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Structural Studies of An Immuno Enhancing Polysaccharide Isolated from an Edible Mushroom, *Volvariella diplasia*

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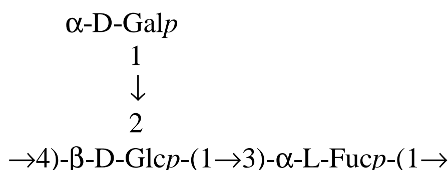
ABSTRACT

A water soluble heteroglycan, isolated from alkali-treated edible mushroom, *Volvariella diplasia*, consists of D-glucose, D-galactose and L-fucose residues in a molar ratio of nearly 1:1:1. This polysaccharide showed macrophage, splenocyte, and thymocyte activation. On the basis of sugar hydrolysis, methylation, periodate oxidation study, and NMR studies (^{1}H, ^{13}C, DQF-COSY, TOCSY, NOESY, ROESY, HMQC, and HMBC), the structure of the repeating unit of the polysaccharide was established as:



Keywords: *Volvariella diplasia*, polysaccharide, NMR spectroscopy, macrophage, splenocyte, thymocyte activation

1.Introduction

Mushrooms are source of nutritious healthy foods that are valued throughout the world a luscious medicine for thousands of years. Currently mushroom derived substances having anti-tumor and immune modulating properties are used as dietary supplements or drugs [1]. Among the different species of *Volvariella, V. bombycina*and *V. diplasia* have been cultivated in India. These are known as straw mushrooms or paddy straw mushrooms. The local people consume them as delicious food materials. Polysaccharides from these mushrooms can reduce blood pressure [2], exhibit a cardiovascular response[3] and affect the biosorptions [4,5] of metal ions. complex organic nitrogen sources like yeast extract, peptone, tryptophan, aspartic acid, serine and casein hydrolysate; inorganic nitrogen sources such as ammonium di-hydrogen phosphate were found to be the best for protein production, germination and germ tube elongation of thespores of *V. diplasia* [6,7].These mushroom polysaccharides showed anti-oxidant activity[8].

The aqueous extract of mushroom *V. diplasia* showed the presence of mannogalactosyl glucose and glucan. The structures of these polysaccharides have been

published Ghosh et al. in journal of *Carbohydrate Research*, 2008 [9,10]. No work has been reported on the polysaccharide, isolated from the alkali extract of the mushroom *V. diplasia.* Therefore, a detailed structural characterization and study of its immune enhancing properties were carried out and reported herein.

2. Result and discussion

2.1. Chemical analysis of the polysaccharide

The pure polysaccharide has specific rotation $[\alpha]_D^{25}$ +10.5 (c 0.84,water). The molecular weight was determined using calibration curve of standard dextrans [11] (T-100,T-70 and T-40) and found to be ~85,000 Da. Total neutral sugars were estimated as 88.5% by phenol-sulfuric acid method[12].

The paper chromatographic analysis [13] of the hydrolyzed product of this polysaccharide showed the presence of, glucose, galactose .and fucose. The GLC analysis of the alditol acetates of the sugars showed the presence of D-glucose, D-galactose and L-fucose. The absolute configuration of the sugar residues were determined by the method of Gerwig et.al.[14] and methylation studies[15], followed by GLC-MS analysis using HP-5 capillary column revealed the presence of 1,2,4,5-tetra-*O*-acetyl-3,6-di-*O*-methyl-D-glucitol,1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol and 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl-L-fucitol in a molar ratio of nearly 1:1:1 (Table 1). This result indicates that (1→2,4)-linked D-glucopyranosyl, terminal D-galactopyranosyl and (1→3)-linked L-fucopyranosyl moieties were present in the polysaccharide. A further linkage conformation was carried out by periodate-oxidation. The GLC analysis of the alditol acetate derived from the periodate oxidation and reduction, followed by themethylation of the polysaccharide showed the presence of1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl-L-fucitol. This result indicated that (1→2,4)-linked D-glucopyranosyl, and terminal D-galactopyranosyl moieties were consumed during oxidation.

2.2. NMR and structural analysis of polysaccharide

IR-Spectroscopic analysis revealed signals at 1143, 1078, 1043,917,880,813 and 778cm^{-1} indicating that the sugar residues of polysaccharide are in the pyranoseform.The^{1}H NMR (500 Hz) spectrum of the polysaccharide (Fig. 1) showed three peaks in the anomeric region at δ 5.21, δ 4.98, and δ 4.51 ppm in a ratio of nearly 1:1:1. The sugar residues were assigned as **A, B** and **C** according to their decreasing anomeric chemical shifts (Table 2). In the ^{13}C NMR (125 MHz) spectrum (Fig. 2, Table 2) three anomeric carbon signals appeared at δ 92.5, δ 99.9 and δ 103.0 ppm in a ratio of nearly 1:1:1. All the ^{1}H and ^{13}C signals were assigned using DQF-COSY, TOCSY, HMQC and HMBC (Fig. 4) NMR experiments.

Residue **A** was assigned as an L-fucopyranosyl unit. This is strongly supported by the appearance of a proton signal at δ 1.24 and a carbon signal atδ 16.2 for a CH_3 group. The appearance of the anomeric proton signal for residue **A** at δ 5.21 and the coupling constant value of $^3J_{1,2}$ (~ 3.75 Hz) clearly indicate that L-fucose is α-linked. This anomeric configuration was further confirmed by ^{1}H-^{13}C coupling constant $^1J_{H\text{-}1,\,C\text{-}1}$ value 171 Hz. The C-1 signal of residue **A** at 92.50 ppm was confirmed by the presence of cross peak **A**C-1, **C**H-4 in HMBC experiment (Fig. 4, Table 4). The carbon signals of

residue **A** were observed at δ 68.27, 80.50,69.36 and 68.75 for C-2, C-3,C-4 and C-5 respectively. The downfield shift of C-3 (δ 80.5) with respect to standard methyl glycosides [16] indicates that residue **A** is (1→3)-α-L-Fuc*p*

	Methylated sugar	T^a	T^b	Characteristics fragments(m/z)	Molar ratio	Mode of linkage
PS	3,6-Me_2-Glc*p*	4.40	3.73	43,59,74,87,99,**129**, 143,173,189,203,233	1	→2,4)- Glc*p*-(1→
	2,3,4,6-Me_4-Gal*p*	1.25	1.19	43,45,59,71,87,**101**, 117,129,145,161,205	1	Gal*p*-(1→
	2,4-Me_2-Fuc*p*	1.12	1.02	44,89,101,**117**,131,159, 173,201	1	→3)-Fuc*p* -(1→
IO_4^- PS	2,4-Me_2-Fuc*p*	1.12	1.02	44,89,101,**117**,131,159, 173,201		→3)- Fuc*p* -(1→

[a]Retention time with respect to that of . 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on a 3% ECNSSM column on a Gaschrom-Q at 170ºC.
[b]Retention time with respect to that of . 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on a 1% OV-225 column on a Gaschrom-Q at 170ºC.
[C]Equipped with a HP-5-fused-silica capillary column using a temperature program from 150ºC (2min) to 200ºC (5min) at 2ºC min^{-1}.

Table 1: GLC and GLC-MS data for the alditol acetates derived from the methylated PS andIO_4^- PS isolated from alkali extract of *V.diplasia.*

Sugar residue	H-1/ C-1	H-2/ C-2	H-3/ C-3	H-4/ C-4	H-5/ C-5	H-6a/ C-6	H-6b
→3)-α-L-Fuc*p*-(1→ **A**	5.21 92.50	3.55 68.27	3.32 80.50	3.86 69.36	4.20 68.75	1.24 16.20	
α-D-Gal*p*-(1→ **B**	4.98 99.90	3.83 69.20	3.57 70.70	4.29 70.30	4.01 71.60	3.86 61.30	3.82
→2,4)-β-D-Glc*p*-(1→ **C**	4.51 103.0	3.31 79.80	3.49 74.60	3.64 77.40	3.72 74.40	4.08 61.17	3.88

[a]The values of chemical shifts were recorded keeping HOD signal fixed at δ 4.74 ppm.
[b]Values of chemical shifts were recorded with reference to acetone as internal standard and fixed at δ 31.05 ppm. at 30 ºC.

Table 2: ^{1}H NMR[a] and ^{13}C NMR[b] chemical shifts of the polysaccharide isolated from alkali extract of *V.diplasia* recorded in D_2O at 30 ºC.

Residue **B** was assigned to Gal*p* as it showed a large coupling constant $J_{H-2,\ H-3}$ (~8.9Hz) and a relatively small coupling constant $J_{H-3,H-4}$ (~3.6 Hz). The anomericproton chemical shift for residue B at δ 4.98 (unresolved) and a carbon shift of δ 99.9 ($J_{H-1,\ C-1}$~170Hz) indicate that galactose is α-linked[17].The anomeric carbon chemical shift of moiety **B** at δ 99.9 was confirmed by the presence of cross peak **B**C-1, **C**H-2 in HMBC experiment (Fig. 4, Table 4). The carbon signals of residue **B** were observed at δ 69.2, 70.7, 70.3, 71.6 and 61.30 corresponding to C-2, C-3, C-4, C-5 and C-6 respectively.Thus considering the results of methylation analysis and NMR experiments, it may be concluded that **B** is anα-glycosidically linked, terminal D-galactose moiety.

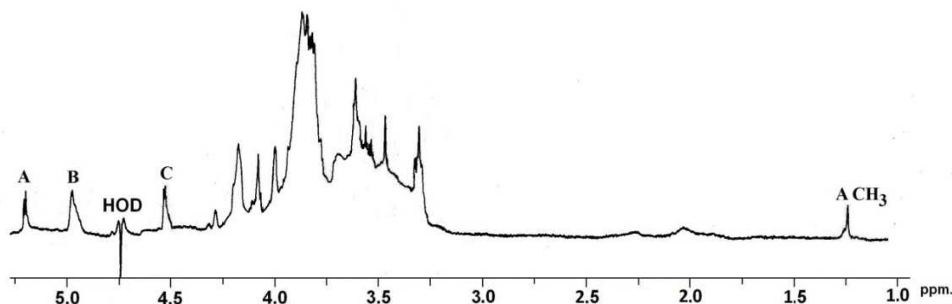
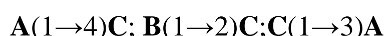


Figure 1:^{1}H NMR spectrum (500 MHz, D_2O, 30 °C) of polysaccharide, isolated fromalkali extract of*V. diplasia.*

The anomeric proton chemical shift for residue **C** is δ 4.51. A large coupling constant $J_{H-1,\ H-2}$ value (~8.29Hz) and $J_{H-1,\ C-1}$ value (161Hz) indicate that it is a β-linked residue. The $J_{H-2,\ H-3}$ value (~ 9.8Hz) and the $J_{H-3,\ H-4}$ (~10 Hz) were observed from the DQF-COSY spectrum for residue **C**, indicating that it is a D-glucosyl moiety. The anomeric carbon signal of residue **C** at δ 103.0 was confirmed by the presence of two cross-peaks **C**C-1, **A**H-3 in the HMBC experiment (Fig. 4, Table 4). The downfield shifts of C-2 (δ 79.8), and C-4 (δ 77.4) signals with respect to the standard value of methyl glycosides [16] are due to the effect of glycosylation. The carbon values of residue **C** (Table 2) indicate that it is a 1,2,4 linked D-glucose.

.The sequence of glycosyl residues of the polysaccharide was determined from NOESY (Fig.3, Table 3) as well as ROESY experiments followed by confirmation with an HMBC experiment

In NOESY experiment, the inter-residual contacts AH-1/ CH-4;BH-1/CH-2;CH-1/AH-3 along with other intra-residual contacts were also observed. thus from NOESY experiment the following sequences are established:



A(1→4)**C**; **B**(1→2)**C**;**C**(1→3)**A**

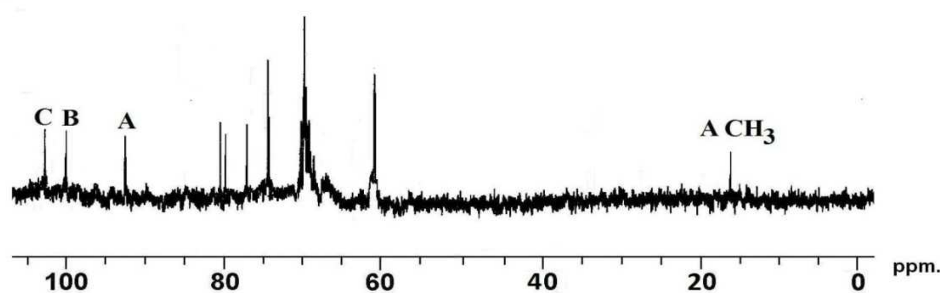


Figure 2: ^{13}C NMR (125 MHz, D_2O, 30°C) spectrum of polysaccharide, isolated fromalkali extract of*V. diplasia*

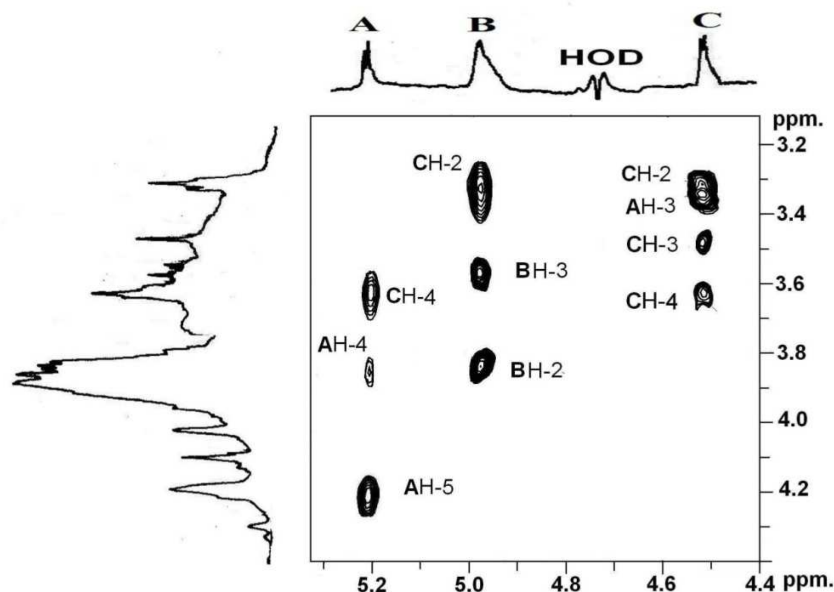
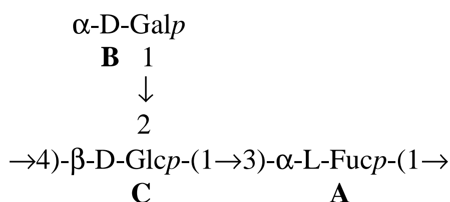


Figure 3: NOESY spectrum of polysaccharide, isolated fromalkali extract of *V. diplasia.*The NOESY mixing time was 300 ms.

The sequence was further confirmed by ^{13}C-^{1}H correlation in HMBC spectrum (Fig. 4, Table 4).Inter residual cross peaks AH-1/CC-4,AC-1/CH-4; BH-1/CC-2,BC-1/CH-2;CH-1/AC-3and CC-1/AH-3 along with other intra residual peaks were also observed.Therefore, based on the results obtained from monosaccharide composition,

methylation studies and NMR experiments the following repeating unit of the polysaccharide of *V. diplasia* was assigned as:



	Anomeric proton	NOE contact to proton	
Glycosyl residue	δ_H	δ_H	Residue, atom
→3)-α-L-Fuc*p*-(1→ **A**	5.21	3.64 3.86 4.20	**C**H-4 **A**H-4 **A**H-5
α-D-Gal*p*-(1→ **B**	4.98	3.31 3.83 3.57	**C**H-2 **B**H-2 **B**H-3
→2,4)-β-D-Glc*p*-(1→ **C**	4.51	3.32 3.31 3.49 3.64	**A**H-3 **C**H-2 **C**H-3 **C**H-4

Table 3: NOE data for the polysaccharide isolated from alkali extract of *V. diplasia*

		H-1/C-1	Observed connectivities		
Residue	Glycosyl linkage	δ_H /δ_C	δ_H /δ_C	Residue,	atom
A	→3)-α-L-Fuc*p*-(1→	5.21 92.50	77.4 3.64 1.24	**C** **C** **A**	C-4 H-4 H-6
B	α-D-Gal*p*-(1→	4.98 99.9	79.8 3.31	**C** **C**	C-2 H-2
C	→2,4)-β-D-Glc*p*-(1→	4.51 103.0	80.5 3.32	**A** **A**	C-3 H-3

Table 4: The significant $^3J_{H,C}$connectivities observed in an HMBC spectrum for the anomeric protons/carbons of the sugar residues of the polysaccharide isolated from alkali extract of *V. diplasia.*

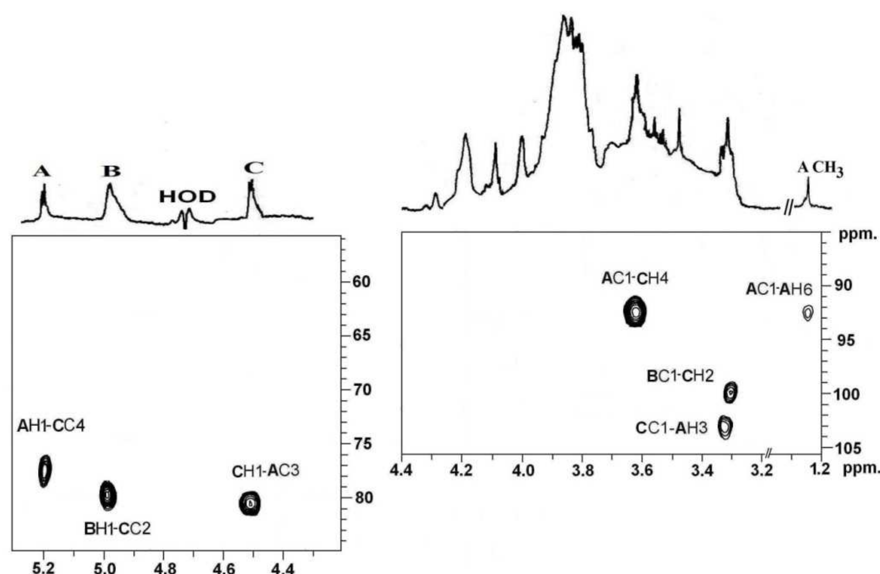


Figure 4: HMBC spectrum of polysaccharide, isolated fromalkali extract ofV. *diplasia.*The delay time in the HMBC experiment was 80 ms.

2.3. Immuno stimulating properties of the polysaccharide

Some biological studies were carried out with polysaccharide. Macrophage activation by polysaccharide was observed in vitro. On treating different concentrations of polysaccharide, an enhanced production of NO was observed in a dose dependent manner with optimum production of 19μM NO per 5x 10^5 macrophages at 100μg/mL(Fig.5).Hence, the effective dose of polysaccharide was observed at 100μg/mL.

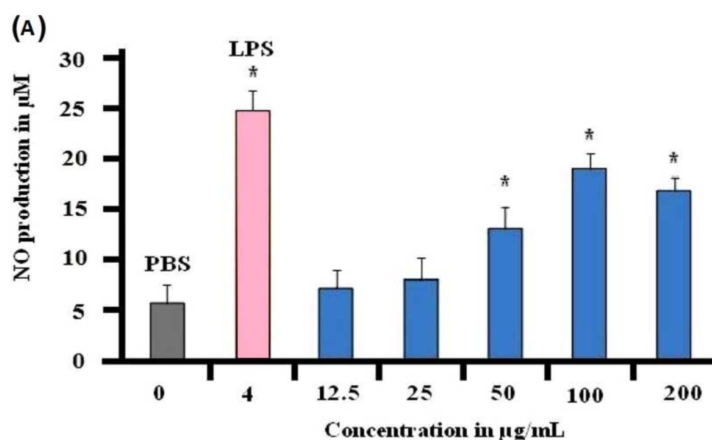


Figure 5: In vitro activation of peritoneal macrophage stimulated with different concentrations of the polysaccharide in term of NO production.

Splenocyte and thymocyte activation tests were carried out in mouse cell culture medium with polysaccharide by the MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide] method [18]Proliferation of splenocytes and thymocytes is an indicator of immune stimulation. Polysaccharide was found to stimulate splenocytes and thymocytes as shown in Figure 6B and 6C, respectively. The splenocytes proliferation index (SPI) and thymocytes proliferation index (TPI) as compared to phosphate stimulatory effect on the immune system. Maximum proliferation index of splenocytes and thymocytes was observed at 100 and 25µg/mL of the polysaccharide, respectively, as compared to other concentrations. Hence, 100µg/mL of polysaccharide can be considered as efficient splenocytestimulator whereas 25µg/mL of the polysaccharide acts asthymocyte stimulator.

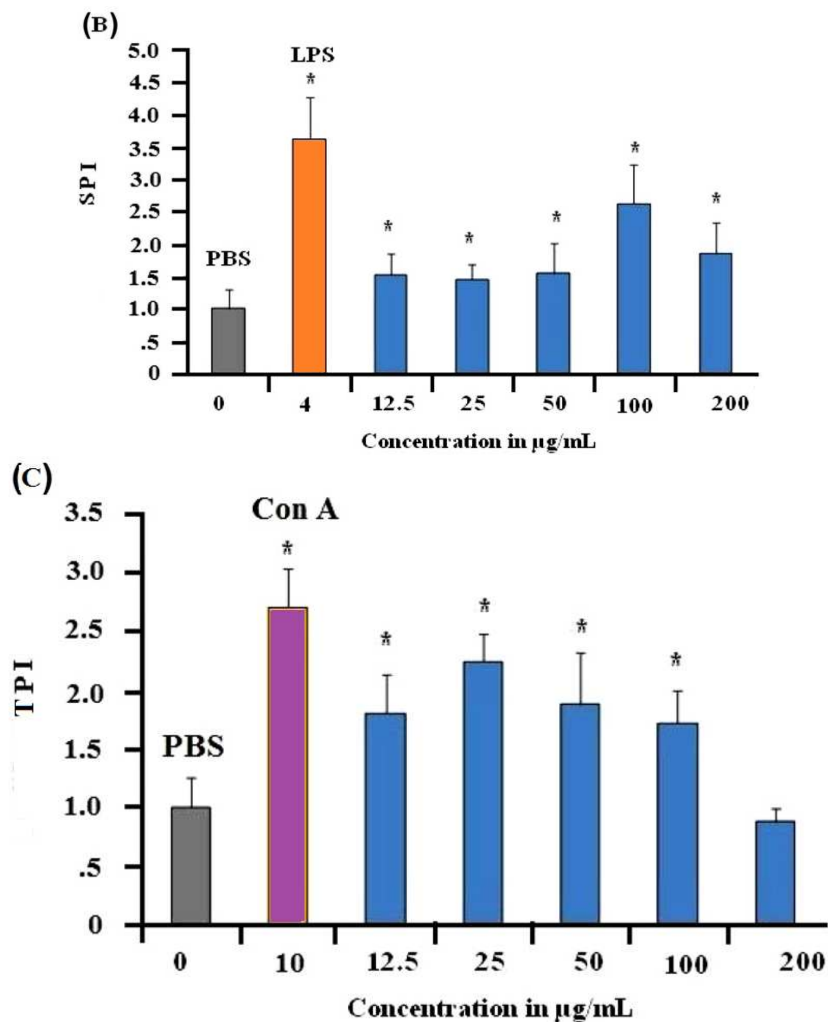


Figure 6: Effect of different concentrations of the polysaccharide on splenocyte **B** and thymocyte **C** proliferation.

3. Conclusion

A water soluble polysaccharide was isolated from alkali extract of mushroom, *V.diplasia*.The structure of this polysaccharide was elucidated on the basis of total hydrolysis, methylation analysis and 1D/2D NMR studies. Theseresult indicated that the repeating unit of the polysaccharide contained a backbone of one (1→3)-linked α-L-fucopyranosyl residue, one (1→2,4)-linked β-D-glucopyranosyl residue and one terminal α-D-galactopyranosyl residue. The polysaccharide activated the macrophages, splenocytes and thymocytes. Hence, on the basis of these activities it could be used as a natural immune enhancing material.

4.Materials and methods

4.1. Isolation and purification of the polysaccharide

The fresh fruiting bodies of *V. diplasia* (1.5 kg) were collected from the local forest, and the fruit body wasgently washed with water and then with distilled water. The mushroom bodies were pulverized for the extraction of polysaccharide by boiling with water for 6 h.The aqueous extract was kept overnight at 4ºC and then filtered through linen cloth. The filtrate was centrifuged at 8000 rpm at 4ºC for 30 min to obtain a clear solution, and then the polysaccharide was precipitated in 1:5 (v/v) EtOH. After keeping the precipitated material in the mixture overnight at 4ºC, it was centrifuged at 4ºC for 1 h, and then the residue was freeze-dried. The dried material was dissolved in 4% NaOH solution and reprecipitated in EtOH. The precipitated material was collected through centrifugation and dissolved in a minimum volume of water, and dialyzed through dialysis tubing of cellulose membrane (Sigma–Aldrich, retaining MW >12,400) against distilled water for 36 h to remove alkali and low-molecular-weight carbohydrate materials. The whole dialyzed solution was then centrifuged at8000 rpm at 4ºC. The water-soluble part was freeze dried, yielding 1.7 g of crude polysaccharide.

The crude polysaccharide (30 mg) was purified by gel permeation chromatography on column (90 × 2.1 cm) of Sepharose 6B in water as eluant (0.4 mL min^{-1}) using Redifrac fraction collector. 95 test tubes (2 mL each) were collected and monitored spectrophotometrically at 490 nm with phenol-sulfuric acid reagent [12] using Shimadzu UV-VIS spectrophotometer, model-1601. A single homogeneous fraction was collected and freeze-dried, yield-20 mg. The purification process was carried out in five lots and polysaccharide fraction was again purified and collected, yield-100 mg.

4.2. Monosaccharide analysis

The polysaccharide sample (3.0 mg) was hydrolyzed with 2M CF_3COOH (2 mL) in a round-bottom flask at100°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 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 MeOH 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 using column (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.Gas-liquid chromatography-

mass spectrometric (GLC-MS) analysis was also performed on Hewlett-Packard 5970A automatic GLC-MS system, using 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^{-1} up to a final temperature of 200°C. Quantitation was carried out from the peak area, using response factors from standard monosaccharides.

4.3. Methylation analysis

The Polysaccharide (4.0 mg) was methylated using Ciucanu and Kerek method[15].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 3 mL water for three times and dried. The methylated products were then hydrolyzed with 90 % formic acid (1mL) at 100 °C for 1 h, reduced with sodium borohydride, acetylated with (1:1) acetic anhydride-pyridine and analyzed by GLC (using columns A and B) and GLC-MS (using HP-5 fused silica capillary column) and the same temperature program indicated above.

4.4. Periodate oxidation study

The Polysaccharide (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 reduced material was divided into two portions. One portion was hydrolyzed with 2M CF_3COOH for 18 h and alditol acetate was prepared as usual. Another portion was methylated by Ciucanu andKerek[15] method and alditol acetate of this methylated product was prepared. Alditol acetates were analyzed by GLC using column A and B.

4.5.Optical rotation

Optical rotation was measured on a Perkin-Elmer model 241 MC spectropolarimeter at 25 °C. .

4.6. Absolute configuration of monosaccharides

The method used was based on Gerwiget.al[14]. The polysaccharide (1.0 mg) was hydrolyzed with CF_3COOH, and then the acid was removed. A solution of 250µL 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 TMSi-derivatives were prepared with N,O-bis (trimethylsilyl) trifluroacetamide (BSTFA). The products were analyzed by GLC using a capillary column SPB-1 (30m × 0.26mm), a temperature program (3 °C/min) from 150 to 210 °C. The 2,3,4,6-tetra-*O*-TMSi- (+)-2-butylglycosides obtained were identified by comparison with those prepared from the D and L enantiomers of different monosaccharides.

4.7. Paper chromatographic studies

Paper partition chromatographic studies were performed on whatmann nos.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[13].

4.8. Determination of molecular weight

The molecular weight of polysaccharide was determined by a gel– chromatographic technique. Standard dextrans[11]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 polysaccharide was then plotted in the same graph and molecular weight of polysaccharide was determined.

5. NMR studies

The polysaccharide was kept over P_2O_5 in vacuum for several days and then exchanged with deuterium [19]by lyophilizing with D_2O (99.96 % atom ^2H, Aldrich) for four times. With a BrukerAvance DPX-500 spectrometer,^1H, TOCSY, DQF-COSY, NOESY and HMBC NMR spectra were recorded in D_2O at 30ºC. The ^1H NMR spectrum was recorded by suppressing the HOD signal (fixed at δ 4.74) using the WEFT pulse sequence[20].The 2D-DQF-COSY experiment was carried out using standard Bruker software at 30 ºC. 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 ^{13}C NMR spectrum of polysaccharide, solution in D_2O was recorded at 30 ºC using acetone as internal standard, fixing the methyl carbon signal at δ 31.05 ppm. The delay time in the HMBC experiment was 80 ms.

6. Biological studies

6.1. Test for macrophage activity by nitric oxide assay

RAW 264.7 growing in Dulbecco's modified Eagle's medium(DMEM) was seeded in 96 well flat bottom tissue culture platesat 5 x 10^5 cells/mL concentration (180 µL)[21].Cells were left overnightfor attachment and treatment of different concentrations (12.5, 25, 50, 100 and 200µg /mL) of polysaccharide. After 48 h oftreatment, culture supernatant of each well was collected andNO content was estimated using Griess reagent (1:1 of 0.1% in1-napthylethylenediamine in 5% phosphoric acid and 1% sulfanilamide in 5% phosphoric acid) [22].Lipopolysaccharide (LPS), L6511of Salmonella enterica serotype typhimurium (sigma, St. Louis,USA) was used as positive control.

6.2. Splenocyte and thymocyte proliferation assay

A single cell suspension of the spleen and thymus were preparedfrom the normal mice under aseptic conditions by homogenizationin Hank's balanced salt solution (HBSS). The suspensionwas centrifuged to obtain cell pellet. The contaminating red blood cells (RBC) were removed by hemolytic Gey's solution. After washingtwo times in HBSS the cells were further suspended in completeRPMI (Roswell Park Memorial Institute) medium. Cellconcentration was adjusted to 1 x 10^6 cells/mL and the viability ofsplenocytes and thymocytes (as tested by trypan blue dyeexclusion) was always over 90%. The cells (180µL) were platedin 96-well flat-bottom tissue culture plates and incubated with20 µL of various concentrations of Polysaccharide (12.5, 25, 50, 100 and 200µg/mL). PBS (Phosphate Buffer Saline, 10 mM, pH-7.4) wastaken as negative control

whereas lipopolysaccharide (LPS, L6511of Salmonella enterica serotype typhimurium, Sigma, 4µg/mL)and Concanavalin A (Con A, 10 µg/mL) served as positive controls. All cultures were set up at 37ºC for 72 h in a humidified atmosphere
of 5% CO_2. Proliferation of splenocytes (SPI) and thymocytes(TPI) was checked by MTT assay method [18].The data are reported asthe mean ± standard deviation of seven different observations andcompared against PBS control [21,23].

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