BIOACTIVE POLYSACCHARIDES FROM EDIBLE MUSHROOMS

A SYNOPSIS

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BY

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SYNOPSIS

The present thesis entitled "**Bioactive Polysaccharides from Edible Mushrooms**" is divided into five chapters:

Chapter-I: It describes the general introduction to carbohydrates, polysaccharides including mushroom polysaccharides, and their biological activities.

Chapter-II represents the experimental methods, which were carried out during the thesis work.

Chapter-III: This chapter is one of the major parts of this thesis, which describes the isolation, purification, structural characterization, biological and antioxidant properties of two polysaccharides isolated from alkaline extract of an edible mushroom *Entoloma lividoalbum*.

These works have been published in

(i) ***International Journal of Biological macromolecules,* 2014**, *63*, 140-149

(ii) ***Carbohydrate Polymers,* 2014,** *114*, 157-165.

Chapter-IV: This chapter contains the structural, immunological, and antioxidant studies of β-glucan isolated from an edible mushroom *Entoloma lividoalbum.*

This work has been published in ***Carbohydrate Polymers,* 2015***,123*, 350-358.

Chapter-V: This chapter represents the isolation, purification, structural characterization and study of immunoactivation of a partially methylated mannogalactan isolated from hybrid mushroom *pfle 1p*.

This work has been published in ***Carbohydrate Research,* 2014**, 395, 1-8.

Chapter-I:

It discusses the introduction of carbohydrates, mushroom, mushroom polysaccharides, and some of their important biological activities. Carbohydrates are essential constituents of all living organisms and have a variety of vital functions. It is the abundant source of dietary fiber and also serves as storage food for supply of energy. Based on number of sugar units present these are classified into four classes: monosaccharides, disaccharides, oligosaccharides, and polysaccharides. A great majority of carbohydrates of nature occur as polysaccharides. Polysaccharides are macromolecules consisting of a large number of monosaccharide units. Now-a-days mushrooms are popularly used as food flavouring substances due to their unique taste, flavor, high nutritive value (proteins, chitin, vitamins and minerals) and medicinal values. They contain a wide range of bioactive molecules including steroids, terpenoids, phenols, neucleotides, glycoproteins, and polysaccharides. Mushroom polysaccharides have drawn the attention of chemists and immunobiologists due to their immunomodulatory, antitumor, antimicrobial, antiflammatory, antiviral, antithrombotic, and antioxidant activities [1]. Several mushroom polysaccharides such as lentinan, PSK, and sonifilan are clinically used as antitumor drugs throughout the world. Among the mushroom polysaccharides, β-D-glucan are most important polysaccharides with immunomodulating and antitumor activity and are known as biological response modifiers (BRM). The biological activities of polysaccharides depend on the molecular structure, molecular weight, size, branching frequency, structural modification, conformation, and solubility. Reactive oxygen species (ROS) are formed continuously as normal by-products of oxygen metabolism process. They play a major role in the development of several human diseases, such as cerebral ischemia, diabetes, Alzheimer, inflammation, rheumatoid arthritis, atherosclerosis and cancer [2]. The antioxidant polysaccharides present in mushrooms are of great interest as potential protective agents against oxidative damage. It is therefore important to determine the exact structure and biological activity of the polysaccharides isolated from mushrooms.

Chapter-II:

This chapter describes the methodologies of isolation, purification and determination of the structure of pure polysaccharides and also their study of specific biological and antioxidant properties. The biological activities of polysaccharides depend on the molecular weight, linking sequences of the monosaccharide residues, and branching pattern. So, it is very important to determine the exact structure of the repeating unit of the polysaccharides isolated from mushroom. Isolation of the pure polysaccharide is the first step for the determination of the structure of polysaccharides. The crude polysaccharide was purified by gel-permeation chromatography (GPC) technique using water as the eluent. The exact structure of the polysaccharides is determined using two types of methods: (1) chemical method that includes total acid hydrolysis, methylation, periodate oxidation and smith degradation studies (2) spectroscopic method comprising of 1D (^{1}H, ^{13}C, DEPT-135) and 2D NMR (DQF-COSY, TOCSY, NOESY, ROESY, HSQC, HMBC).

Different biological studies were also carried out with the polysaccharide fractions. The macrophages 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 polysaccharide by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method. Lipid peroxidation (MDA), the ratio of oxidized glutathione (GSSG) and reduced glutathione (GSH) levels were carried out in normal human lymphocytes. The antioxidant activity of polysaccharide was evaluated through the determination of reducing power, determination of total antioxidant capacity, hydroxyl and superoxide radical scavenging activity. The scavenging activity of the polysaccharide is calculated by the following equation:

$$\text{Scavenging activity (\%)} = \{(A_0-A_1)/ A_0\} \times 100$$

Where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of sample.

Chapter III:

This chapter describes the isolation, purification, structural characterization, antioxidant and biological properties of two polysaccharides isolated from the edible mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička. Alkaline extract of fruit bodies of the edible mushroom *Entoloma lividoalbum* yielded two water soluble polysaccharides, PS-I and PS-II. The molecular weight of PS-I and PS-II were estimated as ~1.94 × 10^5 Da and ~1.48×10^5 Da respectively, from a calibration curve prepared using standard dextran.

Structural analysis of PS-I [3]

GLC analysis of the alditol acetates of PS-I revealed the presence of glucose only. Determination of absolute configuration of the monosaccharide showed that glucose was in D configuration. The GLC-MS analysis of the alditol acetates of methylated product showed 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, and 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol in a molar ratio of approximately 1:3:2:1. These results showed that the (1→6)-D-glucopyranosyl and terminal D-glucopyranosyl residues were consumed during oxidation whereas, (1→3,6)-D-glucopyranosyl and (1→3)-D-glucopyranosyl residues remain unaffected, further confirming the mode of linkages present in PS-I.

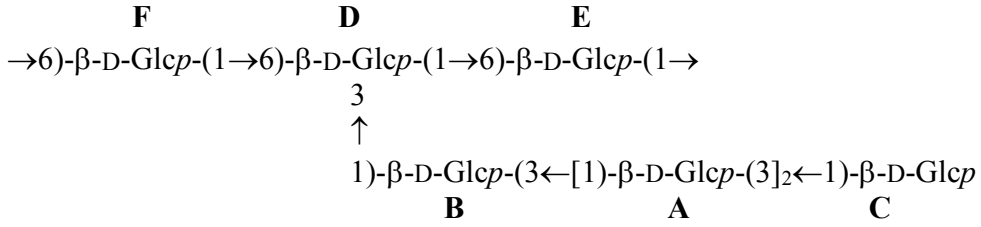
In the ^{1}H NMR spectrum (500 MHz) of PS-I, four anomeric signals at δ 4.77, 4.73, 4.50, and 4.49 were observed at 30 °C in a ratio of nearly 2:1:1:3. The peak at δ 4.77, 4.73, and 4.50 designated as **A**, **B**, and **D**, whereas the peak at δ 4.49 consists of **C**, **E**, and **F** residues. In ^{13}C NMR spectrum (125 MHz) at the same temperature, five signals were observed in the anomeric region at δ 103.0, 102.9, 102.8, 102.7, and 102.6. On the basis of HSQC spectrum, the anomeric carbon signal at δ 102.9 was correlated to both the proton signals δ 4.77 (**A**) and δ 4.73 (**B**) respectively. Again, the anomeric proton signal at δ 4.50 was correlated to the carbon signal at δ 102.6 (**D**) whereas the peak at δ 4.49 was correlated to the anomeric carbon signals at δ 103.0 (**C**), δ 102.8 (**E**), and δ 102.7 (**F**).

The sequence of glycosyl residues (**A** to **F**) were determined from NOESY as well as ROESY (not shown) studies. In NOESY experiment, the inter-residual contacts **A**H-

1/**B**H-3; **B**H-1/**D**H-3; **C**H-1/**A**H-3; **D**H-1/**E**H-6a, 6b; **E**H-1/**F**H-6a, 6b; and **F**H-1/**D**H-6a, 6b along with other intra-residual contacts were also observed. The above NOESY connectivities established the following sequences: **F** (1→6) **D**; **D** (1→6) **E**; **E** (1→6) **F**; **B** (1→3) **D**; **A** (1→3) **B**; and **C** (1→3) **A**.

Finally, these NOESY connectivities were confirmed from HMBC spectrum. In this spectrum the inter-residual cross-peaks between **A**H-1/**B**C-3, **A**C-1/**B**H-3; **B**H-1/**D**C-3, **B**C-1/**D**H-3; **C**H-1/**A**C-3, **C**C-1/**A**H-3; **D**H-1/**E**C-6, **D**C-1/**E**H-6a, 6b; **E**H-1/**F**C-6, **E**C-1/**F**H-6a, 6b; **F**H-1/**D**C-6, **F**C-1/**D**H-6a, 6b along with some intra-residual peaks were also observed.

Hence NOESY and HMBC connectivities confirmed the structure of repeating unit presence in the PS-I as:



NMR experiments were again carried out with Smith degradation product (SDPS) of the PS-I for further confirming the linkages. Smith degradation results in the formation of a glycerol containing tetrasaccharide from the parent polysaccharide and the structure of SDPS were established as:

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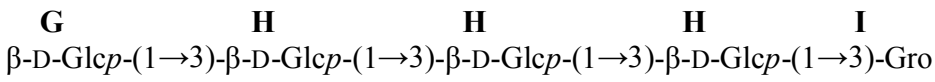


So, all these results indicated that the β-D-glucan (PS-I) isolated from an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička, is a branched glucan with (1→6)-linked backbone where branching occurred at *O*-3 of one unit followed by (1→3)-β-D-Glc*p* and terminal β-D-Glc*p*.

Structural analysis of PS-II [4]

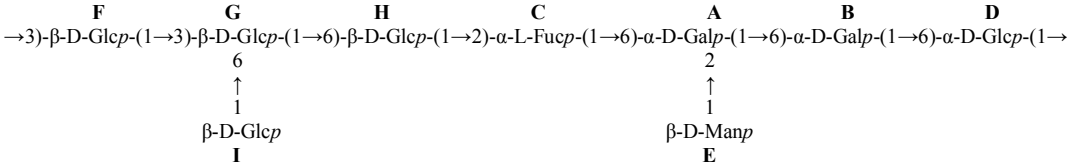
GLC analysis of the alditol acetates of the hydrolyzed product of PS-II revealed the presence of glucose, mannose, galactose, and fucose in a molar ratio of nearly 5:1:2:1. The absolute configuration of the monosaccharide was determined by the method of Gerwig, Kamerling, and Vliegenthart, (1978) and found that glucose, galactose, and mannose had the D configuration but fucose was present in the L configuration. The GLC-MS analysis of the alditol acetates of methylated product showed the presence of terminal mannopyranosyl, terminal glucopyranosyl, (1→2)-fucopyranosyl, (1→3)-glucopyranosyl, (1→6)-glucopyranosyl, (1→3,6)- glucopyranosyl, (1→6)-galactopyranosyl, and (1→2,6)-linked galactopyranosyl in a molar ratio of approximately 1:1:1:1:2:1:1:1. GLC analysis of alditol acetates of the periodate-oxidized, $NaBH_4$-reduced, and hydrolyzed products showed the presence of only glucose, indicating that the D-galactose, D-mannose, and L-fucose moieties were consumed during oxidation. GLC-MS analysis of periodate-oxidized, reduced, methylated PS-II 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 molar ratio of nearly 1:1. These results clearly indicated that the (1→3)-linked and (1→3,6)-linked glucopyranosyl residues remain unaffected whereas all other residues were consumed during oxidation, which further confirmed the mode of linkages present in the PS-II.

The ^{1}H NMR spectrum (500 MHz) of PS-II at 30 °C showed the presence of nine signals in the anomeric region at δ 5.11, 5.04, 5.03, 4.99, 4.77, 4.73, 4.49, 4.48, and 4.47. The sugar residues were designated as **A**, **B**, **C**, **D**, **E**, **F**, **G**, **H**, and **I** according to their decreasing anomeric proton chemical shifts. In ^{13}C NMR spectrum (125 MHz) at the same temperature, seven signals were observed in the anomeric region at δ 103.0, 102.9, 102.7, 102.5, 101.7, 101.6, and 98.3. From the HSQC spectrum, the anomeric carbon signals at δ 103.0, 102.9, 102.7, 102.5, 101.7 and 101.6 were correlated to the anomeric proton signals δ 4.47 (**I**), δ 4.73 (**F**), δ 4.48 (**H**), δ 4.49 (**G**), δ 4.77 (**E**), and δ 5.04 (**B**) respectively. Whereas, the anomeric carbon signal at δ 98.3 was correlated to the anomeric proton signals at δ 5.11 (**A**), δ 5.03 (**C**), and δ 4.99 (**D**).

The sequence of glycosyl residues (**A** to **I**) were determined from ROESY as well as NOESY. In ROESY experiment, the inter-residual contacts **A**H-1/**B**H-6a, 6b; **B**H-1/**D**H-

6a, 6b; **C**H-1/**A**H-6a, 6b; **D**H-1/**F**H-3; **E**H-1/**A**H-2; **F**H-1/**G**H-3; **G**H-1/**H**H-6a, 6b; **H**H-1/**C**H-2; and **I**H-1/**G**H-6a, 6b along with other intra-residual contacts were also observed. The above ROESY connectivities established the following sequences: **A** (1→6) **B; B** (1→6) **D; C** (1→6) **A**; **D** (1→3) **F; E** (1→2) **A; F** (1→3) **G**; **G** (1→6) **H**; **H** (1→2) **C**; and **I** (1→6) **G**. These data clearly indicated the positions of substitution and sequence of sugar residues in the polysaccharide.

Hence, the structure of repeating unit in the PS-II was proposed as:



Finally, Smith degraded material (SDPS) from PS-II was prepared to confirm the linkages of the heteroglycan. Hence, Smith degradation results in the formation of a glycerol containing disaccharide from the parent polysaccharide and the structure of which was established as:

J **K** **L**
β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→3)-Gro

This result further confirmed the repeating unit present in the heteroglycan isolated from the edible mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička.

Antioxidant properties of PS-I

The hydroxyl radical scavenging activity of PS-I gradually increase with the increase of concentration. The hydroxyl radical scavenging rate of PS-I and BHT at 200 μg/mL were found to be 16.6 % and 91.2 % respectively, indicating that PS-I has a moderate antioxidant activity and the activity of PS-I is weak compared with that of BHT. The EC_{50} value of the PS-I was found to be 480 μg/mL. The hydroxyl radical scavenging activity might be explained by hydrogen atom donation ability of the hydroxyl group in PS-I to terminate the free radical mediated oxidative chain reactions.

The scavenging effect of PS-I on superoxide radicals was found concentration-dependent. The percentage inhibition of superoxide generation by 200 µg/mL concentration of PS-I was found to be 64.47 % whereas of BHA was found as 91.01 %. They both showed a concentration dependent scavenging of superoxide radicals and the percentage inhibition of PS-I is close to that of synthetic standard drug BHA at a concentration of 400 µg/mL. The EC_{50} value of the PS-I was found to be 150 µg/mL.

The reducing power of PS-I was concentration-dependent. In this assay, reducing power of PS-I and ascorbic acid increased with increasing sample concentration. At 200 µg/mL, the reducing powers were 0.2765 and 1.16 for PS-I and ascorbic acid respectively. At 600 µg/mL, the reducing powers were 0.58 and 1.27 for PS-I and ascorbic acid respectively. At concentration of 480 µg/mL, PS-I showed reducing power 0.5. This result suggests that PS-I is a good electron donor and may terminate the radical chain reaction by converting free radicals to more stable product.

PS-I inhibited the phosphomolybdenum in the total antioxidant capacity assay. Result showed 1 mg of PS-I is equivalent to 70 ± 15 µg of ascorbic acid. Total antioxidant activity suggests that the electron donating capacity of PS-I and thus it may act as radical chain terminator, by transforming reactive free radicals into more stable non reactive products.

Biological activities of PS-II

The cell viability using PS-II was studied on human lymphocytes with increasing concentrations of PS-II ranging from 50 µg/ml to 400 µg/ml using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT) method. It was observed that the cytotoxicity to normal lymphocytes by PS-II was insignificant. Cell proliferative activity was observed at 50 µg/ml of PS-II with respect to control. These results showed a lower level of cytotoxicity when lymphocytes were treated with PS-II up to 200 µg/ml but even at higher dose 400µg/ml, the polysaccharide showed mild toxicity. Cell culture experiments were carried out and statistical calculations showed the IC50 value was 800 µg/ml, indicating that 200 µg/ml is safe with respect to the other higher doses.

Glutathione is an important antioxidant in cellular system. Hence to understand the glutathione level in cell, both reduced and oxidized form of glutathione were measured.

The reduced glutathione level (GSH) was decreased and the mild augmentation of oxidized form of glutathione level (GSSG) was observed at the dose of 400 µg/ml. It was clearly observed that the alteration of redox ratio (GSH/GSSG) is fully correlated with alteration of drug concentrations (Pearson Co-efficient r= 0.951, Pearson correlation $p < 0.05$). The redox ratio was found concentration dependent. When the dose of the PS-II was increased from 200 to 400 µg/ml, the redox ratio decreased from 1.01 to 0.499 compared to their respective control indicating that 400 µg/ml was toxic. These results indicated that 200 µg/ml is biologically safe and effective dose.

Study of lipid peroxidation is one of the important parameters to assess the cellular damage. It initiates inactivation of cellular components and protective enzymes, and thereby plays a crucial role of oxidative stress in biological systems. Several toxic by-products especially malondialdehyde is released due to lipid peroxidation. Hence, lipid peroxidations in lymphocytes were measured in terms of the concentration of malondialdehyde (MDA) release. The present investigation showed slightly increase of MDA at the dose of 400 µg/ml in comparison to the previous doses indicating that 200 µg/ml is again biologically safe.

Stimulated lymphocytes secreted several factors like NO. The release of NO clearly demonstrated that it was secreted by the lymphocytes when stimulated by PS-II. In presence of the PS-II, single culture of lymphocytes generated significant amount of NO ($p< 0.05$) into the medium after 24h of incubation. The result showed the presence of a high concentration of NO in the co-culture medium of pulsed lymphocytes at 400µg/ml indicating that this dose is cytotoxic. Hence, it is again established that 200 µg/ml is safe and effective dose.

To establish the protective role of PS-II against nicotine toxicity, lymphocytes were treated with nicotine (10 mM) as positive control and different concentrations of PS-II along with nicotine for 24 h in culture media. The significantly ($p < 0.05$) increased cell viability levels were observed up to 200µg/ml. The fluorescent microscopic pictures established the result. The fluorescence images revealed that the PS-II was able to ameliorate the toxic effects of nicotine at the dose of 200 µg/ml, but when the dose was increased to 400 µg/ml, the PS-II lost its ameliorative effects on lymphocytes. The above

result was confirmed by FACS, which established our findings that 400 µg/ml reveled the toxic effects synergistically with nicotine.

It is evident from these experiments that, in vitro application of PS-II does not induce any cellular damage in lymphocytes associated with enhanced MDA level, NO level, GSSG level and decreased GSH level. The cytotoxic profile of PS-II in lymphocytes indicated 200 µg/ml safe and effective, whereas concentrations higher than 200 µg/ml showed significant increase of cytotoxicity. Administration of Nicotine to lymphocytes causes decrease in cell viability which is protected by supplementation of PS-II to nicotine treated cells. These findings suggest the potential use and beneficial role of PS-II for use as antioxidant as well as immunostimulant.

These works were published in

(i) ***International Journal of Biological macromolecules,*** **2014**, *63*, 140-149

(ii) ***Carbohydrate Polymers,*** **2014,** *114*, 157-165.

Chapter-IV:

Fresh fruit bodies of the mushroom *Entoloma lividoalbum* (700 g) were gently washed with water, cut into pieces and boiled at 100 °C with distilled water for 10 h, cooled, centrifuged, supernatant was precipitated in EtOH (1:5) to get crude polysaccharide (900 mg). The crude polysaccharide (25 mg) was purified by gel-permeation chromatography (GPC) on column (90 cm × 2.1 cm) of Sepharose 6B using distilled water as the eluent with a flow rate of 0.5 mL min^{-1}. A single homogeneous fraction was collected and freeze-dried, yielding 15 mg pure polysaccharide. The average molecular weight of ELPS was estimated as ~2×10^5 Da on the basis of standard calibration curve prepared using standard dextrans. GLC analysis of the alditol acetates of this polysaccharide revealed the presence of glucose only. The absolute configuration of glucose was determined as D according to Gerwig et al. The GLC-MS analysis of the alditol acetates of methylated product showed the presence (1→3), (1→6), and (1→3,6)-linked and terminal glucopyranosyl residues in a molar ratio of nearly 1:3:1:1 respectively.

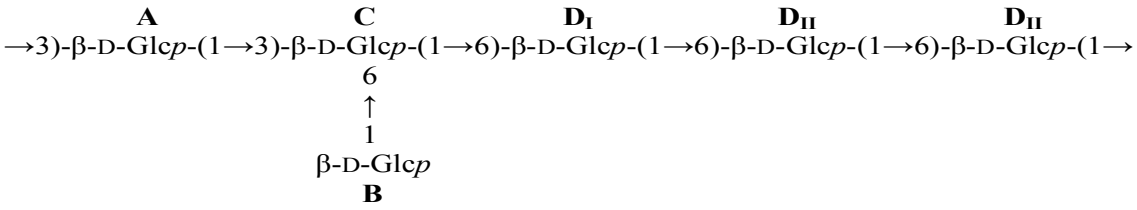
In the anomeric region of the ^1H NMR spectrum (500 MHz) at 30 °C, three signals were observed at δ 4.78, 4.51, and 4.49. The peak at δ 4.78 corresponded to the anomeric

proton of residue **A**. The signal at δ 4.51 corresponded to both the residues **B** and **C** and the signal at δ 4.49 corresponded to residue **D**. ^{13}C NMR spectrum (125 MHz) showed three signals in the anomeric region at δ 102.9, 102.7, and 102.5 at the same temperature. On the basis of HSQC spectrum, the anomeric proton signal at δ 4.51 was correlated to both the carbon signals δ 102.7 and δ 102.5, corresponded to anomeric carbons **B** and **C** respectively. Again, the anomeric proton signals at δ 4.78 and δ 4.49 were correlated to carbon signals at δ 102.7 and δ 102.9, corresponded to anomeric carbon of residues **A** and **D** respectively.

The different linkages that connected these residues (**A** to **D**) were determined from ROESY as well as NOESY experiment. In ROESY experiment, the inter-residual contacts **A**H-1/**C**H-3; **B**H-1/**C**H-6a, **C**H-6b; **C**H-1/**D$_I$**H-6a, **D$_I$**H-6b; **D$_I$**H-1/**D$_{II}$**H-6a, **D$_{II}$**H-6b; and **D$_{II}$**H-1/**A**H-3 along with other intra-residual contacts were also observed (Fig. 6a). The above ROESY connectivities established the following sequences: **A** (1→3) **C**; **B** (1→6) **C**; **C** (1→6) **D$_I$**; **D$_I$** (1→6) **D$_{II}$** and **D$_{II}$** (1→3) **A**.

Finally, the ROESY connectivities were confirmed from HMBC spectrum. In this spectrum the inter-residual cross-peaks between **A**H-1/**C**C-3, **A**C-1/**C**H-3; **B**H-1/**C**C-6, **B**C-1/**C**H-6a, **C**H-6b; **C**H-1/**D$_I$**C-6, **C**C-1/**D$_I$**H-6a, **D$_I$**H-6b; **D$_I$**H-1/**D$_{II}$**C-6, **D$_I$**C-1/**D$_{II}$**H-6a, **D$_{II}$**H-6b; **D$_{II}$**H-1/**A**C-3, **D$_{II}$**C-1/**A**H-3 along with some intra-residual peaks were also observed.

Thus, the HMBC and ROESY connectivities clearly supported the presence of the following repeating unit in the ELPS [5] and the structure is proposed as:



Smith degradation was carried out with the ELPS where a disaccharide containing hydrated glyceraldehyde moiety is produced and the product was analyzed by ^{13}C NMR spectroscopy to confirm further the sequence of the sugar residues present in the

repeating unit. The structure of hydrated glyceraldehyde containing disaccharide unit obtained from ELPS after Smith degradation was established as:

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

Immunostimulating properties of ELPS

In vitro macrophage activation was observed with different concentrations of ELPS. Nitric oxide production was found to increase in dose-dependent manner with optimum production of 22 μM NO per 5×10^5 macrophages at 35 μg/mL of ELPS. Further increase in concentration of ELPS decreased the NO production implying that the effective dose of the ELPS was 35 μg/mL.

Splenocytes include T cells, B cells, dendritic cells, and macrophages that enhance the immunity in living systems. Thymocytes after maturation in thymus are designated as T cells. The splenocyte and thymocyte activation tests were conducted in mouse cell culture medium with the ELPS by the MTT assay method. Proliferation of splenocytes and thymocytes is an indication of immunostimulation. The ELPS was found to stimulate splenocytes and thymocytes. Maximum proliferation index of splenocytes and thymocytes by ELPS were found at 12.5 μg/mL and 100 μg/mL respectively. The decrease in the immunological activities of the polysaccharide after the optimum concentration may be due to insufficient activation signal at the cellular surface. From these findings, it can be concluded that 35 μg/mL, 12.5 μg/mL and 100 μg/mL are the optimum concentration of ELPS for macrophage, splenocytes and thymocytes proliferation respectively.

Antioxidant properties of ELPS

Hydroxyl radical scavenging activity of ELPS was measured at different concentrations (100 to 800 μg/mL) taking butylated hydroxytoluene (BHT) as positive control. These results indicated that the activity of the ELPS gradually increases with the increase of concentrations. The hydroxyl radical scavenging activities of ELPS and BHT were respectively 20.48% and 91.2% at a dose of 200 μg/mL, indicating that antioxidant

activity of ELPS is weak compared to BHT. The IC_{50} value of the ELPS was found to be 400 µg/mL.

The superoxide radical scavenging activities of ELPS and butylated hoydroxyanisole (BHA) were determined to be 56.58% and 89.88%, respectively at the dose of 100 µg/mL. At all concentrations, ELPS showed lower superoxide anion scavenging activity than synthetic standard drug BHA. The IC_{50} value of the ELPS was found to be 75 µg/mL.

The reducing power of ELPS and ascorbic acid increased with increasing sample concentration. The reducing power (absorbance at 700 nm) of ELPS and ascorbic acid were 0.2285 and 1.16 at 200 µg/mL, respectively. At concentration of 470 µg/mL, ELPS showed reducing power 0.5. This result suggests that ELPS has potency to donate electron to reactive free radicals, converting them into more stable non-reactive species and terminating the free radical chain reaction.

This work has been published in ***Carbohydrate Polymers,* 2015***,123*, 350-358.

Chapter-V:

The hybrid mushroom *pfle 1p* was cultivated and collected from Falta experimental farm, Bose Institute, Kolkata. Aqueous extract of the fruit bodies of one of the hybrid mushroom strains, *pfle 1p* yielded two polysaccharides, PS-I and PS-II. The structural characterization and immunostimulating studies of PS-II have been discussed in this chapter. The PS-II showed specific rotation $[\alpha]_D^{28.6}$ +54.7 (*c* 0.91, water). The apparent molecular weight of PS-II was estimated as ~1.65×10^5 Da from a calibration curve prepared with standard dextrans. The GLC analysis of alditol acetates of hydrolyzed product of PS-II confirmed the presence of mannose, galactose, and 3-*O*-methyl-galactose almost in a ratio of 1.0:0.99:1.1. The absolute configuration of all the sugar residues were determined as D. The GLC–MS analysis of partially methylated alditol acetates of PS-II revealed the presence of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-mannitol, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-galactitol and 1,2,5,6-tetra-*O*-acetyl-3,4-di-*O*-methyl-D-galactitol in a ratio of nearly 1:1:1. Thus, PS-II was assumed to consist of terminal D-mannopyranosyl, (1→6)-D-galactopyranosyl and (1→2,6)-D-galactopyranosyl moieties respectively. The GLC-MS analysis of the alditol acetates of

periodate oxidized-reduced and methylated PS-II showed the presence of only 1,2,5,6-tetra-*O*-acetyl-3,4-di-*O*-methyl-D-galactitol. These results clearly indicated that terminal D-mannopyranosyl and (1→6)-linked D-galactopyranosyl residues were consumed during oxidation while (1→2,6)-linked D-galactopyranosyl residue was unaffected by periodate since the C-3 position of the branched galactopyranosyl residue was already occupied by the -*O*Me group. Hence, the mode of linkages in the PS-II was confirmed.

The ^{1}H NMR spectrum showed three peaks in the anomeric region. The peaks were observed at δ 5.12, 4.98 and 4.78 in a ratio of nearly 1:1:1. Rest of the sugar protons were observed in the region of δ 3.37-4.18 and one -*O*CH_3 group signal at δ 3.43. The anomeric peaks were designated **A**, **B** and **C** according to their decreasing proton chemical shifts. In the ^{13}C NMR spectrum three peaks appeared in the anomeric region at δ 101.7, 98.2 and 97.9 in a ratio of nearly 1:1:1. The other carbon signals came in the region δ 79.1-61.1. In addition, there was a signal at δ 56.0, which was assigned for -*O*CH_3 signal. From HSQC spectrum anomeric proton signals at δ 5.12 (**A**), 4.98 (**B**) and 4.78 (**C**) were correlated to the carbon signals at δ 98.2, 97.9 and 101.7 respectively. The chemical shifts of -*O*CH_3 group (δ 3.43/ 56.0) were also assigned from HSQC spectrum.
The different linkages that connected these three residues were determined from NOESY as well as ROESY spectrum. In NOESY spectrum, the inter-residual contacts were observed between **A**H-1/**B**H-6a and **B**H-6b; **B**H-1/**A**H-6a and **A**H-6b; and **C**H-1/**A**H-2 along with other intra-residual contacts.
Finally, these links were confirmed from HMBC spectrum. In this spectrum the inter-residual cross-peaks were observed between **A**H-1/**B**C-6; **A**C-1/**B**H-6a and **B**H-6b; **B**H-1/**A**C-6; **B**C-1/**A**H-6a and **A**H-6b; **C**H-1/**A**C-2; and **C**C-1/**A**H-2 along with other intra-residual contacts.
Thus, the structural motif present in the polysaccharide,PS-II [6] was established as:

A **B**

→6)-3-*O*-Me-α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→

2

↑

1

β-D-Man*p*

C

Immunostimulating properties

Mushroom polysaccharides function as immunostimulator by activating the macrophages. On treatment with different concentrations of this PS-II, it was observed that 49% to 54% of NO production increased up to 50 µg/mL. This was further increased by 70% at 100 µg/mL, but decreased at 200 µg/mL. Hence, the effective dose of this PS-II was observed at 100 µg/mL with optimum production of 10.2 µM NO per 5×10^5 macrophages.

Splenocytes are the cells present in the spleen that include T cells, B cells, dendritic cells, and macrophages that stimulate the immune response in living organism. Thymocytes are hematopoietic cells in thymus which generate T cells. Proliferation of splenocyte and thymocyte is an indicator of immunostimulation. The splenocyte and thymocyte activation tests were carried out in mouse cell culture medium with the PS-II by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method. The PS-II was tested to stimulate splenocytes and thymocytes respectively. Both the splenocyte and thymocyte proliferation indices were found maximum at 50 µg/mL, above and below which it decreases. Hence, it can be concluded that 50 µg/mL is the optimum concentration of the PS-II for splenocyte and thymocyte proliferation.

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REFERENCES

1. Wasser, S. P. (2002). Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. *Applied Microbiology and Biotechnology*, *60*, 258-274.
2. Halliwell, B., & Gutteridge, J. M. C. (1984). Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochemical Journal, 219*, 1-4.
3. Maity, P., Samanta, S., Nandi, A. K., Sen, I. K., Paloi, S., Acharia, K., & Islam, S. S. (2014). Structure elucidation and antioxidant properties of a soluble β-D-glucan from mushroom Entoloma lividoalbum. *International Journal of Biological macromolecules, 63*, 140-149.
4. Maity, P., Nandi, A. K., Sen, I. K., Pattanayak, M., Chattopadhyay, S., Dash, S. K., et al. (2014). Heteroglycan of an edible mushroom *Entoloma lividoalbum:* Structural characterization and study of its protective role for human lymphocytes. *Carbohydrate Polymers, 114*, 157-165.
5. Maity, P., Sen, I. K., Maji, P. K., Paloi, S., Devi, K. S. P., Acharia, K., Maiti, T. K. & Islam, S. S. (2015). Structural, immunological, and antioxidant studies of β-glucan from edible mushroom *Entoloma lividoalbum. Carbohydrate Polymers*, *123*, 350-358.
6. Maity, P., Pattanayak, M., Maity, S., Nandi, A. K., Sen, I. K., Behera, B., et al. (2014). A partially methylated mannogalactan from hybrid mushroom *pfle 1p*: purification, structural characterization and study of immunoactivation. *Carbohydrate Research, 395*, 1-8.