysis with 2 M CF_3COOH followed by GLC analysis showed the presence of only arabinose. GLC analysis of alditol acetate obtained from periodate-oxidized, LAH reduced PS-I showed the presence of galactose and arabinose in a molar ratio 1:1. GLC and GLC–MS analysis of the periodate-oxidized, methylated PS-I showed the presence of 2,4-Me_2-Ara. These results indicated that terminal galactopyranosyl, (1 → 4)-linked galactopyranosyl and (1 → 4)-linked galacturonopyranosyl residues were completely destroyed during oxidation.

3.2. NMR and structural analysis of pectic polysaccharide

The ^{1}H NMR (500 MHz, Fig. 1) spectrum of PS-I at 27 °C showed four signals at δ 5.13, 5.06, 4.93, and 4.62 for five anomeric protons where the signal at δ 5.06 accommodated two protons and each of the other three corresponded one proton. The signal at 3.79 ppm was observed for methyl protons of ester group. The sugar residues were designated as **A**–**E** according to their decreasing anomeric proton chemical shifts (Table 1). In the ^{13}C NMR spectrum (125 MHz, Fig. 2) at 27 °C, three signals appeared at δ 104.8, 100.8,

Table 1
^{1}H NMR[c] and ^{13}C NMR[d] chemical shifts for PS-I, isolated from pods of green bean (*Phaseolus vulgaris* L.) recorded in D_2O at 27 °C.

Glycosyl residue	C-1/H-2	C-2/H-2	C-3/H-3	C-4/H-4	C-5/H-5a, H-5a	C-6/H-6a, H-6b	COOMe
→3)-β-L-Ara*p*-(1→ **A**	104.8 5.13	74.9 4.02	81.0 4.13	72.2 3.80	61.1 3.91[a], 3.94[b]		
α-D-Gal*p*-(1→ **B**	100.8 5.06	68.4 3.72	70.4 3.93	69.5 4.00	70.9 4.12	61.2 3.73[a], 3.69[b]	
→4)-α-D-Gal*p*A6Me -(1→ **C**	100.3 5.06	68.4 3.68	69.2 3.78	78.0 3.98	72.2 4.43	171.2	53.3 3.79[f]
→2,4)-α-D-Gal*p*A6Me-(1→ **D**	100.8 4.93	79.2 3.70	68.0 3.91	79.2 4.10	72.2 4.43	171.2	53.3[e] 3.79[f]
→4)-β-D-Gal*p*-(1→ **E**	104.8 4.62	70.9 3.66	73.7 3.77	78.0 4.15	74.9 3.74	61.2 3.98[a], 3.96[b]	

[a,b]Interchangeable.
[c] Values of the ^{1}H chemical shifts were recorded with respect to the HOD signal fixed at δ 4.73 at 27 °C.
[d] Values of the ^{13}C chemical shifts were recorded with reference to acetone as the internal standard and fixed at δ 31.05 at 27 °C.
[f] Value of methyl protons of ester group.
[e] Value of the methyl carbon of ester group.

Table 2
The NOESY data for PS-I, isolated from pods of green bean (*Phaseolus vulgaris* L.).

Anomeric proton		δ	NOE contact protons
Glycosyl residue	δ		Residue, atom
→3)-β-L-Ara*p*-(1→ **A**	5.13	4.15	**E** H-4
α-D-Gal*p*-(1→ **B**	5.06	4.13 3.72	**A** H-3 **B** H-2
→4)-α-D-Gal*p*A6Me-(1→ **C**	5.06	4.10 3.68	**D** H-4 **C** H-2
→2,4)-α-D-Gal*p*A6Me-(1→ **D**	4.93	3.98 3.70 3.91	**C** H-3 **D** H-2 **D** H-3
→3)-β-D-Gal*p*-(1→ **E**	4.62	3.70 3.77 3.74	**D** H-2 **E** H-3 **E** H-5

and 100.3 where each of the signals at δ 104.8 and 100.8 accommodated two carbons. The peak at 100.3 ppm corresponded to the anomeric carbon of residue **C**. The signal at 100.8 corresponded to the anomeric carbons of residues, **B** and **D**. The peak at 104.8 ppm was correlated to the anomeric carbons of **A** and **E** residues. The methyl carbon signal of ester group appeared at δ 53.3 ppm. All the ^{1}H and ^{13}C signals (Table 1) were assigned using DQF-COSY, TOCSY, and HMQC NMR experiments.

Residue **A** was assigned as β-L-Ara*p* as it showed large coupling constant values $^{3}J_{2,3}$ ~8.3 Hz, $^{3}J_{3,4}$ ~6 Hz, and relatively small coupling constant $^{3}J_{1,2,}$ as well as two H-5 signals (δ 3.91, and 3.94). The anomeric proton chemical shift for residue **A** at δ 5.13 ppm and carbon chemical shift of 104.8 ppm ($^{1}J_{C,H}$ ~170 Hz) indicated that it was a β-linked anomer. The down field shift of C-3 (δ 81.0 ppm) and slightly upfield shift of C-2 (74.9 ppm) with respect to the standard value of methyl glycoside (Agrawal, 1992; Rinaudo & Vincendon, 1982) indicated that it was (1 → 3)-β-L-Ara*p*.

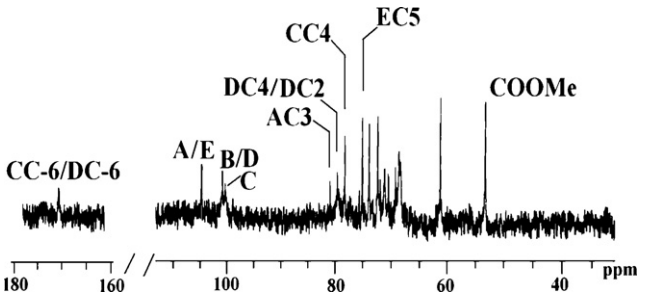


Fig. 2. ^{13}C NMR spectrum (125 MHz, D_2O, 27 °C) of PS-I, isolated from pods of green bean (*Phaseolus vulgaris* L.).

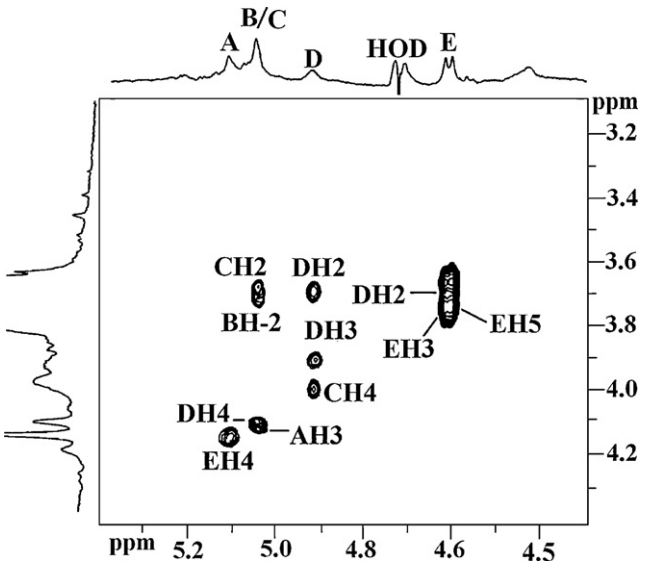


Fig. 3. Part of NOESY spectrum of PS-I, isolated from pods of green bean (*Phaseolus vulgaris* L.). The NOESY mixing time was 300 ms.

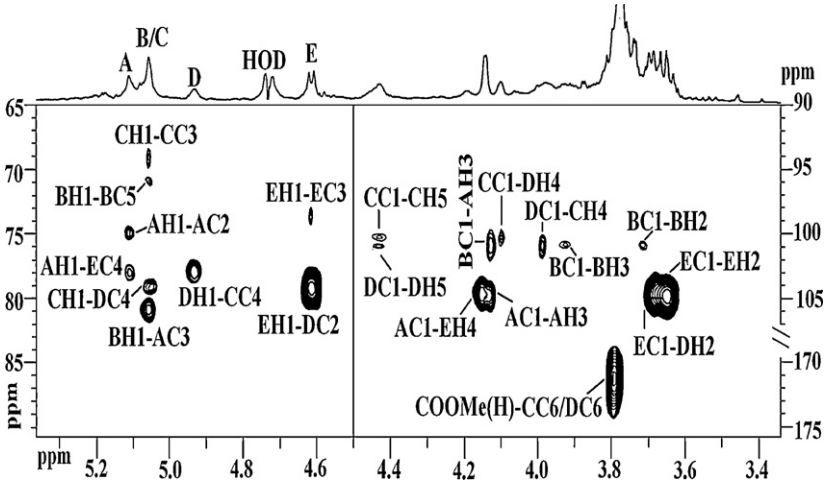


Fig. 4. The HMBC spectrum of PS-I, isolated from pods of green bean (*Phaseolus vulgaris* L.). The delay time in the HMBC experiment was 80 ms.

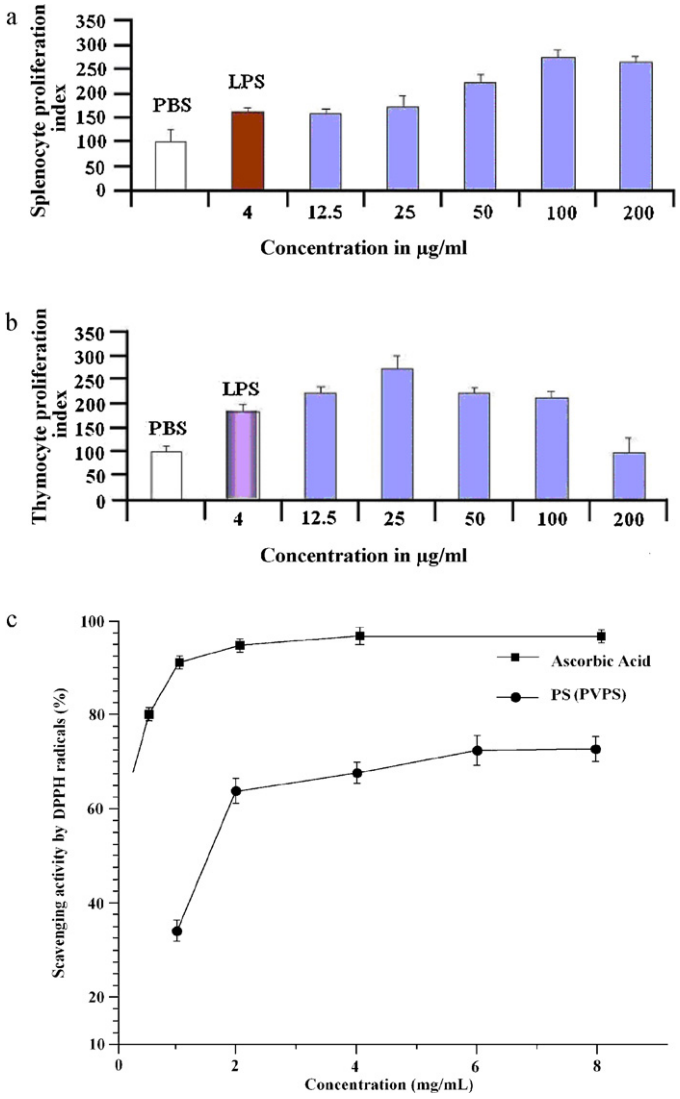


Fig. 5. Effect of different concentrations of PS-I on (a) splenocyte and (b) thymocyte proliferation. (c) Scavenging ability of PS-I isolated from pods of green bean (*Phaseolus vulgaris* L.) against DPPH radical. Results were presented as means ± SD ($n = 3$).

The anomeric proton chemical shift for residue **B** at δ 5.06 ppm ($^3J_{1,2}$ ~3.2 Hz) and carbon chemical shift at δ 100.8 ppm ($^1J_{C,H}$ ~170 Hz) indicated that **B** was α-linked anomer. A large $^3J_{2,3}$ (8.5 Hz) and relatively small $^3J_{3,4}$ (3.7 Hz) coupling constant indicated that **B** had galacto configuration. The chemical shift of C-2, C-3, C-4, C-5 and C-6 of this residue were similar to the standard value of methyl glycosides which indicated that residue **B** was α-linked terminal D-galactose.

The spin system of residue **C**, which consisted of only five protons with relatively high chemical shift of the H-5 signal (δ 4.43 ppm) and weak coupling of H-4 with both H-3 and H-5 indicated that the residue **C** was D-Gal*p*A. The anomeric signals for moiety **C** at δ 5.06 ppm and $^1J_{C,H}$ ~171 Hz indicated that D-galacturonosyl residue was α-linked. The C-4 peak of residue **C** at δ 78.0 showed a downfield shift compared to that of standard methyl glycosides due to the α-glycosylation effect. The carbon signals of residue **C** were observed at δ 68.4, 69.2, 72.2, and 171.2 corresponding to C-2, C-3, C-5, and C-6 (carbonyl carbon), respectively.

In case of residue **D**, an anomeric proton chemical shift appeared at δ 4.93 ppm and this spin system also consisting of only five protons with a weak coupling constants, $^3J_{3,4}$ and $^3J_{4,5}$ indicated its galacto configuration. The signal of H-1 and the characteristic $^3J_{1,2}$ coupling constant value (<3 Hz), and $^1J_{C,H}$ ~171 Hz showed that the residue **D** was α-D-Gal*p*A. The downfield shift of C-2 (79.2 ppm), C-4 (79.2 ppm) signals, with respect to the standard value of methyl glycosides indicated that residue **D** was (1 → 2,4)-linked moiety. The carbon signals of this residue, observed at δ 68.0, 72.2, and 171.2 were correlated to C-3, C-5, and C-6 (carbonyl carbon), respectively. The carbonyl groups of both galacturonic acids were present as methyl ester which was confirmed by the appearance of intra-residual coupling between the ester carbonyl carbon (δ 171.1) and the methyl protons (3.79 ppm) in the HMBC experiment. This observation indicated that residue **C** was the methyl ester of a 1,4-linked α-D-Gal*p*A and residue **D** was the methyl ester of a 1,2,4-linked α-D-Gal*p*A.

All the proton chemical shifts of unit **E** (H-1–H-6) were identified from DQF-COSY as well as the TOSCY spectra. A coupling constant value $^3J_{1,2}$ (~8.4 Hz) and $^1J_{C,H}$ (~162 Hz) indicated that it was β-linked residue. A large $^3J_{2,3}$ ~9.3 Hz and also small $^3J_{3,4}$ ~3.1 indicated that residue **E** had galacto configuration. The chemical shifts for anomeric proton and carbon at 4.62 ppm and 104.8 ppm respectively supported its β-configuration. The downfield shift of C-4 signal of residue **E** indicated that it was present as (1 → 4)-linked moiety in the repeating unit of PS-I.

Table 3
The significant $^{3}J_{H,C}$ connectivities observed in an HMBC spectrum for the anomeric protons/carbons of the sugar residues of PS-I, isolated from pods of green bean (*Phaseolus Vulgaris* L.).

Residue	Sugar linkage	H-1/C-1		Observed connectivities	
		δ_H/δ_C	δ_H/δ_C	Residue	Atom
A	→3)-β-L-Ara*p*-(1→	5.13	78.0	**E**	C-4
			74.9	**A**	C-2
		104.8	4.15	**E**	H-4
			4.13	**A**	H-3
B	α-D-Gal*p*-(1→	5.06	81.0	**A**	C-3
			70.9	**B**	C-5
		100.8	4.13	**A**	H-3
			3.72	**B**	H-2
			3.93	**B**	H-3
C	→4)-α-D-Gal*p*A6Me-(1→	5.06	79.2	**D**	C-4
			69.2	**C**	C-3
		100.3	4.10	**D**	H-4
			4.43	**C**	H-5
D	→2,4)-α-D-Gal*p*A6Me-(1→	4.93	78.0	**C**	C-4
		100.8	3.98	**C**	H-4
			4.43	**D**	H-5
E	→3)-β-D-Gal*p*-(1→	4.62	79.2	**D**	C-2
			73.7	**E**	C-3
		104.8	3.70	**D**	H-2
			3.66	**E**	H-2

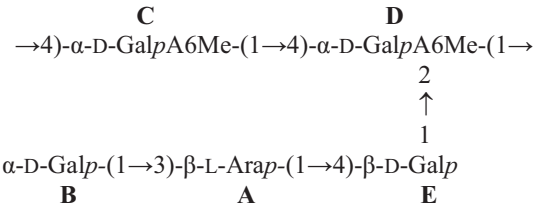
Residue	Sugar linkage	COOMe (δ_H)	(δ_C)	Observed connectivities Residue	Atom
C	→4)-α-D-Gal*p*A6Me-(1→	3.79	171.2	**C**	C-6
D	→2,4)-α-D-Gal*p*A6Me-(1→	3.79	171.2	**D**	C-6

The sequence of glycosyl residues of the polysaccharide was determined from NOESY (Fig. 3 and Table 2) as well as ROESY (figure not shown) experiments. The inter-residual NOE contacts between **A**H-1 to **E** H-4; **B** H-1 to **A** H-3; **C** H-1 to **D** H-4; **D** H-1 to **C** H-4; and **E** H-1 to **D** H-2 were observed and the following sequences were established:

A-(1 → 4)-**E**; **B**-(1 → 3)-**A**; **C**-(1 → 4)-**D**; **D**-(1 → 4)-**C**;

E-(1 → 2)-**D**

Long-range ^{13}C–^{1}H correlations of HMBC experiment (Fig. 4) confirmed the sequences of sugar residues deduced from both ROESY and NOESY experiments. The intra- and inter-residual cross peaks in the HMBC spectra are shown in Fig. 4 and Table 3. The inter-residual cross couplings, **A**H-1/**E**C-4; **A**C-1/**E**H-4; **B**H-1/**A**C-3; **B**C-1/**A**H-3; **C**H-1/**D**C-4; **C**C-1/**D**H-4; **D**H-1/**C**C-4; **D**C-1/**C**H-4; **E**H-1/**D**C-2; and **E**C-1/**D**H-2 were observed in HMBC experiment. Hence, from the NOESY and HMBC experiment the structure of the pentasaccharide repeating unit of the pectic polysaccharide (PS-I) was established as:



3.3. Splenocyte and thymocyte proliferation assay

The splenocyte and thymocyte activation tests were carried out in mouse cell culture medium with PS-I by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method. Proliferation of splenocytes and thymocytes is an indicator of immunostimulation (Ohno et al., 1993). PS-I was tested to stimulate the splenocytes and thymocytes as shown in Fig. 5(a) and (b), respectively. At 100 μg/mL of PS-I, splenocyte proliferation index was observed maximum as compared to other concentrations. Hence, 100 μg/mL of PS-I can be considered as efficient splenocyte proliferators. Again 25 μg/mL of that sample showed maximum effect on thymocyte proliferation. The splenocyte and thymocyte proliferation index as compared to PBS (Phosphate Buffer Saline) control if closer to 100 or below indicates low stimulatory effect on immune system. From these observations it was clear that this PS-I can act as an efficient immunostimulator.

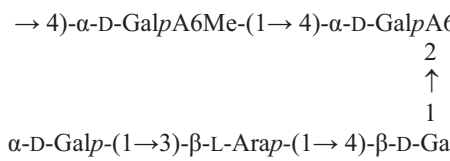
3.4. Assay for antioxidant activity

Fig. 5(c) demonstrates DPPH scavenging activity caused by different concentrations of PS-I. A maximum of 72.5% DPPH radical scavenging activity was observed at 8 mg/mL of PS-I. The EC_{50} value of PS-I for DPPH radicals was 1.56 mg/mL. The scavenging activity of PS-I increased steadily from 2 to 8 mg/mL, while it reached a maximum plateau from 0.5 to 2 mg/mL for ascorbic acid, which indicated the scavenging activity of PS-I against DPPH radical was less than that of ascorbic acid but it is appreciable and effective as an antioxidant material.

4. Conclusion

A water soluble pectic polysaccharide (PS-I) was isolated from aqueous extract of the pods of green bean and purified by gel-filtration chromatography. The reported pectin contained [→4)-α-D-Gal*p*A6Me-(1 → 4)-α-D-Gal*p*A6Me-(1→] as a back bone in which branching occurred at C-2 position of one methyl galacturonate moiety by α-D-Gal*p*-(1 → 3)-β-L-Ara*p*-(1 → 4)-β-D-Gal*p*-(1→. This molecule showed significant antioxidant activity as

well as thymocyte and splenocyte activation. On the basis of chemical analysis and NMR studies the structure of the repeating unit of PS-I was established as:



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