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Pectic polysaccharide from immature onion stick (*Allium cepa*): Structural and immunological investigation

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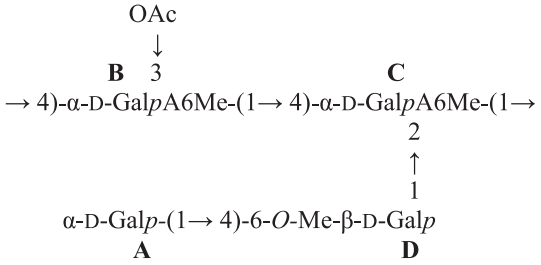
ABSTRACT

The structure of a water-soluble pectic polysaccharide (PS) isolated from immature onion stick (*Allium cepa*) was investigated using acid hydrolysis, methylation analysis, periodate oxidation study, and NMR studies (^{1}H, ^{13}C, DQF-COSY, TOCSY, NOESY, ROESY, HSQC, and HMBC). The results of the above experiments indicated that the PS contained D-galactose, 6-*O*-Me-D-galactose, 3-*O*-acetyl-D-methyl galacturonate and D-methyl galacturonate in a molar ratio of nearly 1:1:1:1 and possesses a backbone of [→4)-α-D-Gal*p*A6Me-(1 → 4)-α-D-Gal*p*A6Me-(1→] in which one methyl galacturonate was substituted at *O*-3 position by an acetyl group and the neighboring methyl galacturonate being substituted at *O*-2 with a side chain, α-D-Gal*p*-(1 → 4)-6-*O*-Me-β-D-Gal*p*-(1→. The probable structure of repeating unit of the pectic polysaccharide was established as:



The pectic polysaccharide showed in vitro splenocyte, thymocyte as well as macrophage activations.

1. Introduction

Pectic polysaccharides, a component of dietary fiber (Lunn & Buttriss, 2007) in fruits and vegetables, are excellent gelling and thickening agents for the production of jams and jellies in food industries and also display different pharmaceutical activities (Voragen, Pilnik, Thibault, Axelos, & Renard, 1995). They exhibit anti-inflammatory activity by oral administration (Ovodova et al., 2009; Popov, Popova, Ovodova & Ovodov, 2005; Popov et al., 2007) and prevent hyperlipidemia, as well as bowel cancer (Lim, Yamada, & Nonaka, 1998; Willats, McCartney, Mackie, & Knox, 2001). Ginseng pectin decreases blood glucose levels in normal and hyperglycemic mice (Konno, Sugiyama, Kano, Takahashi, & Hikino, 1984; Suzuki & Hiking, 1989), inhibit tumor growth and metastasis (Kim, Kang, & Kim, 1990; Shin et al., 2004), modulate the immune system (Du, Jiang, Wu, Won, & Choung, 2008; Han, Song, Yun, & Yi, 2005) and also protect animals from the lethal effects of ionizing radiation (Kim et al., 2007; Song et al., 2003). The branching and presence of acetyl groups on galacturonic acid are very important for the expression in bioactivities (Kravtchenko, Penci, Voragen, & Pilnik, 1993). A pectic polysaccharide isolated from the pods of green beans (*Phaseolus vulgaris* L) exhibited immunoenhancing activity as well as antioxidant property (Patra, Das, Behera, Maiti, & Islam, 2012). Immunoenhancing effects on T and B lymphocytes by a pectic polysaccharide, isolated from the stems of *Dendrobium nobile* was also reported (Wang, Luo, & Zha, 2010). People all over the India consume immature onion stick as a

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delicious vegetable mainly at winter seasons. The presence of diallyl sulfide and flavonoid compounds in onion bulbs exhibit different pharmacological effects like inhibition of tumor and microbial cells, scavenging of free radicals and protection of cardiovascular diseases (Block, Calvey, Gillies, Gillies, & Uden, 1997; Elattar & Virji, 1999; Jang et al., 1997; Stavric, 1997). Onion bulbs promote bile production; reduce sugar and lipid levels (Augusti, 1990). It is reported that onion bulbs (Golovchenko, Khramova, Ovodova, Shashkov, & Ovodov, 2012) contained pectic polysaccharide in which the main constituents of the linear regions are joined by galacturonan and rhamnogalacturonan. Oxalate-citrate extracted fraction of onion cell walls was reported to compose of a range of pectic polysaccharides with varying proportions of neutral side-chains (Mankarios, Hall, Jarvis, Threlfall, & Friend, 1980). The present work is devoted to the elucidation of the detailed structural features and immunological activities of a pectic polysaccharide (PS) isolated from immature onion stick (*Allium cepa*) and reported herein.

2. Materials and methods

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

The immature onion sticks (600 g) were collected from the local market, washed with water, cut into small pieces and boiled in 300 ml of distilled water for 8 h. The whole mixture was kept overnight at 4 °C and filtered through linen cloth. The filtrate was centrifuged at 8000 rpm (using a Heraeus Biofuge stratos centrifuge) for 1 h 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 five times with ethanol and then freezes dried followed by dialysis through cellulose membrane (Sigma–Aldrich, retaining > M_w 12,400) against distilled water for 36 h to remove low-molecular weight materials. The aqueous solution was then collected from the dialysis bag and freeze-dried, to yield crude polysaccharide (1.5 g).

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 with a flow rate of 0.5 mL/min using Redifrac fraction collector. Forty 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. A single fraction was collected and freeze-dried, to yield 12 mg pure polysaccharide (PS). The purification process was carried out in several lots and 95 mg PS was collected.

2.2. Monosaccharide analysis

2.2.1. Alditol acetate analysis

4.0 mg PS 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 $NaBH_4$, followed by acidification with dilute CH_3COOH. It was then co-distilled with pure CH_3OH to remove excess boric acid. The reduced sugars 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 and GLC–MS using Hewlett-Packard 5970A automatic GLC–MS system, fitted with an HP-5 capillary column (25 m × 25 mm). 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. Quantification was carried out from the peak area, using response factors from standard monosaccharide's using inositol as standard.

2.2.2. Preparation of carboxyl reduced polysaccharide

PS (3.5 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 reaction mixture was maintained on ice for at least 1 h. The excess borohydride was decomposed 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 was precipitated by adding 3–4 volume of 95% (v/v) ethanol (2 mL). The sample was reprecipitated two more times with 95% ethanol, centrifuged and the sedimented material was freeze-dried. The carboxyl-reduced PS (Maness, Ryan, & Mort, 1990) was hydrolyzed with 2 M CF_3COOH for 18 h at 100 °C, and after usual treatment, the sugars were analyzed by GLC and GLC–MS on a Hewlett-Packard Model 5730A.

2.3. Methylation analysis

PS (6.0 mg) was methylated using the procedure described by 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 water for several times by taking 5 mL of water in each time and then dried. The methylated product was then hydrolyzed with 90% formic acid (1 mL) at 100 °C for 1 h, and excess HCOOH was evaporated by co-distillation with distilled water. The hydrolyzed product was then reduced with sodium borohydride, acetylated with (1:1) acetic anhydride-pyridine, and then the alditol acetates of the methylated sugars were analyzed by GLC (using columns A and B) and GLC–MS (using HP-5 fused silica capillary column).

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 reducing agent 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, and analyzed by GLC and GLC–MS.

2.4. Periodate oxidation

The PS (6.0 mg) was oxidized by 2 mL 0.1 M sodium metaperiodate at room temperature in the dark for 48 h. The excess of $NaIO_4$ was destroyed by adding of ethylene glycol, and the solution was dialyzed against distilled water. The dialyzed material was reduced by $NaBH_4$ for 15 h and treated with acetic acid to make the solution slightly acidic. 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 hydrolyzed material was analyzed by GLC (as alditol acetates).

2.5. Colorimetric estimations

Colorimetric estimations were carried out on a Shimadzu UV–vis spectrophotometer, model 1601.

2.6. Optical rotation

Optical rotation was measured on a Jasco Polarimeter model P-1020 at 25.7 °C.

2.7. Paper chromatographic studies

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 (v/v/v, 4:1:5, upper phase) and (*Y*) EtOAc–pyridine–H_2O (v/v/v, 8:2:1). The spray reagent used was alkaline silver nitrate solution.

2.8. Determination of molecular weight

The molecular weight (Hara, Kiho, Tanaka, & Ukai, 1982) of PS was determined by a gel-chromatographic technique. Standard dextrans T-40, T-70, and T-200 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 was then plotted on the same graph, and the average molecular weight of PS was determined.

2.9. GLC and GLC–MS experiments

All gas–liquid chromatography experiments were performed on a Hewlett-Packard 5730A instrument. A gas chromatograph having a flame ionization detector and glass columns (1.8 m × 6 mm) packed with (A) 3% ECNSS-M on Gas Chrom Q (100–120 mesh) and column (B) 1% OV-225 on Gas Chrom Q (100–120 mesh) at 170 °C. All the GLC–MS experiments were carried out 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.

2.10. Absolute configuration of monosaccharide

The method used was based on Gerwig, Kamarling, and Vliegenthart (1978). At first, PS (2.5 mg) was hydrolyzed with CF_3COOH, and then the acid was removed by co-distillation with water. 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 monosaccharide.

2.11. NMR studies

Prior to NMR-spectroscopic analysis, sample PS 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. The ^1H and ^{13}C NMR experiments were carried out at 500 MHz and 125 MHz, respectively with a Bruker Avance DPX-500 spectrometer. The ^1H, ^{13}C, 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 4.74 ppm) using the WEFT pulse sequence (Hård, Zadelhoff, Moonen, Kamerling, & Vliegenthart, 1992). Acetone was used as an internal standard (δ 31.05 ppm) for ^{13}C spectrum. 2D (DQF-COSY) NMR experiment was performed using standard Bruker software. 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.

2.12. Test for macrophage activity by nitric oxide assay

Peritoneal macrophages (5 × 105 cells/mL) after harvesting were cultured in complete RPMI (Roswell Park Memorial Institute) media in 96-well plate (Ohno, Hasimato, Adachi, & Yadomae, 1996; Sarangi, Ghosh, Bhutia, Mallick, & Maiti, 2006). The purity of macrophages was tested by adherence to tissue culture plates. The polysaccharide was added to the wells in different concentrations. The cells were cultured for 24 h at 37 °C in a humidified 5% CO_2 incubator. Production of nitric oxide was estimated by measuring nitrite levels in cell supernatant with Greiss reaction (Green et al., 1982). Equal volumes of Greiss reagent (1:1 of 0.1% in 1-napthylethylenediamine in 5% phosphoric acid and 1% sulfanilamide in 5% phosphoric acid) and sample cell supernatant were incubated together at room temperature for 10 min. Absorbance was observed at 550 nm.

2.13. 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 (Roswell 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 with lipopolysaccharide of 4 μg/mL. PBS (Phosphate Buffer Saline, 10 mM, pH 7.4) is taken as negative control whereas lipopolysaccharide (LPS, L6511 of Salmonella enterica serotype typhimurium, Sigma, 4 μg/mL) and Concavalin A (Con A, 10 μg/mL) served as positive controls. 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 et al., 2006).

3. Result and discussion

3.1. Isolation, purification and chemical analysis of PS

The hot aqueous-extract of immature onion stick (600 g) was, at first cooled, then filtered, and precipitated in alcohol. The residue was dissolved in minimum volume of distilled water, dialyzed, centrifuged, and then freeze dried to yield 1.5 g of crude polysaccharide. Crude polysaccharide on fractionation through Sepharose-6B using water as eluant, yielded only one homogeneous fraction (PS). For more purification, PS was again fractionized through sepharose-6B in aqueous medium.

The pure pectic polysaccharide has a specific rotation of $[\alpha]_D^{25.7}$ + 105 (*c* 0.104, water). The total carbohydrate of PS was calculated to be 80.6% using phenol-sulfuric acid method (York et al., 1985). The molecular weight (Hara et al., 1982) of PS was found to be ~1.8×10^2 kDa. Paper chromatographic analysis (Hoffman et al.,

1972) of the hydrolyzed PS showed the presence of galacturonic acid, galactose and a slow moving spot nearer to galactose. GLC analysis of the alditol acetates showed the presence of galactose and 6-*O*-methyl galactose in the molar ratio of nearly 1:1 but the carboxy-methyl-reduced (Maness et al., 1990) PS on hydrolysis, followed by GLC analysis of corresponding alditol acetate showed the presence of galactose and 6-*O*-methyl galactose in the molar ratio of nearly 3:1. From the above GLC analysis it was concluded that the PS contained galacturonic acids, galactose and 6-*O*-methyl galactose in the molar ratio of nearly 2:1:1. The absolute configuration of the sugar units present in the PS were determined by the method of Gerwig et al. (1978) and it was found that galactose and galacturonic acid have D configuration. The modes of linkages of PS were determined by methylation analysis using Ciucanu and Kerek (1984) method followed by hydrolysis and alditol acetates preparation. The GLC and GLC–MS of alditol acetates of methylated product showed the presence of 2,3,4,6-Me_4-Gal, and 2,3,6-Me_3-Gal in a molar ratio of nearly 1:1. This result indicated the presence of non-reducing-end galactopyranosyl, and (1 → 4)-linked galactopyranosyl moieties. The methylated carboxyl-reduced (Abdel-Akher & Smith, 1950) PS showed the presence of the above peaks along with two new peaks corresponding to 2-Me-Gal, and 3-Me-Gal in a molar ratio of nearly 1:1:1:1. This observation indicated the presence of 1,4-linked but 3,4 substituted Gal*p*A of which 3-position was linked by OAc, confirmed by NMR-data and 1,4-linked but 2,4 substituted Gal*p*A. Thereafter, for more linking information periodate-oxidation (Goldstein et al., 1965; Hay et al., 1965) was carried out with the pectic polysaccharide. The periodate-oxidized reduced material upon hydrolysis with trifluoro acetic acid followed by GLC-analysis gave no information. But, a part of the periodate-oxidized PS on hydrolysis showed the presence of galacturonic acid in the paper chromatography examination (Hoffman et al., 1972). The GLC and GLC–MS analysis of the alditol acetates of the periodate-oxidized carboxyl reduced (Abdel-Akher & Smith, 1950) methylated PS showed the presence of 3,6-Me_2-Gal and 2,3,6-Me_3-Gal. These result indicated that terminal Gal*p* and (1 → 4)-linked methoxy-Gal*p* residues were destroyed by the diol oxidizing agent and galacturonic acids were present as 1,4-linked but 2,4 substituted and 1,4-linked but 3,4 substituted of which 3-position was linked by OAc moities, respectively.

3.2. NMR and structure analysis of PS

The ^{1}H NMR (500 MHz, Fig. 1a) spectrum of PS at 27 °C exhibits four signal at δ 5.22, 5.07, 4.94, and 4.62 in a molar ratio of nearly 1:2:1:1. The signal at 5.07 ppm corresponded to two protons whereas the signals at 5.22, 4.94, and 4.62 ppm were referred by three protons. The signal at 2.04 ppm was indicative of the CH_3 proton of an acetic ester. The carbomethoxy proton resonated at δ 3.78. The sugar residues were designated as **A**–**D** according to their decreasing anomeric chemical shifts (Table 1). The signal at 5.07 ppm also corresponded to H-3 of residue **B**.

In the ^{13}C NMR spectrum (125 MHz, Fig. 1b) at 27 °C, three signals were appeared at δ 104.7, 100.8, and 100.4 where signal at 100.8 ppm consists of two anomeric carbon of residues, **B** and **C** and the rest two anomeric signals at δ 104.7 and 100.4 corresponded to the anomeric carbons of residues **D** and **A** respectively. Moreover, δ 20.4, 53.2, and 61.1 were assigned for CH_3 of acetic ester, carbomethoxy carbon, and *O*-methyl carbon, respectively. All the ^{1}H and ^{13}C signals (Table 1) are assigned from DQF-COSY, TOCSY, and HSQC (Fig. 2) experiments. From DQF-COSY experiment the proton–proton coupling constants value was determined.

From methylation data, residue **A** was assigned as terminal D-galactopyranose. The galacto-configuration was confirmed from large coupling constant value $J_{H\text{-}2,H\text{-}3} \sim 8.6$ Hz and relatively small $J_{H\text{-}3,H\text{-}4} \sim 3.4$ Hz. The α-configuration of residue **A** was

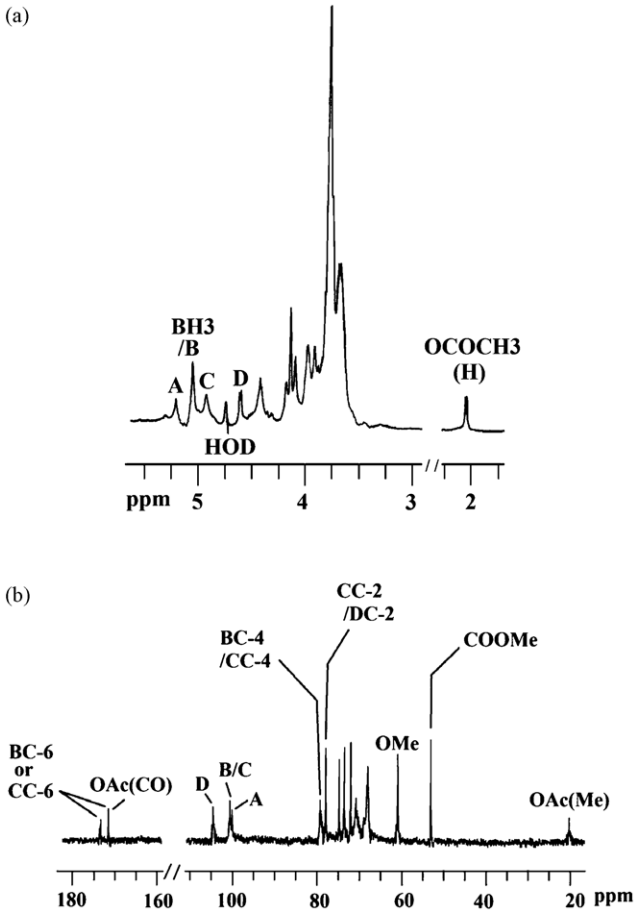


Fig. 1. (a) ^{1}H NMR spectrum (500 MHz, D_2O, 27 °C) of PS, isolated from immature onion stick (*Allium cepa*). (b) ^{13}C NMR spectrum (125 MHz, D_2O, 27 °C) of PS, isolated from immature onion stick (*Allium cepa*).

proved from an anomeric proton signal δ 5.22, coupling constants $J_{H\text{-}1,H\text{-}2} \sim 3.3$ Hz, and $J_{C\text{-}1,H\text{-}1} \sim 170$ Hz. The anomeric carbon signal of residue **A** appears at100.4 ppm. The chemical shift of C-2, C-3, C-4, C-5, and C-6 of this residue were more or less same with the standard value of methyl glycosides (Agrawal, 1992; Rinaudo & Vincendon, 1982) which indicated that the residue **A** was α-linked non-reducing-end D-galactose.

The spin system of residue **B**, which has only five protons with a relatively high chemical shift of the H-5 signal (4.44 ppm)

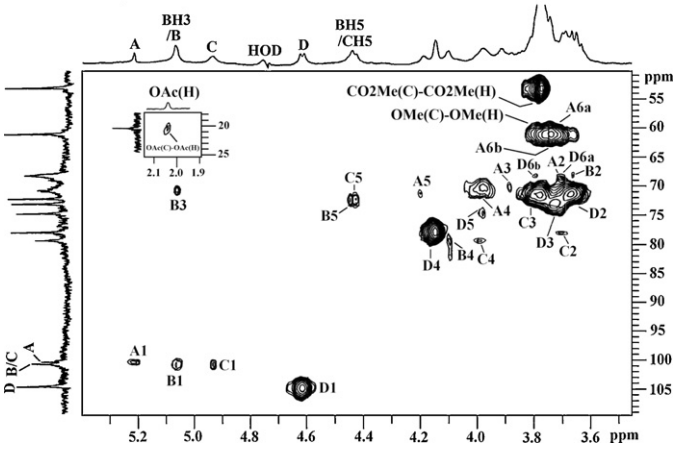


Fig. 2. HSQC spectrum of PS, isolated from immature onion stick (*Allium cepa*).

Table 1
^{1}H NMR[a] and ^{13}C NMR[b] chemical shifts (δ, ppm) for PS, isolated from immature onion stick (*Allium cepa*) recorded in D_2O at 27 °C.

Glycosyl residue	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6a,H-6b/C-6	COOMe	3-*O*-COMe	6-*O*-Me
A α-D-Gal*p*-(1→	5.22 100.4	3.70 68.8	3.89 70.3	4.00 70.9	4.20 71.2	3.72, 3.77 61.1			
→4)-α-D-Gal*p*A6Me-(1→ 3 ↑ OAc **B**	5.07 100.8	3.68 68.2	5.07 70.9	4.11 79.4	4.44 72.2	171.9[e] or 173.5[e]	3.78[c] 53.2[f]	2.04[d] 20.4[g] 171.9[h]	
C →2,4)-α-DGal*p*A6Me-(1→	4.94 100.8	3.71 78.0	3.81 70.9	3.99 79.4	4.44 72.2	171.9[e] or 173.5[e]	3.78[c] 53.2[f]		
D →4)-6-OMe-β-D-Gal*p*-(1→	4.62 104.7	3.66 70.9	3.75 73.1	4.15 78.0	3.98 74.9	3.70, 3.79 68.2			3.80 61.1

[a] Values of the ^{1}H chemical shifts were recorded and assigned with respect to the HOD signal fixed at δ 4.74 at 27 °C.
[b] Values of the ^{13}C chemical shifts were recorded with reference to using acetone as the internal standard and fixed at δ 31.05 at 27 °C.
[c] Value of methyl protons of ester group.
[d] Value of the methyl protons of the acetyl group.
[e] ^{13}C chemical shifts value of the carbonyl group of the ester group.
[f] Methyl carbon value of the ester group.
[g] Methyl carbon value of the acetyl group.
[h] Value of the carbonyl group of the acetyl group.

and small coupling constants, $J_{H\text{-}3,H\text{-}4}$ and $J_{H\text{-}4,H\text{-}5}$ indicated that it was D-Gal*p*A. The anomeric proton chemical shift at δ 5.07, carbon chemical shift at δ 100.8 and coupling constant value $J_{C\text{-}1,H\text{-}1} \sim 171$ Hz, indicated that D-galacturonosyl residue was α-linked. The C-4 peak of residue **B** at δ 79.4 showed a downfield shift compare to that of the standard methyl glycosides due to the α- glycosylation effect. The carbon signals of residue **B** were observed at δ 68.2, 70.9, 72.2, and 171.9 or 173.5 (not assigned) corresponding to C-2, C-3, C-5 and C-6 (carbonyl carbon), respectively.

The residue **C** had an anomeric proton chemical shift at δ 4.94 and this spin system also consisting of only five protons with a weak coupling of H-4 with both H-3 and H-5 indicated that it was a D-Gal*p*A. The singlet of H-1 and the characteristics $J_{H\text{-}1,\ H\text{-}2}$ coupling constant value (<3 Hz), and $J_{C\text{-}1,H\text{-}1} \sim 171$ Hz showed that the residue **C** was α-configuration. The downfield shift of C-2 (78.0 ppm), C-4 (79.4 ppm) signals, with respect to the standard value of methyl glycosides indicated that residue **C** was 1,4-linked but 2,4 substituted moiety. The carbon signals of this residue were observed at δ 70.9, 72.2, and 171.9 or 173.5 (not assigned) corresponding to C-3, C-5, and C-6 (carbonyl carbon), respectively. The carbonyl groups of both galacturonic acids were present as methyl ester. The presence of a carboxy-methyl group in residues **B** and **C** was confirmed by the appearance of intra-residual coupling between the ester carbonyl carbon (171.9 ppm or 173.5 ppm) and the carboxy methyl proton (3.78 ppm) in the HMBC experiment. From this observation, we concluded that residue **B** was methyl ester of a 1,4-linked α-D-Gal*p*A and residue **C** was methyl ester of a 1,4-linked but 2,4 substituted α-D-Gal*p*A.

In the case of residue **D**, the anomeric proton chemical shift appeared at δ 4.62. A large coupling constant value $J_{H\text{-}1,H\text{-}2}$ (~8.5 Hz) and $J_{C\text{-}1,H\text{-}1}$ value (162 Hz) indicated that it was a β-linked residue. The $J_{H2,H\text{-}3}$ (~9.2 Hz) and $J_{H\text{-}3,H\text{-}4}$ (~3.3 Hz) indicated that its galacto-configuration. Chemical shift of all the protons (H-1 to H-6) of this residue were identified from 2D-COSY and TOCSY spectra. On the basis of the proton assignments, the chemical shift of C-1 to C-6 was obtained from ^{1}H–^{13}C HSQC spectra (Fig. 2). The methoxy group at C-6 of residue **D** was confirmed by the appearance of cross couplings between the methoxy proton (δ 3.80 ppm) and the C-6 atom of galactose [**D** OCH_3(H), **D** C-6] and the methoxy carbon δ 61.1 and its H-6a, H-6b atoms [**D** OCH_3(C), **D** H-6a] and [**D** OCH_3(C), **D** H-6b] in the HMBC experiment. The downfield shift of C-4 of residue **D** indicated that it was present as (1 → 4)-linked moiety in the repeating unit of PS.

The sequence of glycosyl residues of PS was determined from NOESY (Fig. 3a and, Table 2) as well as ROESY (Fig. 3b) experiments followed by confirmations with an HMBC experiment. In NOESY experiment, the inter-residual contacts from **A** H-1 to **D** H-4, **B** H-1 to **C** H-4, **C** H-1 to **B** H-4, and **D** H-1 to **C** H-2 along with other intra residual contacts established the following sequences: **A**-(1 → 4)-**D**; **B**-(1 → 4)-**C**; **C**-(1 → 4)-**B**; **D**-(1 → 2)-**C**.

The acetyl group at C-3 of residue **B** was also confirmed from the result of HMBC experiment (Fig. 4 and Table 3). The intra- and inter-residual cross peaks were assigned in HMBC experiment. The inter-residual cross couplings **A** H-1/**D** C-4; **A** C-1/**D** H-4; **B** H-1/**C** C-4; **B** C-1/**C** H-4; **C** H-1/**B** C-4; **C** C-1/**B** H-4; **D** H-1/**C** C-2; **D** C-1/**C** H-2 were observed in HMBC experiment. The attachments of acetyl group with 3-position of residue **B** was confirmed by the presence of cross-peak **B** H-3, **B** OAc (carbonyl carbon). Intra-residual

Table 2
The NOESY data for PS, isolated from immature onion stick (*Allium cepa*).

Anomeric proton			NOE contact protons
Glycosyl residue	δ_H	δ_H	Residue, atom
α-D-Gal*p*-(1→**A**	5.22	4.15 3.70	**D** H-4 **A** H-2
→4)-α-D-Gal*p*A6Me-(1→ 3 ↑ OAc **B**	5.07	3.99 3.68 4.44	**C** H-4 **B** H-2 **B** H-5
→2,4)-α-D-Gal*p*A6Me-(1→**C**	4.94	4.11 4.44 3.71	**B** H-4 **C** H-5 **C** H-2
→4)-6-OMe-β-D-Gal*p*-(1→**D**	4.62	3.71 3.75 3.98	**C** H-2 **D** H-3 **D** H-5

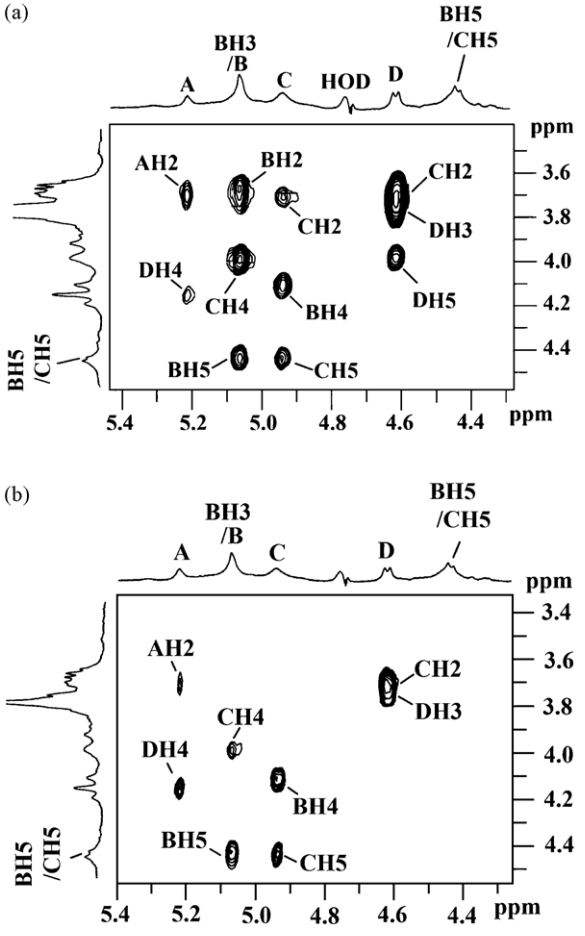


Fig. 3. (a) Part of NOESY spectrum of PS, isolated from immature onion stick (*Allium cepa*). The NOESY mixing time was 300 ms. (b) Part of ROESY spectrum of PS, isolated from immature onion stick (*Allium cepa*). The ROESY mixing time was 300 ms.

coupling between methoxy carbon and H-6a, and H-6b proton of residue **D** [OMe (**C**), **D** H-6a] and [OMe (**C**), **D** H-6b] were observed. Again, cross-peak was also observed between carbomethoxy protons (3.78 ppm) and ester carbonyl carbon [171.9 ppm or 173.5 ppm (not assigned)] of residues **B** and **C**. Hence from the NOESY and HMBC experiments the structure of the tetrasaccharide repeating

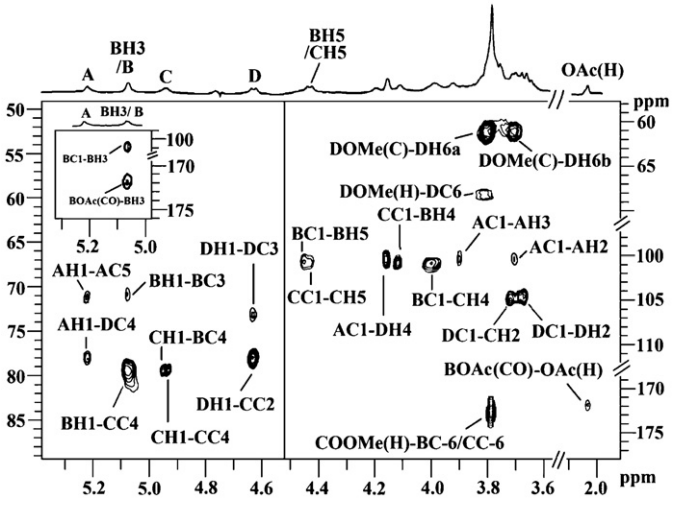


Fig. 4. The HMBC spectrum of PS, isolated from immature onion stick (*Allium cepa*). The delay time in the HMBC experiment was 80 ms.

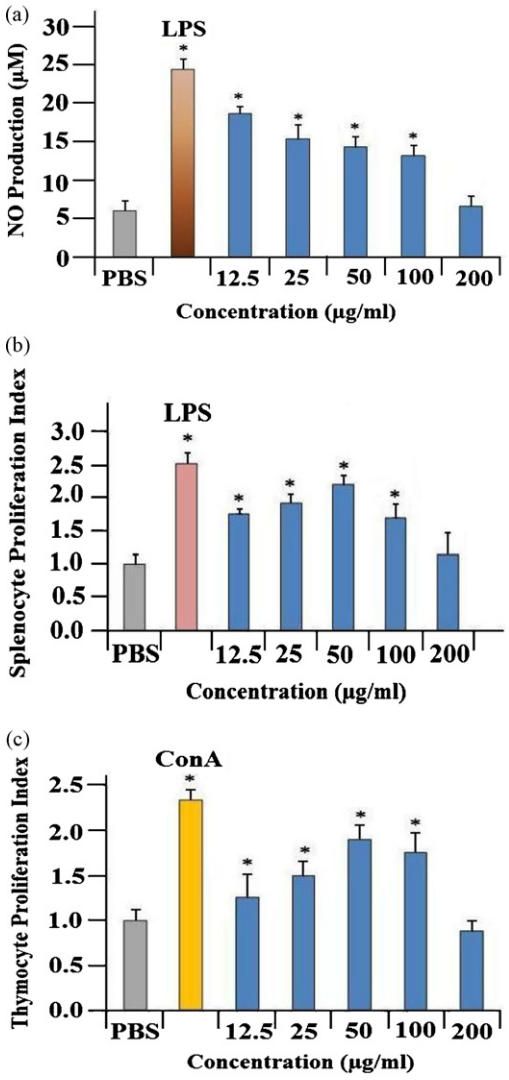
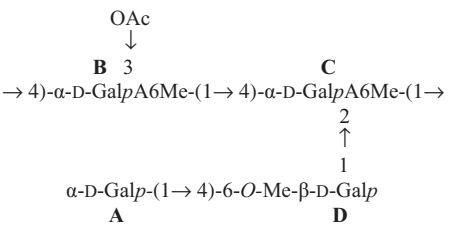


Fig. 5. (a) In vitro activation of peritoneal macrophage stimulated with different concentrations of PS in terms of NO production. Effect of different concentrations of PS on (b) splenocyte and (c) thymocyte proliferation.

unit of the pectic polysaccharide (PS) was established as:



3.3. Assay for macrophage activity by NO production

Macrophage activation by this pectic polysaccharide has been studied by nitric oxide (NO) production in culture supernatant in vitro. Upon treatment with different concentrations of this pectic polysaccharide, an enhanced production of NO was observed with optimum production of 18.5 μM NO per 5×10^5 macrophages at 12.5 μg/mL (Fig. 5a) and then gradually decreases. Hence 12.5 μg/mL was the effective dose of pectic polysaccharide for NO production. Many pectic polysaccharides of different sources

Table 3
The significant $^3J_{H,C}$ connectivities observed in an HMBC spectrum for the anomeric protons/carbons of the sugar residues of PS, isolated from immature onion stick (*Allium cepa*).

Residue	Sugar linkage	Observed connectivities	
		Inter	Intra
A	α-D-Gal*p*-(1→	**A**H-1/**D**C-4 **A**C-1/**D**H-4	**A**C-1/**A**H-2 **A**C-1/**A**H-3 **A**H-1/**A**C-5
B	→4)-α-D-Gal*p*A6Me-(1→ 3 ↑ OAc	**B**H-1/**C**C-4 **B**C-1/**C**H-4	**B**C-1/**B**H-5 **B**H-1/**B**C-3 **B**C-1/**B**H-3
C	→2,4)-α-D-Gal*p*A6Me-(1→	**C**H-1/**B**C-4 **C**C-1/**B**H-4	**C**C-1/**C**H-5 **C**H-1/**C**C-4
D	→4)-6-OMe-β-D-Gal*p*-(1→	**D**H-1/**C**C-2 **D**C-1/**C**H-2	**D**H-1/**D**C-3 **D**C-1/**D**H-2
B	→4)-α-D-Gal*p*A6Me-(1→ 3 ↑ OAc		**B**CO_2Me(H)/**B**C-6 **B**OAc(CO)/**B**OAc(H) **B**OAc(CO)/**B**H-3
C	→2,4)-α-D-Gal*p*A6Me-(1→		**C**CO_2Me(H)/**C**C-6
D	→4)-6-OMe-β-D-Gal*p*-(1→		**D**OMe(H)/**D**C-6 **D**OMe(C)/**D**H-6a, **D**H-6b

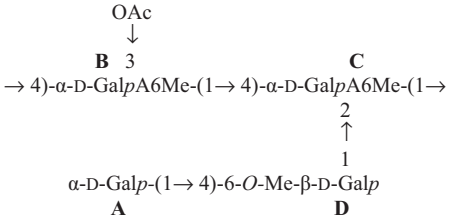
exhibited such stimulating effect on NO production. A RG-I type of polysaccharide isolated from *Nerium indicum* flowers showed dose dependent stimulating effect on the production of NO by macrophages RAW 264.7 cells, Dong et al., 2010. Pectic polysaccharide from *Biophytum petersianum* Klotzch was reported to induce NO release from rat R2 macrophage cell line, Inngjerdingen et al. (2008). An acidic polysaccharide fraction isolated from the roots of *Angelica sinecis* (olive) Diels significantly enhanced NO production in murine peritoneal macrophage, Yang, Zhao, Wang, and Mei (2007).

3.4. Splenocyte and thymocyte proliferation assay

Splenocyte and thymocyte proliferations are the measurement of immunoactivation (Ohno et al., 1993). The activation of splenocyte and thymocyte tests was carried out in mouse cell culture medium with the pectic polysaccharide by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide(method. The stimulation of the splenocytes (Fig. 5b) and thymocytes (Fig. 5c) were examined by this PS and the asterisks on the columns indicated the statistically significant differences compared to PBS (Phosphate Buffer Saline) control. The splenocyte and thymocyte proliferation index as compared to PBS (Phosphate Buffer Saline) control if closer to one or below indicates low stimulatory effect on immune system. Splenocyte and thymocyte proliferation index were found to be maximum at 50 μg/mL of the pectic polysaccharide as compared to other concentrations. Hence, 50 μg/mL of the pectic polysaccharide can be considered as the optimum concentration for splenocyte and thymocyte proliferation. From the above observations it was clear that this pectic polysaccharide could act as an efficient immunostimulating agent.

4. Conclusion

The structural characterization of a water-soluble pectic polysaccharide (PS) was carried out using acid hydrolysis, methylation analysis; periodate oxidation and NMR spectral data. The results indicated that α-(1 → 4)-D-methylgalactouronate fragments occupied the main chain of the pectic polysaccharide in which branching occurred at *O*-2 position of one methyl galacturonate moiety by α-D-Gal*p*-(1 → 4)-6-*O*-Me-β-D-Gal*p*-(1→ and other methyl galacturonate moiety contained an acyl group at *O*-3 position. This molecule showed significant splenocyte, thymocyte and macrophage activations. On the basis of the chemical and spectroscopic analysis the structure of the repeating unit of the pectic polysaccharide was established as:



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