Carbohydrate Research 347 (2012) 107–113

Contents lists available at SciVerse ScienceDirect

Carbohydrate Research

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

Glucans from alkaline extract of a hybrid mushroom (backcross mating between *PfloVv12* and *Volvariella volvacea*): structural characterization and study of immunoenhancing and antioxidant properties

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ARTICLE INFO

Article history:
Received 27 July 2011
Received in revised form 26 October 2011
Accepted 28 October 2011
Available online 6 November 2011

Keywords:
Hybrid mushroom
Polysaccharide
NMR spectroscopy
Immunostimulation
Antioxidant property

ABSTRACT

Two different glucans (water-soluble PS-I, water-insoluble PS-II) were isolated from the alkaline extract of the fruit bodies of hybrid mushroom. PS-I was found to consist of only (1→6)-linked β-D-glucopyranose. PS-II was composed of terminal, (1→3,4)-linked, and (1→3)-linked β-D-glucopyranosyl moieties in a molar ratio of nearly 1:1:1. PS-I showed macrophages, splenocytes, and thymocytes activation as well as antioxidant property. On the basis of sugar analysis, methylation analysis, and NMR studies (^{1}H, ^{13}C, DEPT-135, TOCSY, DQF-COSY, NOESY, ROESY, HMQC, and HMBC), the structure of the repeating unit of these glucans were established as:

PS-I →6)-β-D-Glc*p*-(1→

(Water-soluble glucan)

PS-II →3)-β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→
4
↑
1
β-D-Glc*p*

(Water-insoluble glucan)

1. Introduction

Mushrooms have been used as a food or food flavoring materials because of their unique and subtle flavor[1] for a long time. Edible mushrooms are highly nutritious[2] and used as a source of physiologically beneficial and nontoxic medicines.[3] Currently mushroom-derived substances having anti-tumor and immunomodulating properties are used as dietary supplements or drugs.[4] Characters within the gene pool of any one particular edible mushroom species is limited and therefore for further improvement of quality of edible mushrooms, introgression of characters from different mushroom strains are needed and that can be done through production of hybrid mushrooms.[5] In our laboratory, water-soluble polysaccharides have already been isolated from the aqueous extract of fruit bodies of somatic hybrid mushrooms *PfloVv5FB*[6] and *PfloVv1aFB*[7] and characterized. Water-soluble branched α,β-(1→3)(1→6) and insoluble β-(1→3)(1→6)-glucans from *Pleurotus florida*[8,9] and another glucan from *V. volvacia*[10] were reported from parent mushrooms. The aqueous extract of this hybrid mushroom showed the presence of β-(1→6) glucan and manno galactosyl

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doi:10.1016/j.carres.2011.10.043

glucose (communicated) but its alkaline extract showed the presence of linear soluble β-(1→6) and also insoluble branched β-(1→3)(1→4) glucans. Here, we report the structural characterization of both the glucans and the study of immunoenhancing and antioxidant properties of the β-(1→6) glucan isolated from alkaline extract of a novel hybrid mushroom (backcross mating between *PfloVv12* and *Volvariella volvacea*).[5] *PfloVv12*, was initially obtained through intergeneric protoplast fusion between *P. florida* and *V. volvacea*.

2. Results and discussion

2.1. Structural investigation of water-soluble glucan (PS-I)

The alkaline extract of fruit bodies of hybrid mushroom was cooled, centrifuged, and precipitated in ethanol. The residue was dialyzed until alkali free, centrifuged, and freeze–dried to yield 300 mg of water-soluble and 900 mg of water-insoluble crude polysaccharides. The water-soluble crude material was purified, yielding a single fraction (PS-I). PS-I had a specific rotation $[\alpha]_D^{25}$ −26.6 (*c* 0.6, water). Molecular weight[11] of PS-I was estimated as ~2.2×10^5 Da. Paper chromatography[12] analysis of the hydrolyzed PS-I showed the presence of only one spot of glucose. This was also confirmed by GLC analysis of alditol acetate of hydrolyzed product of PS-I. The absolute configuration of the glucose residue was determined as D by the method of Gerwig et al.[13] The mode of linkages of the sugar moieties present in PS-I was determined by using Ciucanu and Kerek[14] method followed by hydrolysis and alditol acetate preparation. The GLC–MS analysis of the partially methylated alditol acetates of PS-I revealed the presence of only one peak of 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-glucitol. This result indicated the presence of only (1→6)-linked glucopyranosyl residue in the glucan (PS-I). Further GLC analysis of alditol acetates of periodate-oxidized,[15,16] $NaBH_4$ reduced, methylated polysaccharide showed the disappearance of the sugar units. This result is in agreement with the presence of (1→6)-linked sugar residue in PS-I.

The 500 MHz ^{1}H NMR spectrum (Fig. 1, Table 1) and ^{13}C NMR (125 MHz) spectrum (Fig. 2, Table 1) showed the anomeric signals at 4.54 ppm and 103.4 ppm, respectively. From the above anomeric signals it was confirmed that PS-I consist of only one sugar unit in a repeating manner, designated as **A**. The coupling constant $J_{H\text{-}1,H\text{-}2}$ value (~8.5 Hz) and $J_{C\text{-}1,H\text{-}1}$ value (~160 Hz) suggested that it was β-linked residue. The large $J_{H\text{-}2,H\text{-}3}$ and $J_{H\text{-}3,H\text{-}4}$ values (~10.0 Hz) confirmed its glucopyranosyl configuration. The proton chemical shifts from H-1 to H-6 were assigned from the DQF-COSY and TOCSY spectrum. On the basis of the proton assignments, the chemical shifts of C-1 to C-6 were obtained from the ^{1}H–^{13}C HMQC spectrum. The carbon signals at 73.5, 76.0, 70.0, 75.4, and 69.3 ppm correspond to C-2, C-3, C-4, C-5 and C-6, respectively, of the glucopyranoside residue. The C-6 signal of the glucopyranoside residue at 69.3 ppm was shifted to 7.5 ppm downfield compared to the standard methyl glycosides due to the α-glycosilation[17,18] effect. These results indicated that residue **A** was (1→6)-linked β-D-glucopyranosyl residue. The (1→6)-linking was also confirmed from the DEPT-135 NMR spectrum (Fig. 3).

The sequence of glycosyl residues of PS-I was determined from the NOESY (Fig. 4, Table 2) as well as ROESY experiments followed by confirmation with a HMBC (Fig. 5, Table 3) experiment. For explanation of NOESY and HMBC experiments two units of glucose (**A** and **A′**) were considered. NOESY experiment showed inter-residual contact from **A** H-1 to **A′** H-6a and **A′** H-6b along with other intra-residual contacts. Hence, the following sequence in PS-I was established as:

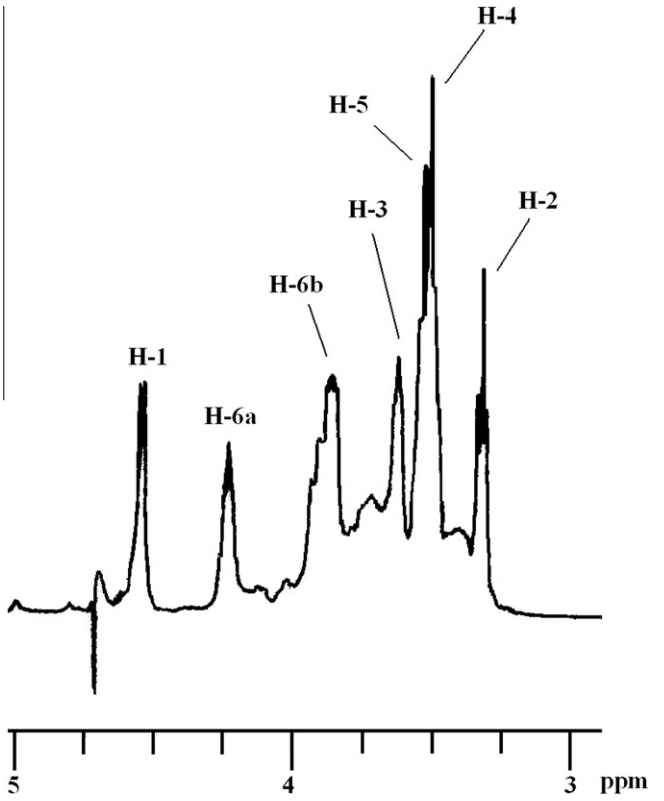


Figure 1. ^{1}H NMR spectrum (500 MHz, D_2O, 27 °C) of PS-I, isolated from fruit bodies of hybrid mushroom.

Table 1
^{1}H NMR and ^{13}C NMR chemical shifts (ppm) of PS-I isolated from the fruit bodies of hybrid mushroom[a,b] recorded 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
→6)-β-D-Glc*p*-(1→	4.54	3.34	3.64	3.50	3.52	4.24, 3.87
A	103.4	73.5	76.0	70.0	75.4	69.3

[a] 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.
[b] Values of the ^{1}H chemical shifts were recorded with respect to the HOD signal fixed at δ 4.74 at 27 °C.

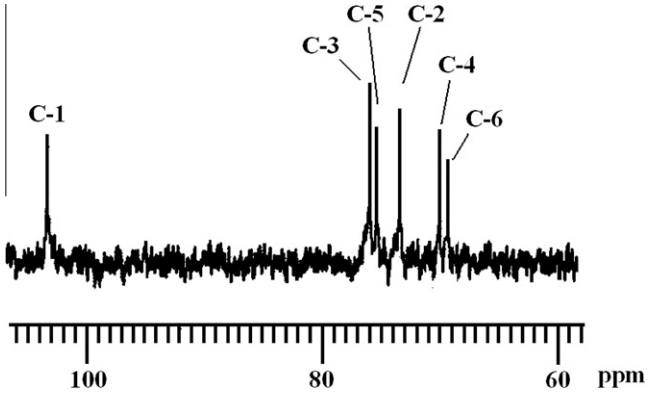


Figure 2. ^{13}C NMR spectrum (125 MHz, D_2O, 27 °C) of PS-I, isolated from fruit bodies of hybrid mushroom.

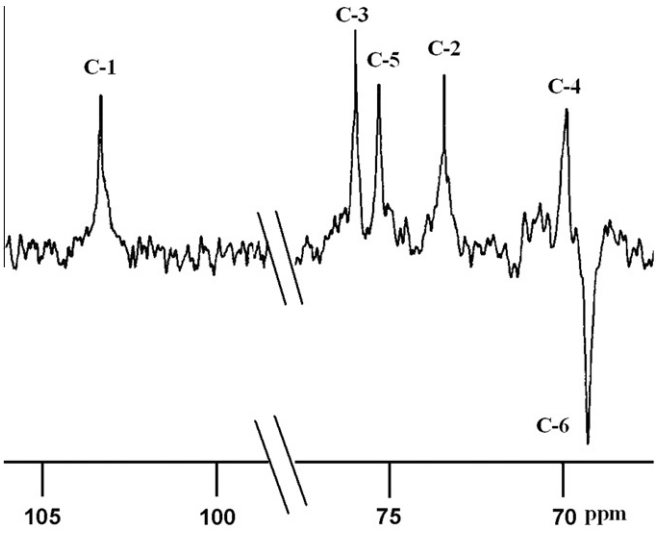


Figure 3. DEPT-135 NMR spectrum (D_2O, 27 °C) of PS-I, isolated from fruit bodies of hybrid mushroom.

$$\underset{\mathbf{A}}{\rightarrow 6)\text{-}\beta\text{-}D\text{-}Glc\mathit{p}\text{-}(1\rightarrow} \underset{\mathbf{A'}}{6)\text{-}\beta\text{-}D\text{-}Glc\mathit{p}\text{-}(1\rightarrow}$$

Long range ^{13}C–^{1}H HMBC experiment confirmed the above sequences deduced from NOESY experiment. The cross-peaks of both anomeric proton and carbon of the sugar moiety were examined and both inter- and intra-residual connectivities were observed from the HMBC experiment (Fig. 5, Table 3). Cross-peaks were found between H-1 (4.54 ppm) of residue **A** and C-6 (69.3 ppm) of residue **A′** (**A** H-1, **A′** C-6) and C-1 (δ 103.4 ppm) of residue **A** and H-6a (4.24 ppm) and H-6b (3.87 ppm) of residue **A′** (**A** C-1, **A′** H-6a and **A** C-1, **A′** H-6b).

Thus, from all these observations the structure of the repeating unit of the glucan (PS-I) was established as:

$$\underset{\mathbf{A}}{\rightarrow 6)\text{-}\beta\text{-}D\text{-}Glc\mathit{p}\text{-}(1\rightarrow}$$

2.2. Structural investigation of water insoluble glucan (PS-II)

The molecular weight of the glucan (PS-II) was determined from a calibration curve prepared with standard dextrans[11] as ~2.0×10^5 Da. Paper chromatographic analysis[12] of the hydrolyzed PS-II was performed and showed only one spot of glucose. The presence of only glucose was further confirmed by the GLC analysis of alditol acetates of PS-II. Thus, above experiments confirmed that PS-II was a glucan. The absolute configuration of the sugar units was determined as configuration D by the method of Gerwig et al.[13] The mode of linkages of the sugar moieties were determined by methylation analysis using the method of Ciucanu and Kerek,[14] followed by hydrolysis and alditol acetates preparation. GLC–MS analysis of the partially methylated alditol acetates revealed the presence of three peaks of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-glucitol, 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methyl-glucitol, and 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-glucitol in a molar ratio of nearly 1:1:1. Above results indicated the presence of terminal, (1→3,4)-linked, and (1→3)-linked glucopyranosyl moieties in the water-insoluble glucan. Further GLC–MS analysis of alditol acetates of periodate-oxidized,[15,16] $NaBH_4$ reduced, methylated polysaccharide showed the peaks of 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methyl-glucitol and 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-glucitol in a molar ratio of nearly 1:1. This result indicated that terminal D-glucosyl moiety was consumed during periodate oxidation, while (1→3,4)-linked, (1→3)-linked residues were retained.

Table 2
NOESY data for PS-I isolated from the fruit bodies of hybrid mushroom

Anomeric proton		NOE contact protons	
Glycosyl residue	δ (ppm)	δ (ppm)	Residue, atom
→6)-β-D-Glc*p*-(1→	4.54	4.24	**A′** H6a
A		3.87	**A′** H6b
		3.64	**A** H-3
		3.52	**A** H-5
		3.34	**A** H-2

The ^{13}C NMR spectrum (Fig. 6, Table 4) of PS-II was carried out at 27 °C. The anomeric carbon signal at 103.6 ppm confirmed the β-conformation of the D-glucopyranosyl moieties. The anomeric carbon signal of terminal, (1→3,4)-linked, and (1→3)-linked D-glucopyranosyl moieties appeared at the same position (103.6 ppm) and the moieties were designated as **X**, **Y**, and **Z**, respectively. C-2 to C-6 values of residue **X** corresponds nearly to the standard methyl glycosides.[17,18] It was assigned as a terminal β-D-glucopyranosyl moiety.

In case of residue **Y**, the C-3 signal at 87.0 ppm shifted to 10.2 ppm downfield, the C-4 signal at 77.6 ppm shifted to 6.0 ppm downfield and the slightly upfield shift of C-2 and C-5 signal compared to the standard methyl glycosides confirmed that it was (1→3,4)-linked β-D-glucopyranosyl moiety.

In ^{13}C spectrum, the C-3 value of residue **Z** at 87.3 ppm shifted 10.5 ppm downfield compared to the standard methyl glycosides indicated that it was (1→3)-linked β-D-glucopyranosyl moiety. Since, residue **Y** is the most rigid part of the backbone of the glucan, its C-3 value (δ 87.0 ppm) appeared at the upfield compared to the C-3 value (87.3 ppm) of residue **Z**. Therefore, based on all the

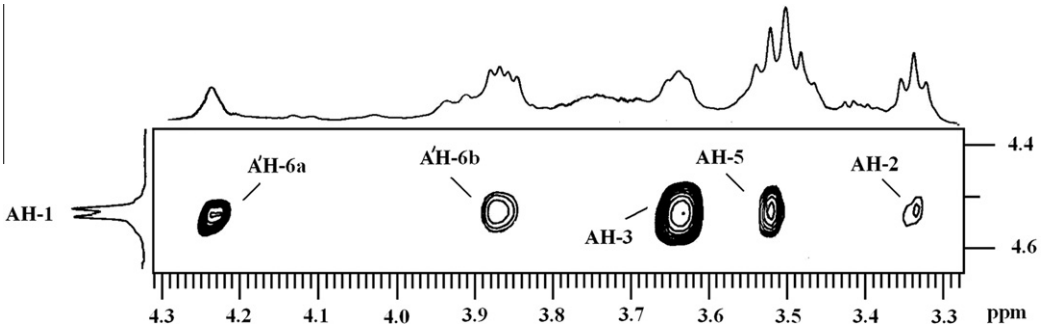


Figure 4. The NOESY spectrum of PS-I isolated from fruit bodies of hybrid mushroom. The NOESY mixing time was 300 ms.

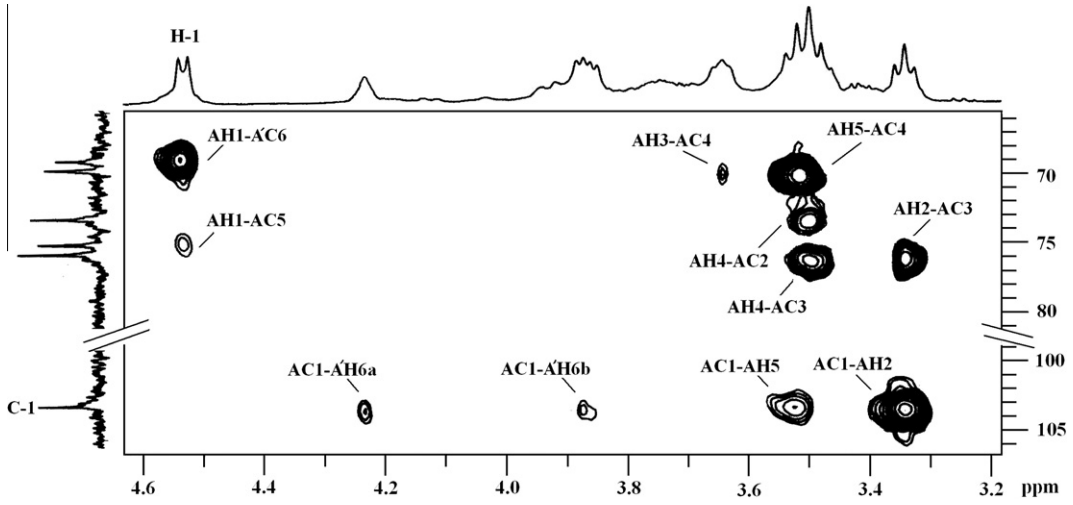


Figure 5. The HMBC spectrum of PS-I isolated from fruit bodies of hybrid mushroom The delay time in the HMBC experiment was 80 ms.

Table 3
The significant $^{3}J_{H,C}$ connectivities observed in a HMBC spectrum for the anomeric protons/carbons of the sugar residues of PS-I of the fruit bodies of hybrid mushroom

Residue	Sugar linkage	H-1/C-1 δ_H/δ_C (ppm)	Observed connectivities		
			δ_H/δ_C (ppm)	Residue	Atom
A	→6)-β-D-Glc*p*-(1→	4.54	69.3	A′	C-6
			75.4	A	C-5
		103.4	4.24	A′	H-6a
			3.87	A′	H-6b
			3.52	A	H-5
			3.34	A	H-2

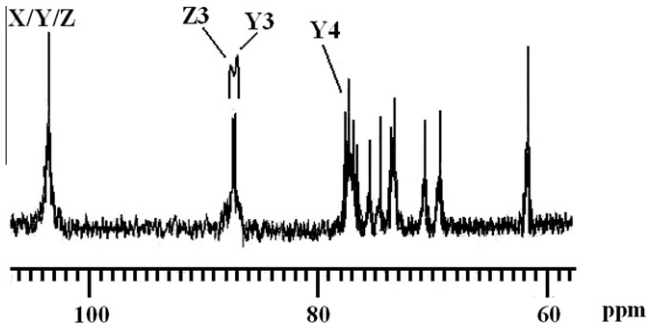


Figure 6. ^{13}C NMR spectrum (125 MHz, D_2O, 27 °C) of PS-II, isolated from fruit bodies of hybrid mushroom.

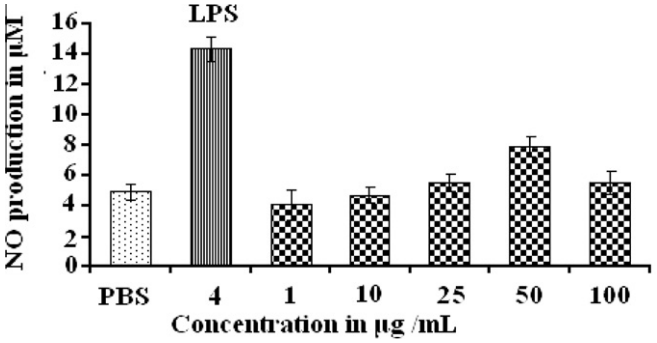


Figure 7. In vitro activation of peritoneal macrophage stimulated with different concentrations of the polysaccharide (PS-I) in terms of NO production.

Table 4
The chemical shifts in the ^{13}C NMR[a] spectrum of PS-II, a water-insoluble glucan isolated from the fruit bodies of hybrid mushroom in Me_2SO-d_6 at 27 °C

Residue	Sugar linkage	C-1	C-2	C-3	C-4	C-5	C-6
	β-D-Glc*p*-(1→ (lit.[14])	104.0	74.1	76.8	70.6	76.8	61.8
X	β-D-Glc*p*-(1→	103.6	74.6	77.2	70.8	76.5	61.8
Y	→3,4)-β-D-Glc*p*-(1→	103.6	73.4	87.0	77.6	75.4	61.8
Z	→3)-β-D-Glc*p*-(1→	103.6	73.7	87.3	69.3	76.9	61.8

[a] Values of chemical shifts were recorded with reference to acetone as internal standard and fixed at δ 31.05 ppm at 27 °C.

chemical and spectroscopic evidence, the structure of trisaccharides repeating unit of water-insoluble β-glucan was established as:

Z **Y**
→3)-β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→
4
↑
1
β-D-Glc*p*
X

2.3. Biological characterization

Some biological studies were carried out with PS-I. Macrophage activation of the polysaccharide was observed in vitro. Upon treatment with different concentrations of this PS-I, enhanced produc-

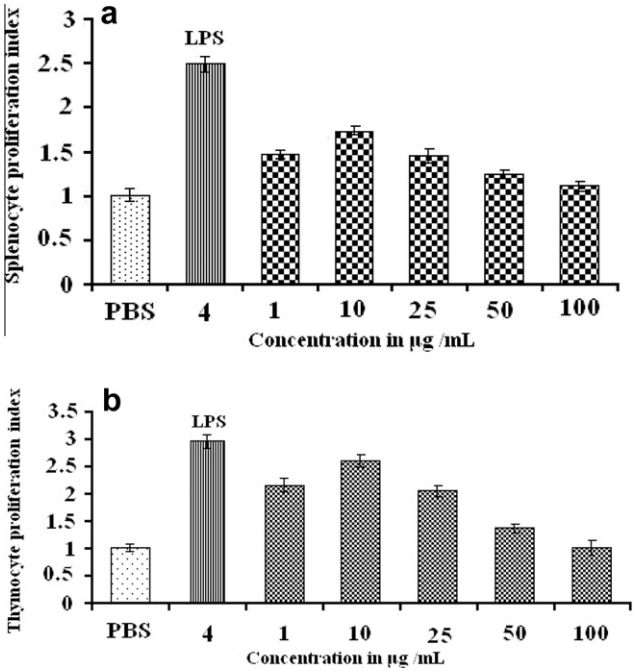


Figure 8. Effect of different concentrations of the polysaccharide (PS-I) on splenocyte (A) and thymocyte (B) proliferation.

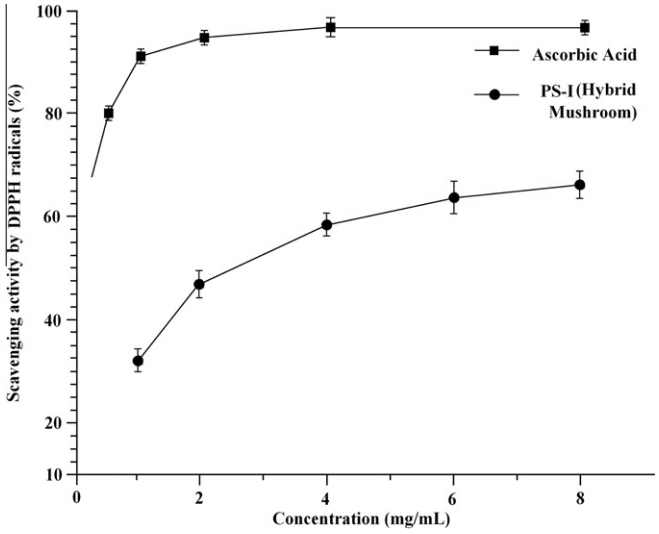


Figure 9. Ability of PS-I against DPPH radical. Results were presented as Means ± SD (n = 3).

tion of NO was observed in a dose-dependent manner with optimum production of 8.2 μM NO per 5×10^5 macrophages at 50 μg/mL (Fig. 7). As depicted from the Figure 7, there is no increase of NO production at concentrations 1 and 10 μg/mL. The NO production increases at 25 μg/mL by 10% which was further increased by 50% at 50 μg/mL and decreased thereafter at 100 μg/mL. Hence, the effective dose of PS-I was observed at 50 μg/mL. From our previous experiences, it is evident that biological phenomenon like NO production needs an optimum concentration for optimum activity below which it is incapable of producing sufficient strength of stimulus and above which it may produce some inhibitory signals as observed in our previous reports.[19–22] In this present case of PS-I similar trend of effect is observed in a dose dependent manner.

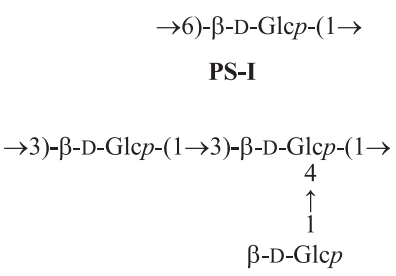
Splenocyte and thymocyte activation tests were carried out in mouse cell culture medium with PS-I by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method.[23] Proliferation of splenocytes and thymocytes is an indicator of immunostimulation. PS-I was found to stimulate splenocytes and thymocytes as shown in Figure 8A and B, respectively. The splenocyte and thymocyte proliferation index as compared to phosphate buffer saline (PBS) control either closer to 1 or below indicated low stimulatory effect on the immune system. At 10 μg/mL of PS-I, splenocyte and thymocyte proliferation index were found maximum as compared to other concentrations. Hence, 10 μg/mL of PS-I can be considered as an efficient splenocyte and thymocyte stimulator.

From these experiments it was observed that NO production occured at higher concentration of PS-I than that of splenocytes and thymocytes. The possible reasons of differences in effective concentration of NO production to that of splenocytes and thymocytes proliferations may be due to the following reasons. Dectin-1, CR3 and TLRs are the receptors at the cell surface to which polysaccharides like glucans bind for producing a down-stream signal like proliferation and NO production.[24–26] Further there are reports that binding of polysaccharides to cells activate nuclear transcription factors such as NF-κB[27,28] which translocates to nucleus and enhances transcription as well as translation of genes required for proliferation or NO production. Biological processes like this proliferation and NO production needs one sufficient strength of signal at the cell surface level for optimum down stream effect and the strength of signal depends on various factors like cell type, availability and abundance of specific receptors on the cell surface, nature of stimulant (polysaccharide). Again strength of stimuli generated by polysaccharides depends on their molecular mass, the degree of branching, conformation and chemical modification.[29,30] These factors may be responsible for the observed effects of PS-I in present study that induce maximum NO production at higher concentrations but stimulates splenocyte and thymocyte proliferation at lower concentrations. Further studies on mechanism of action of PS-I are needed to get more insight on its action. It is note worthy to mention that several mushroom polysaccharides also showed similar type of immunostimulation as reported in our previous works.[31,32]

Figure 9 demonstrated DPPH scavenging activity[33] caused by different concentrations of PS-I. A maximum of 66.5% DPPH radical scavenging activity was observed at 8 mg/mL of PS-I. The EC_{50} value of the PS-I for DPPH radicals was 2.5 mg/mL. The scavenging activity of the PS-I increased steadily from 0.5 to 6 mg/mL, while it reached a maximum plateau from 0.5 to 2 mg/mL for ascorbic acid, which indicates the scavenging activity of PS-I against DPPH radical was less than that of ascorbic acid.

3. Conclusion

Water-soluble (PS-I) and insoluble (PS-II) glucans were isolated from alkaline extract of fruit bodies of a hybrid mushroom. The water-soluble polysaccharide was purified by gel-filtration chromatography which showed high splenocyte, thymocyte and macrophage activation as well as antioxidant property in a particular concentration. The water insoluble polysaccharide was purified by repeated precipitation in alcohol. On the basis of chemical and NMR analysis, the structure of the repeating unit of the polysaccharides was established as:



4. Experimental

4.1. Isolation and purification of the polysaccharide

Hybrid mushroom was cultivated and collected from Falta campus, Bose Institute, Kolkata 700054. Fresh fruit bodies of hybrid mushroom (600 g) were washed with distilled water and then boiled with 6% NaOH for 45 min. The crude polysaccharide was isolated by applying the methods reported earlier,[34] yield 300 mg (water-soluble) and 900 mg (water-insoluble). Purification of the water-soluble polysaccharide was carried out by gel-permeation chromatography on a Sepharose 6B column (90 × 2.1 cm) using a Redifrac fraction collector loading 30 mg crude polysaccharide for each run. Ninety five Test tubes (each contain 2 mL eluant) were collected and monitored by the phenol–sulfuric acid procedure[35] at 490 nm using Shimadzu UV–vis spectrophotometer, model-1601. A single fraction (test tube nos. 16–28) was collected and freeze–dried, yielding 14 mg of material. The purification process was carried out for several lots and polysaccharide fraction was again purified and collected. The water insoluble polysaccharide (PS-II) was purified dissolving in 6% NaOH and repeated precipitation in alcohol (1:5 v/v) followed by centrifugation.

4.2. General methods

The optical rotation was measured on a Jasco polarimeter, model P-1020 at 25 °C. For monosaccharide analysis, the polysaccharide sample (3.0 mg) was hydrolyzed with 2 M CF_3COOH (2 mL), and the analysis was carried out as described earlier.[34] The molecular weight of the polysaccharide was determined as reported earlier.[11,34] Paper chromatographic[12] was carried out as described in our previous papers.[8] The absolute configuration of the monosaccharide constituent was assigned according to Gerwig et al.[13] The polysaccharide was methylated according to Ciucanu and Kerek method.[14] Gas liquid chromatographic (GLC) analysis was performed by using a Hewlett-Packard Model 5730 A, having a flame ionization detector and glass columns (1.8 m × 6 mm) packed with 3% ECNSS-M (A) on Gas Chrom Q (100–120 mesh) and 1% OV-225 (B) on Gas Chrom Q (100–120 mesh). All GLC analyses were performed at 170 °C. Gas liquid chromatography–mass spectrometric (GLC–MS) analysis was also performed on Shimadzu GLC–MS Model QP-2010 Plus automatic system, using ZB-5MS capillary column (30 m × 0.25 mm). The program was isothermal at 150 °C; hold time 5 min, with a temperature gradient of 2 °C/min up to a final temperature of 200 °C. Periodate oxidation was performed as described in an earlier report.[34] NMR experiments were carried out as reported in our previous papers.[34,36,37] DEPT-135 NMR experiment was carried out at 27 °C.

4.3. Test for macrophage activity by nitric oxide assay

Peritoneal macrophages (5×10^5 cells mL^{-1}) after harvesting were cultured in complete RPMI (Rose well Park Memorial Institute) media in 96-well plates.[38,39] The purity of macrophages was tested by adherence to tissue culture plates. The polysaccharide was added to the wells in different concentrations (1–100 μg/mL). 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.[40] 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. Lipopolysaccharide (LPS), L6511 of *Salmonella enterica* serotype typhimurium (sigma, St. Louis, USA) was used as positive control.

4.4. Splenocyte and thymocyte proliferation assay[39,41]

A single cell suspension of spleen and thymus was prepared from the normal mice under aseptic conditions by frosted slides in PBS (Phosphate Buffered Saline). The suspension was centrifuged to obtain cell pellet. The contaminating red blood cells were removed by hemolytic Gey's solution. After washing two times in PBS the cells were resuspended in complete RPMI 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-bottomed plates and incubated with 20 μL of various concentrations (1–100 μg/mL) of the polysaccharide. The same lipopolysaccharide as used in macrophage activation was also used here as positive control. The 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.[23] Data were reported as the mean ± standard deviation of five different observations and compared against PBS control.

4.5. DPPH radical scavenging activity of PS-I

The antioxidant activity of the 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 et al.[33] with some modification. Samples were dissolved in distilled water at 0.2, 0.5, 1, 2, 4, and 8 mg/mL. One milliliter test sample was mixed with two milliliter 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:

$$Q = (A_0 - A_c)/A_0 \times 100\%$$

Q = percentage reduction of the DPPH. Where A_0 = initial absorbance. A_c = absorbance after added sample concentration c.

Acknowledgments

The authors are grateful to Prof. S. Roy, Director, IICB, Dr. A.K. Sen (Jr.), IICB Kolkata, for providing instrumental facilities. Mr. Barun Majumder of Bose Institute, Kolkata, is acknowledged for preparing NMR spectra. DST, Govt of India is acknowledged for sanctioning a project (Ref. No: SR/S1/OC- 52/2006 dated 19/02/2007). One of the authors (R.S.) is grateful to UGC, New Delhi for offering junior research fellowship.

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