Synopsis

The present thesis entitled "**Structural and biological studies of mushroom polysaccharides**" is mainly based on the determination of the structure as well as some important biological activities of different polysaccharides, isolated from the edible mushrooms *Termitomyces robustus* var. and *Ramaria botrytis*.

Chapter-1: It represents the introduction of carbohydrates, mushroom and its polysaccharides and some of their important biological activities. Carbohydrates are essential constituents of all living organisms having a variety of vital functions. These are generally classified into three groups: monosaccharides, oligosaccharides, and polysaccharides. The great bulk of the carbohydrates in nature are present as polysaccharides that are large and complex molecules. The polysaccharides serve two principal functions: (1) these are used by both plants and animals to store glucose as a source of future food energy, and (2) they provide some of the mechanical structure of cells.

Mushroom is a fleshy, aerial umbrella-shaped, fruiting body of macrofungi. Nutritionally they are of a valuable source of health food, which is low in fat and calories, and rich in carbohydrates, protein, fibre, important vitamins and minerals including iron, potassium, selenium and zinc. Mushrooms contain a wide variety of bioactive molecules including terpenoids, steroids, phenols, nucleotides, glycoproteins and polysaccharides. Among these, polysaccharide is the most potent mushroom derived material having various bioactive properties. Biological activities of mushroom polysaccharides included antitumor, immunomodulating, antioxidative, antiinflammatory, antiviral, antimicrobial, and hypoglycaemic effects but the most important aspects of mushroom polysaccharides are their immunomodulatory and antitumor effects. The main active components of the mushroom polysaccharides were proved to be the glucans, specifically β-D-glucans. β-D-glucans are important for their outstanding ability to enhance and stimulate the immune systems and are thus regarded as typical biological response modifiers (BRMs). They possess antitumor effect through host defence mechanism against tumor without side effect. Except for β-D-glucans several polysaccharides having different composition and structure also showed biological effects. Currently, several mushroom polysaccharides isolated from

Lentinus edodes, *Schizophyllum commune*, *Agaricus blazei*, *Grifola frondosa*, *Ganoderma lucidum* are widely used clinically as anti-tumor agents and many of them have been commercialized throughout the world. The biological activities of polysaccharides depend on the molecular structure, molecular weight, size, branching frequency, structural modification, conformation, and solubility. It is therefore very important to determine the exact structure and study of biological activity of the polysaccharides isolated from edible mushrooms.

Chapter-2: This chapter describes the methodologies of isolatation, purification and structure determination of the pure polysaccharides along with the study of their physical properties and biological activities. The crude polysaccharide was purified by gel-permeation chromatographic (GPC) technique using water as the eluant. The neutral sugars present in the polysaccharide were identified and estimated by Gas-liquid-chromatography (GLC). The total carbohydrate was estimated by the phenol-sulfuric acid method. The absolute configurations of sugars were identified using the method of Gerwig et al. The alditol acetates of partially methylated sugars derived from polysaccharide (using Ciucanu and Kerek Method) were identified by GLC-MS to know the mode of linkages present in the polysaccharide. Periodate oxidation study was carried out to confirm the mode of linkages of the sugar residues present in a polysaccharide. Chemical degradation (Smith degradation) was carried out to generate different oligosaccharides from the polysaccharide. Besides the above chemical methods, ^{1}H, ^{13}C, DEPT, DQF-COSY, TOCSY, NOESY, HSQC and HMBC NMR experiments were carried out to confirm the repeating unit present in the polysaccharide.

Biological investigations were also carried out with different polysaccharide fractions. The macrophage activation induced by the polysaccharides was studied by nitric oxide (NO) production using Griess reagent. The activation of splenocyte and thymocyte tests were carried out in mouse cell culture medium with polysaccharides by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method.

Chapter-3: This chapter illustrates the isolation and characterization of the immunostimulating β-glucans of an edible mushroom *Termitomyces robustus* var. Fresh fruit bodies of *T. robustus* var., collected from Vidyasagar University campus were washed

thoroughly with distilled water and then boiled with water for 6 h followed by centrifugation, precipitation in EtOH and freeze drying to yield 1.5 g crude polysaccharide. On treatment with 4% NaOH followed by centrifugation and dialysis the crude polysaccharide yielded two fractions, water soluble (PS-I) and insoluble (PS-II). PS-I was further purified by using gel permeation chromatography. PS-II was further dissolved in 4% NaOH solution and insoluble material was removed by centrifugation. The filtrate was dialyzed several times against distilled water followed by precipitation, centrifugation and freeze drying to yield pure PS-II. Structural studies of PS-I and PS-II were performed using acid hydrolysis, methylation analysis, periodate oxidation studies along with NMR analysis (^{1}H, ^{13}C, DEPT, TOCSY, DQF-COSY, NOESY, HSQC, and HMBC). Both polysaccharides (PS-I and PS-II) showed macrophage activation and splenocyte as well as thymocyte proliferation.

Analysis of PS-I: The pure polysaccharide showed a specific rotation of $[\alpha]_D^{25}$ -31.6 (*c* 0.6, water). The molecular weight of the polysaccharide was estimated from a calibration curve prepared with standard dextrans as ~ 1.82×10^5 Da. The paper chromatographic studies and the GLC analysis of the alditol acetates of the hydrolyzed product indicated the presence of glucose only. The absolute configuration of the monosaccharide was determined by the method of Gerwig et al, and it was found that glucose had D-configuration. The polysaccharide was methylated by the method of Ciucanu and Kerek, followed by hydrolysis and then alditol acetate preparation. The alditol acetates on analysis by GLC and GLC-MS was found to contain 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol. From these results the linkage of the constituent sugar was identified as (1→6)-linked D-Glc*p* in glucan. The FT-IR spectrum of the polysaccharide showed band at 900 cm^{-1} indicating β-configuration.

The ^{1}H NMR (500 MHz) spectrum and ^{13}C NMR (125 MHz) spectrum at 27 °C showed the anomeric signals at 4.50 ppm and 103.4 ppm respectively. All the proton signals were assigned from the correlation of DQF-COSY and TOCSY spectra. The coupling constant values, $^3J_{1,2}$ ~ 8.5 Hz and $^2J_{C\text{-}1,H\text{-}1}$ ~ 160 Hz clearly indicated that the sugar residue was β-linked. The large $^3J_{2,3}$ and $^3J_{3,4}$ coupling constant (~ 10 Hz) confirmed the glucopyranosyl configuration. With the help of HSQC all the carbon signals were assigned. The downfield shift of C-6 (69.2 ppm) with respect to the standard values of methyl glucoside indicated

that it was (1→6)-linked moiety, supported by the strong NOE contacts from H-1 to both H-6a and H-6b.

From the HMBC experiment the sequence of glucosyl residues in PS-I was confirmed. For explanation, two units of glucose (**A** & **A′**) were considered. The cross-peaks were found between **A** H-1/ **A′**C-6 as well as **A** C-1/**A′** H-6a; **A** C-1/ **A′** H-6b and vice-versa were also observed.

→6)-β-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→

A **A′**

On the basis of chemical and NMR studies the structure of the repeating unit present in the PS-I was established as:

→6)-β-D-Glc*p*-(1→

Analysis of PS-II: The analysis of alditol acetates of PS-II by PC and GLC showed the presence of only glucose. The absorption at 890 cm^{-1} in the FT-IR spectroscopy indicated that PS-II had β-glucopyranosidic linkages. The absolute configuration of glucose was determined as D according to method by Gerwig et al. The glucan was methylated according to the method of Ciucanu and Kerek. GLC and GLC-MS analyses of alditol acetates obtained from the hydrolyzate of the methylated polysaccharide revealed the presence of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol and 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol in a ratio of nearly 1:2:1 respectively. These results indicated the presence of terminal, (1→3), and (1→3,6)-linked D-glucopyranosyl moieties respectively. Further, GLC-MS analysis of the alditol acetates of the periodate-oxidised, reduced, methylated polysaccharide showed the presence of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol and 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol in a ratio of nearly 2:1. The absence of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol indicated that the terminal D-glucopyranosyl moiety was consumed during oxidation.

The ^{13}C NMR spectrum (125 MHz) of this water insoluble glucan (PS-II) at 30˚C in d_6-DMSO showed only one anomeric signal at 103.4 ppm. Three anomeric proton signals appearing at 4.22, 4.53, and 4.50 ppm in ^{1}H NMR (500 MHz) spectrum in d_6-DMSO at

30˚C were designated for residues **X**, **Y**, and **Z,** respectively. Resonances for anomeric protons at low and anomeric carbons at high frequency indicated that all D-glucosyl units in this glucan were β-linked which was further supported having $^{3}J_{1,2}$ ~ 6 Hz. All the proton chemical shifts were assigned using DQF-COSY, TOCSY, and HSQC NMR spectra. From the above experimental data the residues (**X**, **Y,** and **Z**) were found to have following linkages;

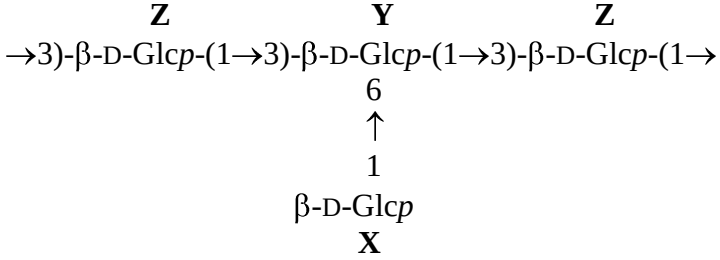
Residue **X**: terminal β-D-glucopyranosyl moiety

Residue **Y**: (1→3,6)-β-D-glucopyranosyl moiety

Residue **Z**: (1→3)-β-D-glucopyranosyl moiety

The C-6 linkage was further confirmed from DEPT experiment which showed the complete disappearance of the signal at 68.9 ppm but no appreciable downward displacement of the peak was observed in comparison to free $–CH_2$ of residues **X** and **Z**. The peaks appearing at 86.4, 86.8, and 87.5 ppm in ratio of nearly (1:1:1) corresponded to C-3 linkages of **Y** and two **Z** residues indicating that the main chain consisted of three units. Since, **Y** is the most rigid part of the backbone of the present glucan, its C-3 signal (86.4 ppm) appeared at the upfield region in comparison to the C-3 values of two **Z** residues. The C-3 value (87.5 ppm) of one **Z** residue attached glycosidically appeared 0.7 ppm downfield with respect to another unit (86.8 ppm) due to neighbouring effect with rigid part **Y.**

Based on all these chemical and NMR experimental results, the structure of the repeating unit of the polysaccharide was established as:



Both PS-I and PS-II were found to activate the macrophages. The stimulation of macrophages by PS-I and PS-II were studied by NO production in culture supernatant in vitro. Upon treatment with different concentrations of the PS-I and PS-II, there were an

enhanced production of NO in a dose dependent manner with optimum production of 17.04 μM and 13.1 μM NO per 5×10^5 macrophages at 80 μg/mL and 100 μg/mL, respectively.

Stimulation of splenocytes and thymocytes is an indicator of immunoactivation. The splenocyte and thymocyte activation tests were carried out in mouse cell culture medium with the polysaccharide by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method. Both splenocyte and thymocyte proliferation indices for PS-I and PS-II were maximum at 10 μg/mL as compared to other concentrations. Hence, both the glucans can be considered as efficient splenocyte and thymocyte stimulator at10 μg/mL.

This work has been published in ***Carbohydrate Research***, **2012**, *357*, 83-89. It is also highlighted in ***Nature India*** (doi:10.1038/nindia.2012.76; Published online 18 May 2012).

Chapter-4: This chapter deals with the structural studies of an immunoenhancing glucan of an ectomycorrhizal fungus *Ramaria botrytis*. The fresh fruit bodies of edible fungus, *Ramaria botrytis* (500 g) were boiled with distilled water. The supernatant was rejected, and the residue part was extracted with 4% NaOH followed by centrifugation, alcohol precipitation, dialysis yielded two fractions, water-soluble (240 mg) and water-insoluble (300 mg). The total sugar of the water-soluble crude polysaccharide was measured using phenol-sulfuric acid method and found to be 98.5%. The water-soluble crude polysaccharide (30 mg) on fractionation through a Sepharose 6B column yielded one homogeneous fraction. The apparent molecular weight of this polysaccharide ~1.3×10^5 Da, was estimated by gel permeation chromatography with reference to dextrans of known molecular weights. The molecular weight of the repeating unit of the polysaccharide is 826 Da. So, the degree of polymerization of this polysaccharide is 157. The structure of alkali treated, water soluble polysaccharide isolated from fruiting bodies of *Ramaria botrytis* was investigated using acid hydrolysis, methylation analysis, periodate oxidation, Smith degradation, partial hydrolysis study, and NMR studies (^{1}H, ^{13}C, DEPT, DQF-COSY, TOCSY, HSQC, and HMBC).

GLC analysis of the alditol acetates of this polysaccharide revealed the presence of glucose only. The polysaccharide showed a specific rotation of $[\alpha]_D^{25.7}$ -18.4 ° (*c* 0.24, water). The negative optical rotation indicated that the glucosyl residues had β-anomeric configuration. The absolute configuration of the monosaccharide present in the glucan was determined by the method of Gerwig et al, and it was found that glucose had D-

configuration. The polysaccharide was methylated according to the method of Ciucanu and Kerek, followed by hydrolysis and then alditol acetate preparation. GLC and GLC-MS analyses of alditol acetates obtained from the hydrolyzate of the methylated polysaccharide revealed the presence of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol, 1,5,6-tri-*O*-acetyl-2,3,4,-tri-*O*-methyl-D-glucitol, 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol in a molar ratio of nearly 1:1:2:1, respectively. These results indicate the presence of nonreducing end, (1→3)-, (1→6)-, and (1→3,6)-linked D-glucopyranosyl moieties in the glucan. According to this result, any of the three types of repeating unit is possible for this glucan: a (1→6)-linked backbone, a (1→3)-linked backbone or an alternatively (1→3)-, (1→6)-linked backbone. Therefore, periodate oxidation and mild hydrolysis were performed for determination of the backbone present in the polysaccharide repeating unit. The GLC analysis of the alditol acetates of the periodate-oxidised, reduced PS showed the presence of D-glucose only and periodate-oxidised, reduced, methylated PS showed the presence of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol, 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol in a molar ratio of nearly 1:1. Mild hydrolysis was carried out with the periodate-oxidised, reduced PS to get Smith degradation product (SDPS). The GLC analysis of the alditol acetates of Smith degraded hydrolyzed products showed the presence of D-glucose and D-glycerol. The GLC-MS analysis of the methylated SDPS revealed the presence of 1,5-di-*O*-acetyl 2,3,4,6-tetra-*O*-methyl D-glucitol and 1,3,5-tri-*O*-acetyl 2,4,6-tri-*O*-methyl D-glucitol in a molar ratio of 1:1. Partial hydrolysis of the glucan was carried out with 0.1 M TFA to know the sequence of β-D-glucosyl moieties in the repeating unit. As a result of this hydrolysis, two fractions were obtained; partially hydrolysed oligosaccharide (F1) and partially hydrolysed polysaccharide (F2). The oligosaccharide (F1) was reduced by $NaBH_4$ and subjected to methylation analysis. Methylation analysis showed the presence of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol and 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol in a molar ratio of 1:1, indicating the presence of a nonreducing end and a (1→3)-linked-D-glucopyranosyl moiety attached to the backbone. Methylation analysis of F2 revealed the presence of 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol only. This result clearly indicated that the backbone chain F2 of the glucan consists of (1→6)-D-glucopyranosyl moieties, branched with the oligomeric unit F1.

The ^{1}H NMR (500 MHz) spectrum at 30 ˚C showed four anomeric proton signals at 4.68, 4.47, 4.45, and 4.44 ppm which were designated as **A**, **B**, **C**, and **D** residues according to their decreasing chemical shifts. The ^{13}C NMR (125 MHz) spectrum and the DEPT-135 spectrum at the same temperature showed the presence of four anomeric carbon signals at 103.0, 102.8, 102.7, and 102.5 ppm which corresponded to the **C**, **D**, **A**, and **B** residues respectively as revealed from HSQC spectrum. All the ^{1}H and ^{13}C signals were assigned on the basis of correlation of TOCSY, DQF-COSY, and HSQC NMR spectra. The proton-proton coupling constant values were measured from DQF-COSY experiment. The large $J_{H\text{-}2,H\text{-}3}$ and $J_{H\text{-}3,H\text{-}4}$ coupling constant values (~10 Hz) of all residues from **A** to **D** supported the presence of glucopyranosyl configuration in the polysaccharide. All the residues (**A**-**D**) with anomeric proton and carbon signals ranging from 4.68-4.44 ppm and 103.0-102.5 ppm respectively revealed that sugar residues were present in β-configuration. The β-anomeric configuration of all residues was further supported from the coupling constant values, $J_{H\text{-}1,H\text{-}2}$ ~8 Hz and $J_{C\text{-}1,H\text{-}1}$ ~161 Hz. The downfield shifts of C-3 (84.2 ppm) of both residues **A** and **B** and C-6 (68.2 ppm) of residue **B** with respect to standard value of methyl glycosides indicated that residues **A** and **B** were (1→3)- and (1→3,6)-linked β-D-Glc*p* respectively. The downfield shift of C-6 (68.9 ppm) of residues **C** supported that they were (1→6)-linked β-D-Glc*p*. All the carbon chemical shifts of residue **D** were nearly close to the standard values of methyl glycoside of β-D-Glc*p*. This observation clearly indicated that the residue **D** was non-reducing end β-D-Glc*p*.

From the above experimental data the residues (**A**-**D**) were found to have following linkages;

Residue **A**: (1→3)-β-D-glucopyranosyl moiety
Residue **B**: (1→3,6)-β-D-glucopyranosyl moiety
Residue **C**: (1→6)-β-D-glucopyranosyl moiety
Residue **D**: terminal β-D-glucopyranosyl moiety

From the HMBC experiment, the cross peaks of both the anomeric proton and carbon of each of the sugar moieties were examined and inter- and intra-residual connectivities were observed. Inter residual cross-peaks **A**H-1/**B**C-3; **A**C-1/**B**H-3; **B**H-1/**C**C-6; **B**C-1/**C**H-6a, **C**H-6b; **C**H-1/**B**C-6; **C**C-1/**B**H-6a, **B**H-6b; **D**H-1/**A**C-3; **D**C-1/**A**H-3 along with other intra-residual peaks were observed.

For confirming the linkages, an NMR experiment was carried out with Smith degraded product (SDPS). The ^{13}C NMR (125 MHz) spectrum at 30 ˚C of SDPS showed two anomeric carbon signals at 102.7 and 102.8 ppm corresponding to β-D-Glc*p*-(1→ (**E**) and →3)-β-D-Glc*p*-(1→ (**F**) residues respectively. The carbon signals C-1, C-2, and C-3 of the glycerol moiety were assigned as 68.5, 72.1, and 62.5 ppm respectively. The nonreducing β-D-Glc*p*-(1→ unit (**E**) was produced during Smith degradation and was generated from (1→3)-β-D-Glc*p* (**A**) due to complete oxidation of the β-D-Glc*p*-(1→ residue (**D**) and the (1→3)-β-D-Glc*p* (**F**) was produced from the (1→3,6)-β-D-Glc*p* (**B**) residue due to oxidation followed by Smith degradation of the (1→6)-β-D-Glc*p* moiety (**C**). The glycerol (**G**) moiety was generated from the (1→6)-β-D-Glc*p* moiety (**C**) which was linked glycosidically with the rigid part (**B**) and be attached to the (1→3)-β-D-Glc*p* moiety (**F**).

E **F** **G**
β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→3)-Gro

Hence, from chemical investigations and NMR spectral analysis of the native, Smith-degraded and partial hydrolyzed product, the repeating unit of the glucan was established as:

C **B** **C**
→6)-β-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→
3
↑
1
β-D-Glc*p*-(1→3)-β-D-Glc*p*
D **A**

Macrophage activation by this glucan has been studied by nitric oxide (NO) production in culture supernatant in vitro. Upon treatment with different concentrations of glucan, an enhanced production of NO was observed with optimum production of 25.4 µM NO per 5 × 10^5 macrophages at 100 µg/mL and then decreases. Hence 100 µg/mL was the effective dose of glucan for NO production.

The activation of splenocyte and thymocyte tests were carried out in mouse cell culture medium with glucan by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method. Both splenocyte and thymocyte proliferation indices were found to be

maximum at 50 μg/mL of the glucan as compared to other concentrations. Hence, 50 μg/mL of the glucan can be considered as the optimum concentration for splenocyte and thymocyte proliferation. From the above observations it was clear that this glucan can act as efficient immunostimulating agent.

This work has been published in ***Carbohydrate Research***, **2013**, *374*, 59-66. It is also highlighted in ***Nature India*** (doi:10.1038/nindia.2013.65; Published online 21 May 2013).

Chapter-5: It includes the structural investigation of water-insoluble glucans from the edible fungus *Ramaria botrytis.* This edible fungus, *Ramaria botrytis* were collected from Darjeeling, Himalaya hill region. The fresh fruit bodies of edible mushroom, *Ramaria botrytis* (500 g) were boiled with distilled water. The supernatant was rejected, and the residue part was extracted with 4% NaOH followed by centrifugation, alcohol precipitation, dialysis, and freeze drying to yield two parts, water soluble and insoluble. The water insoluble crude polysaccharide was then extracted with 5% NaOH containing 0.05% $NaBH_4$ for 20 h at room temperature. The extract was neutralized by mixing 1M AcOH. Two fractions (PS-I and PS-II) were collected and lyophilized. Now, the structures of the PS-I and PS-II were analyzed in the following way.

Analysis of PS-I: PS-I on acid hydrolysis by 2 M CF_3 COOH showed the presence of glucose only, which was detected by paper chromatography (PC) as well as by GLC analysis. The absolute configurations of the sugar unit present in the PS-I was determined by the method of Gerwig et al, and it was found that glucose had D-configuration. The mode of linkage of PS-I was determined by methylation analysis using method of Ciucanu and Kerek followed by hydrolysis and alditol acetates preparation. The GLC-MS of alditol acetates of the partially methylated product showed the presence of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol. This result indicated the presence of (1→3)-linked D-glucopyranosyl moiety.

The ^{1}H NMR spectrum (500 MHz) recorded in DMSO-d_6 at 30˚C showed one signal in the anomeric region at 5.07 ppm. This indicated that the PS-1 was composed of only one monosaccharide as repeating unit, supported by the appearance of one signal in the anomeric region at 100.3 ppm in ^{13}C NMR (125 MHz) spectrum. The coupling constant values $J_{H\text{-}1,H\text{-}2}$ (~3 Hz) and $J_{C\text{-}1,\ H\text{-}1}$ (~170 Hz), in addition to their anomeric proton and carbon signals at 5.07 ppm and 100.3 ppm respectively indicated that the sugar residue was

a α-linked. All the ^{1}H and ^{13}C signals were assigned using 2D ^{1}H NMR (DQF-COSY, TOCSY) and 2D ^{1}H-^{13}C NMR (HSQC) experiments. The signal at 83.3 ppm was assigned to C-3 of a (1→3)-α-D-glucosyl residue. The HSQC spectrum correlated with seven protons and six carbon signals. The PS-1 was dissolved in DMSO-d_6 prior to the NMR analysis. Thus, ^{1}H NMR spectrum contained hydroxyl proton signals. The resonances of hydroxyl groups did not generate cross-peaks in the HSQC spectrum.

From the above experimental evidences, it was concluded that PS-I was a glucan composed of a (1→3)-linked α-D-glucopyranosyl repeating unit as:

→3)-α-D-Glc*p*-(1→

Analysis of PS-II: Acid hydrolysis, followed by GLC analysis and paper chromatographic study, showed that the PS-II was pure glucan. The absolute configuration of monosaccharide units was determined as D according to the method by Gerwig et al. The mode of the linkages of sugar residues of PS-II was determined by methylation analysis using the method of Ciucanu and Kerek followed by formolysis and alditol acetates preparation. GLC and GLC-MS analyses of alditol acetates obtained from the hydrolysis of the methylated polysaccharide revealed the presence of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol; 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol; and 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol in a ratio of nearly 1:3:1. The GLC analysis of the alditol acetates of the periodate-oxidized reduced polysaccharide was found to contain only glucose, and periodate-oxidized reduced methylated polysaccharide showed the presence of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol and 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol in a ratio of nearly 3:1. The absence of 1, 5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol indicated that the terminal D-glucopyranosyl moiety was consumed during oxidation.

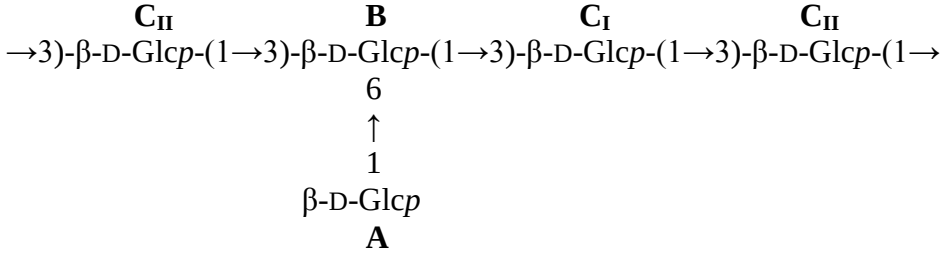
The ^{1}H NMR spectrum (500 MHz) of the water-insoluble glucan at 30 °C in DMSO-d_6 showed two signals at anomeric region. The ^{13}C NMR spectrum (125 MHz) of the following glucan at 30 °C in DMSO-d_6 showed only one anomeric signal at 103.4 ppm. Resonance for anomeric protons at low and anomeric carbon at high frequency indicated that all D-glucosyl units in this glucan were β-linked. Coupling constant values $J_{H\text{-}1,\ H\text{-}2}$ ~8 Hz, and $J_{C\text{-}1,\ H\text{-}1}$ ~161 Hz also supported the β-linkage. All the proton chemical shifts were

assigned using DQF-COSY, TOCSY, and HSQC experiments. The HSQC spectrum contained C1/H1 cross-peaks at 103.4/4.20 ppm was assigned to the nonreducing end unit **A** and other C-1/H-1 cross-peaks at 103.4/4.52 ppm were designated as **B**, and **C** and assigned to (1→3,6)- and (1→3)-β-D-Glc*p* respectively.

Residue **A**: terminal β-glucopyranosyl moiety
Residue **B**: (1→3,6)-β-D-glucopyranosyl moiety
Residue **C**: (1→3)-β-D-glucopyranosyl moiety

The C-6 linkage was further confirmed from DEPT-135 experiment which showed the complete disappearance of the signal at 68.8 ppm but no appreciable downward displacement of the peak was observed in comparison with free C-6 of residues **A** and **C**. The peaks appearing at 86.4, 86.8, and 87.5 ppm in ratio of nearly (1:2:1) corresponded to C-3 linkages of **B**, two $\mathbf{C_{II}}$, and one $\mathbf{C_I}$ residues respectively indicating that the main chain consisted of four units. Since, B is the most rigid part of the backbone of the present glucan, its C-3 signal (86.4 ppm) appeared at the upfield region in comparison with the C-3 values of three **C** residues. The C-3 value (87.4 ppm) of $\mathbf{C_I}$ residue attached glycosidically appeared 0.4 ppm downfield with respect to the two $\mathbf{C_{II}}$ residues (86.8 ppm) due to the neighbouring effect of rigid part **B**.

Finally, the structure of the repeating unit of the polysaccharide was established as:



This work has been communicated.