ISOLATION, PURIFICATION AND CHARACTERIZATION OF BIOACTIVE POLYSACCHARIDES FROM AN EDIBLE MUSHROOM, *RUSSULA ALBONIGRA*

A SYNOPSIS

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BY

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SYNOPSIS

The main aim of the present thesis entitled "**Isolation, purification and characterization of bioactive polysaccharides from an edible mushroom, *Russula albonigra***" is to determine the structure as well as some biological activity of different polysaccharides isolated from the fruiting bodies of an edible mushrooms *Russula albonigra* (Krombh.) Fr. The entire thesis is divided into five chapters.

Chapter-1 describes the general introduction to carbohydrates, polysaccharides, and especially mushroom polysaccharides and their biological activities.

Chapter-2 represents the experimental methods, which were carried out during the thesis work.

Chapter-3 is one of the major part of the thesis, which describes the isolation, purification, determination of the structure, and as well as study of immunoenhancing properties of the α,β-glucan (PS-I) isolated from hot aqueous extract of the fruiting bodies of the edible mushrooms *Russula albonigra* (Krombh.) Fr. This work has been published in ***Carbohydrate Research***, **2012**, *363*, 43-50.

Chapter-4 contains structural elucidation and biological investigation of heteroglycan (PS-II), isolated from hot aqueous extract of the edible mushroom, *Russula albonigra* (Krombh.) Fr. This work has been published in ***Carbohydrate Polymers*, 2013**, *94*, 918-926.

Chapter-5 is another major and important part of the thesis, deals with the isolation and purification of β-glucan (PS), isolated from alkaline extract of the edible mushroom, *Russula albonigra* (Krombh.) Fr. and also study of immunostimulation as well as antioxidant activities. This work has been published in ***Carbohydrate Polymers*, 2014**, *99*, 774-782.

Chapter-1: In this chapter the general introduction to carbohydrates and polysaccharides emphasizing on mushroom polysaccharides and their biological activities are discussed. Carbohydrates are the most abundant and diverse class of organic compounds occurring in nature. They are essential constituents of all living organisms and have a variety of vital functions. These are generally classified into four groups: monosaccharide, disaccharide, oligosaccharide, and polysaccharide. The great bulk of the carbohydrates in nature are present as polysaccharides which are large and complex molecules. The polysaccharides serve two principal functions: (1) used both by plants and animals to store glucose as a source of future food energy, and (2) provide some of the mechanical structure to protect the cells.

A mushroom is a fleshy spore bearing fruiting body of a fungus, found in soil or on decomposing leaves, and compost. Nutritionally they are a valuable source of food which is low in fat but rich in carbohydrates, protein, fibre, vitamins and minerals including iron, potassium, selenium and zinc. Mushrooms contain a wide variety of bioactive molecules including lectins, terpenoids, steroids, phenols, nucleotides, glycoproteins and polysaccharides. Study on biological activities of mushroom polysaccharides included antitumor, immunostimulating, antioxidative, antiflammatory, antimicrobial, and hypoglycaemic properties, but the most important features are their immunostimulation and antitumor effects. The main active components for the mushroom polysaccharides were proved to be the glucans, specifically β-D-glucans which are important for their outstanding ability to enhance and stimulate the immune systems and thus regarded as typical biological response modifiers (BRMs) [1]. They exert antitumor effect through host defence mechanism against tumor without side effect. Currently, several mushroom polysaccharides isolated from *Lentinus edodes*, *Schizophyllum commune, Agaricus blazei, Grifola Frondosa, Trametes versicolor,* and *Pleurotus ostreatus* are widely used clinically as antitumor 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 important to determine the exact structure and biological activity of the polysaccharides isolated from mushrooms.

Polysaccharide Chemistry is a broad topic that stands at the intersection of Organic Chemistry, Biochemistry and Polymer Chemistry. In recent years, polysaccharides from both mushroom and plant have drawn the attention of chemist and immunobiologists on account of their immunostimulation and antitumor as well as antioxidant properties. Free radicals are formed as part of body's normal metabolic process. The imbalance between pro-oxidant and anti-oxidant due xenobiotics, x-ray, radiation, pollution and even stress have been implicated in the pathogenesis of atherosclerosis, ischemic disease, hypertension, Alzheimer's disease, Parkinson, inflammation, rheumatoid arthritis, cancer and diabetes mellitus. Antioxidants act as a major defense against radical mediated toxicity by protecting the damage caused by free radicals such as ROS (Reactive oxygen species).

Chapter-2: This chapter describes the methodologies of isolatation, purification and determination of the structure of pure polysaccharides and also study of their specific biological activity. The crude polysaccharide was purified by gel permeation chromatography (GPC) using water as the eluent. Several chemical reactions were carried out including total acid hydrolysis, methylation study, periodate oxidation, and Smith degradation to determine the structure of the polysaccharide. The neutral sugars obtained from acid hydrolysis of the polysaccharide were identified and estimated by Gas-liquid-chromatography (GLC). The absolute configurations of sugars were determined using the method of Gerwig et al. [2] The polysaccharide was methylated by the Ciucanu and Kerek method [3], followed by identification using Gas-liquid-chromatography-Mass spectroscopy (GLC-MS) to know the mode of linkages of the sugars present in the polysaccharide. Periodate oxidation was carried out to confirm the mode of linkages of the sugar residues. Smith degradation reaction of the polysaccharide was carried out to prepare some oligosaccharide, the determination of the structure of which gives some idea about the structure of the polysaccharide under investigation. 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.

Different biological analysis was 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 polysaccharide by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method. The antioxidant activity of polysaccharide was evaluated through the chelating ability of ferrous ions, determination of reducing power, inhibition of β carotene bleaching assay, 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 is the absorbance of the blank (without test sample) and A_1 is the absorbance in the presence of the sample.

Chapter-3: This chapter describes the isolation, purification, structural characterization and immunostimulating properties of α,β-glucan isolated from hot aqueous extract of the fruiting bodies of an edible mushrooms *Russula albonigra* (Krombh.) Fr.

Structural analysis of PS-I [4]

PS-I was hydrolyzed with 2M trifluoroacetic acid and then alditol acetates were prepared for GLC analysis. GLC analysis of alditol acetate of hydrolyzed product of PS-I confirmed the presence of glucose only. The absolute configuration of the glucose residue was determined as D by the method of Gerwig et al. The mode of linkages of the PS-I was determined by the methylation analysis using the Ciucanu and Kerek method followed by hydrolysis and alditol acetate conversion. The GLC–MS analysis of partially methylated alditol acetates revealed the presence of 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol and thus, PS-I was deduced to consist of (1→3,6), (1→3)-linked, and terminal glucopyranosyl residues in a molar ratio of nearly 1:3:1. GLC analysis of alditol acetates of the periodate-oxidized, $NaBH_4$-reduced, methylated PS-I

showed the presence of 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol and 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol in a molar ratio of nearly 1:3. These results clearly indicated that the terminal glucopyranosyl residues were consumed during oxidation whereas, (1→3,6)-linked and (1→3)-linked glucopyranosyl residues remain unaffected which further confirmed the mode of linkages present in the PS-I.

The ^{1}H NMR (500 MHz) spectrum at 30 ºC showed five signals in the anomeric region at δ 5.10, 5.04, 4.97, 4.51, and 4.49 in a ratio of nearly 1:1:1:1:1. They were designated as residues **A**, **B**, **C**, **D**, and **E** according to their decreasing proton chemical shifts. In the ^{13}C and DEPT-135 NMR (125 MHz) spectrum at 30 ºC five anomeric signals appeared at δ 102.4, 102.2, 100.2, 98.0, and 97.8 in a ratio of nearly 1:1:1:1:1. Based on the result of the HSQC experiment, the anomeric carbon signals at δ 102.4, 102.2, 100.2, 98.0, and 97.8 corresponded to the anomeric proton signals at δ 4.51, 4.49, 5.10, 5.04, and 4.97, respectively. All the ^{1}H and ^{13}C signals were assigned using DQF-COSY, TOCSY, HSQC experiments. Coupling constants were measured from DQF-COSY spectrum. The large $J_{H\text{-}2,H\text{-}3}$ and $J_{H\text{-}3,H\text{-}4}$ coupling constant values of 8~10 Hz in residues **A**, **B**, **C**, **D** and **E** support the presence of the glucopyranosyl configuration in the polysaccharide.

Based on the coupling constant, $J_{H\text{-}1,H\text{-}2}$ ~ 3 Hz and $J_{C\text{-}1,H\text{-}1}$ of ~ 170 Hz the residues **A**, **B**, and **C** were established as α-anomer. In residue **A**, all carbon chemical shift values matched with the standard values of methyl glycosides. Thus considering the results of methylation analysis and NMR spectroscopy, it was concluded that residue **A** was α-linked terminal D-glucopyranosyl moiety. On the other hand, both the Residues **B** and **C** showed downfield shift **B**C-3 (δ 81.0) and **C**C-3 (δ 81.2) with respect to standard values of methyl glycosides which indicated that they were (1→3)-linked-α-D-glucopyranosyl moiety. The residue **C** was situated adjacent to **D**, and other residue **B** was away from it. So, C-3 (δ 81.2) of residue **C** showed δ 0.2 downfield shift than that of C-3 **(**δ 81.0) of residue **B** due to neighbouring effect of the rigid part **D** and C-1 chemical shift value of both residues were slightly different due to different chemical environment while other carbon signals remain almost same.

Residues **D** and **E** were established as β-anomer from coupling constant values $J_{H\text{-}1,H\text{-}2}$ ~8 Hz, and $J_{C\text{-}1,H\text{-}1}$ ~ 160 Hz. The downfield shift of C-3 (δ 84.3) and C-6 (δ 68.8) of **D**

with respect to standard values indicated that **D** was linked at C-3 and C-6. The linking of residue **D** at C-6 was further confirmed from DEPT-135 spectrum. These observations indicated that **D** was (1→3,6)-linked-β-D-glucopyranosyl moiety. On the other hand, the downfield shift of C-3 (δ 85.0) of residue **E** with respect to standard values of methyl glycosides indicated that it was (1→3)-linked-β-D-glucopyranosyl moiety. Since, residue **D** was the most rigid part of the backbone of the PS-I, it's C-3 (δ 84.3) appeared at the upfield region in comparison to the C-3 (δ 85.0) of residue **E**.

The sequences of glucosyl moieties were determined from ROESY (Fig.4, Table 2) as well as NOESY (not shown) experiments. In ROESY experiment, the inter-residual contacts **A**H-1/**B**H-3; **B**H-1/**D**H-6a, **D**H-6b; **C**H-1/**E**H-3; **D**H-1/**C**H-3 and **E**H-1/**D**H-3 along with some other intra residual contacts were also observed. The above ROESY connectivities established the following sequences:

A **B**
α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→ ;

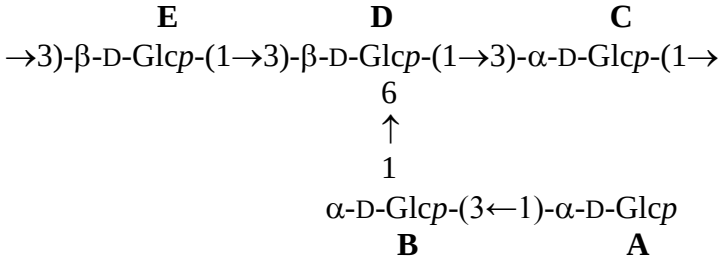
D **C**
→3)-β-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→ ;
6
↑

B **D**
→3)-α-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→ ;
3
↑

E **D**
→3)-β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→ ;
6
↑

C **E**
→3)-α-D-Glc*p*-(1→3)-β-D-Glcp-(1→;

A long range HMBC experiment was carried out to confirm the ROESY connectivities. In HMBC experiment, inter residual couplings **A**H-1/**B**C-3, **A**C-1/**B**H-3, **B**H-1/**D**C-6, **B**C-1/ **D**H-6a, **D**H-6b, **C**H-1/**E**C-3, **C**C-1/**E**H-3, **D**H-1/**C**C-3, **D**C-1/**C**H-3, **E**H-1/**D**C-3, and **E**C-1/**D**H-3 along with some intra residual couplings were also observed. Thus, the HMBC and ROESY connectivities confirmed the repeating unit in the PS-I isolated from an edible mushroom *R. albonigra* (Krombh.) Fr. Thus, based on all these chemical and spectroscopic evidences, the structure of repeating unit of the polysaccharide was established as:



Immunological studies of PS-I

Macrophage activation of the PS-I was observed *in vitro*. An enhanced production of NO was observed in a dose-dependent manner on treatment with different concentrations of the PS-I and found optimum production of 24.5 µM NO per 5 x 10^5 macrophages at 100 µg/mL. Proliferation of splenocytes and thymocytes is an index of immunostimulation. Both splenocyte and thymocyte proliferation indices were found maximum at 50 µg/mL of the PS-I compared to other concentrations. To estlablish the immunoenhancing activity of PS-I, HeLa cancer cells which is not a part of the immune system was used as control and treated with different concentrations (12.5 to 200 µg/mL) of the PS-I.

This work has been published in ***Carbohydrate Research***, **2012**, *363*, 43-50.

Chapter-4: This chapter describes the isolation, purification, structural characterization and immunostimulating properties of a heteroglycan isolated from hot aqueous extract of the fruiting bodies of an edible mushrooms *Russula albonigra* (Krombh.) Fr.

Structural analysis of PS-II [5]

The sugar analysis of PS-II by paper chromatography and GLC of alditol acetates showed that it was found to consist of glucose, galactose, manose, 2-*O*Me-fucose, and fucose in a molar ratio of nearly 2:2:1:1:1. The absolute configuration of the monosaccharides was determined by the method of Gerwig et al and it was found that glucose, galactose, and manose had the D configuration but 2-*O*Me-fucose and fucose were present in the L configuration. The mode of linkages of the PS-II was determined by the methylation analysis using the method described by Ciucanu & Kerek followed by hydrolysis and alditol acetate conversion. The GLC–MS analysis of partially methylated alditol acetates revealed the presence of 6-deoxy-2,3,4-Me_3-Fuc, 2,3,4,6-Me_4-Man, 6-deoxy-3,4-Me_2-Fuc, 2,4,6-Me_3-Glc, 2,6-Me_2-Glc, 2,3,4-Me_3-Gal, and 3,4-Me_2-Gal in a nearly equal molar ratio. The above result indicated that non reducing end 2-*O*Me-L-fucopyranosyl, terminal D-manopyranosyl, (1→2)-linked L-fucopyranosyl, (1→3)-linked D-glucopyranosyl, (1→3,4)-linked D-glucopyranosyl, (1→6)-linked D-galactopyranosyl, and (1→2,6)-linked D-galactopyranosyl moieties were present in the PS-II in a nearly equal molar ratio. These linkages were further confirmed by periodate oxidation experiment. GLC analysis of alditol acetates of periodate-oxidized, reduced, and hydrolyzed products showed the presence of only D-glucose, indicating that the D-galactose, D-manose, 2-*O*-Me-L-fucose, and L-fucose moieties were consumed during oxidation. The GLC and GLC-MS analysis of periodate-oxidized and methylated PS-II showed the presence of 2,4,6-Me_3-Glc and 2,6-Me_2-Glc in a molar ratio of nearly 1:1. This observation clearly indicated that (1→3)-linked and (1→3,4)-linked D-glucopyranosyl moieties remain unaffected whereas all other moieties were consumed during oxidation, which further confirmed the mode of linkages present in the PS-II.The ^{1}H NMR (500 MHz) spectrum at 30 ºC showed five signals in the anomeric region at δ

5.10, 5.04, 4.97, 4.52, and 4.50 in a ratio of nearly 1:2:2:1:1. Hence, the signals at δ 5.10, 4.52, and 4.50 indicated the presence of only one residue while the signals at δ 5.04 and 4.97 corresponded to two residues. The sugar residues were designated as **A**-**G** according to their decreasing anomeric proton chemical shifts. In the ^{13}C and DEPT-135 NMR (125 MHz) spectrum at 30 ºC five anomeric signals appeared at δ 102.5, 102.2, 100.8, 98.0, and 97.8 in a ratio of nearly 1:2:1:1:2. Based on the result of the HSQC experiment, the anomeric carbon signals at δ 102.5, 100.8, and 98.0 corresponded to the anomeric carbons of **G**, **A**, and **B** residues, respectively whereas the signal at δ 102.2 corresponded to the anomeric carbon of **C** and **F** residues while the peak at δ 97.8 was correlated to the anomeric carbon of **D** and **E** residues. All the ^{1}H and ^{13}C signals were assigned using DQF-COSY, TOCSY, HSQC experiments. Coupling constants were measured from DQF-COSY spectrum.

Based on the coupling constant, $J_{H\text{-}1,H\text{-}2}$ ~ 3.1 Hz and $J_{C\text{-}1,H\text{-}1}$ ~ 171 Hz the residues **A** and **B** were established as α-anomer. A large $J_{H\text{-}2,H\text{-}3}$ (~ 9 Hz) and small $J_{H\text{-}3,H\text{-}4}$ (< 5 Hz) indicated that those were D-galactosyl unit. In residue **A**, the downfield shift of C-2 (δ 75.6) and C-6 (δ 66.5) with respect to standard values of methyl glycosides indicated that the moiety **A** was (1→2,6)-linked unit. On the other hand, in residue **B**, the downfield shift of C-6 (δ 66.7) with respect to standard values of methyl glycosides indicated that it was (1→6)-linked unit. The linking at C-6 of the both residue **A** and **B** were further confirmed from DEPT-135 spectrum. Hence, these observations confirmed that residue **A** was a (1→2,6)-linked-α-D-galactopyranosyl moiety and the residue **B** was a (1→6)-linked-α-D-galactopyranosyl moiety.

The anomeric proton signal of residue **C** at δ 5.04 with low values of $J_{H\text{-}1,H\text{-}2}$, $J_{H\text{-}2,H\text{-}3}$ (~ 3.5 Hz) and $J_{C\text{-}1,H\text{-}1}$ of ~ 170 Hz clearly indicated that it was a α-linked mannopyranosyl moiety. This was further confirmed from the large coupling constant value $J_{H\text{-}3,H\text{-}4}$ ~7.5 Hz and $J_{H\text{-}4,H\text{-}5}$ ~ 10 Hz. The carbon chemical shifts of residue **C** from C-1 to C-6 corresponded nearly to the standard values of methyl glycoside of α-D-mannose indicating residue **C** was terminal α-D-mannopyranosyl moiety.

Residues **D** and **E** were assigned to L-fucopyranosyl unit. This was strongly supported by the appearance of a proton signal at δ 1.24, carbon signal at δ 15.6 for a CH_3 group,

and the relatively small $J_{H\text{-}3,H\text{-}4}$ (< 3 Hz). The appearance of the anomeric proton and carbon signals for both residues at δ 4.97 and 97.8, respectively, as well as the coupling constant value $J_{H\text{-}1,H\text{-}2}$ ~ 3.75 Hz clearly indicated that those were α-anomer. The anomeric configuration was further confirmed by $^{1}H\text{-}^{13}C$ coupling constant $J_{C\text{-}1,H\text{-}1}$ ~ 171 Hz. In residue **D**, the downfield shift of C-2 (δ 78.2) with respect to standard values indicated that the moiety **D** was linked at C-2 position with $-OCH_3$ group. This was further confirmed by the appearance of cross coupling between the methoxy proton (δ 3.43) and the C-2 atom of residue **D** and between methoxy carbon (δ 56.0) and its H-2 atom in the HMBC experiment. On the other hand, the downfield shift of C-2 (δ 78.0) with respect to standard values of methyl glycosides indicated that the residue **E** was also linked at C-2 position with residue **A** which further confirmed by the ROESY as well as HMBC experiment. So the moiety **E** was (1→2)-linked unit. The C-2 chemical shift values of the residues **D** and **E** were slightly different due to slight difference in chemical environment while other carbon signals remain almost same. Thus, it may be conclude that the residue **D** was a non reducing end 2-*O*Me-α-L-fucopyranosyl moiety and the residue **E** was a (1→2)-linked-α-L-fucopyranosyl moiety.

Residues **F** and **G** were established as β-anomer from coupling constant values $J_{H\text{-}1,H\text{-}2}$ (~8 Hz), and $J_{C\text{-}1,H\text{-}1}$ (~160 Hz) and the large coupling constant values $J_{H\text{-}2,H\text{-}3}$ and $J_{H\text{-}3,H\text{-}4}$ (~ 10 Hz) of the residues **F** and **G** confirmed their glucopyranosyl moiety. The downfield shift of C-3 (δ 84.5) and C-4 (δ 75.2) with respect to standard values indicated that moiety **F** was linked at C-3 and C-4. These observations indicated that **F** was (1→3,4)-linked-β-D-glucopyranosyl moiety. On the other hand, the downfield shift of C-3 (δ 85.0) with respect to standard values of methyl glycosides indicated that moiety **G** was linked at C-3. Thus it may be concluded that **G** was (1→3)-linked-β-D-glucopyranosyl moiety. Since, the residue **F** was rigid part in comparison to that of residue **G**. So the C-3 (δ 84.5) value of residue **F** appeared at the upfield region than that of the C-3 (δ 85.0) of residue **G**.

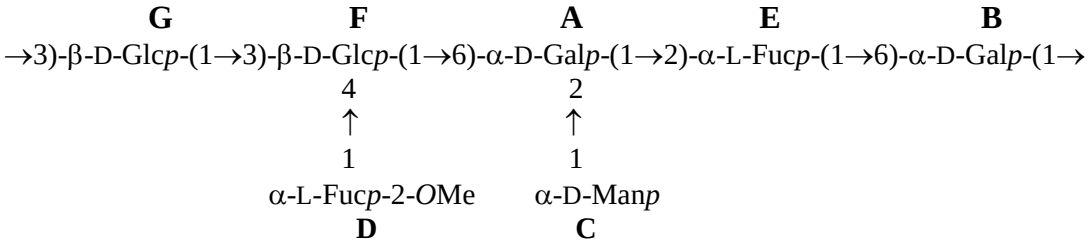
The sequences of glycosyl moieties were determined from ROESY as well as NOESY experiments. In ROESY experiment, the inter-residual contacts **A**H-1/**E**H-2; **B**H-1/**G**H-3; **C**H-1/**A**H-2; **D**H-1/**F**H-4; **E**H-1/**B**H-6a, **B**H-6b; **F**H-1/**A**H-6a, **A**H-6b and **G**H-1/**F**H-3

along with some other intra residual contacts were also observed. The above ROESY connectivities established the following sequences:

A-(1→2)-**E**; **B**-(1→3)-**G**; **C**-(1→2)-**A**;

D-(1→4)-**F**; **E**-(1→6)-**B**; **F**-(1→6)-**A**; **G**-(1→3)-**F**;

A long range HMBC experiment was carried out to confirm the ROESY connectivities. In HMBC experiment, inter-residual couplings **A**H-1/**E**C-2, **A**C-1/**E**H-2, **B**H-1/**G**C-3, **B**C-1/**G**H-3, **C**H-1/**A**C-2, **C**C-1/**A**H-2, **D**H-1/**F**C-4, **D**C-1/**F**H-4, **E**H-1/**B**C-6, **E**C-1/**B**H-6a, **B**H-6b, **F**H-1/**A**C-6, **F**C-1/**A**H-6a, **A**H-6b, **G**H-1/**F**C-3, and **G**C-1/**F**H-3 along with some intra residual couplings were also observed. Thus, the HMBC and ROESY connectivities confirmed the presence of heptasaccharide repeating unit in the PS-II isolated from an edible mushroom *R. albonigra* (Krombh.) Fr. which is shown as:



Immunological studies of LPS free polysaccharide (LFPS-II)

A negative (-) LAL test indicated that LFPS-II which was obtained after passing the PS-II through polymixin-B matrix, was free from bacterial endotoxin. Macrophage activation by LFPS-II was observed *in vitro*. Enhanced production of NO was observed in a dose-dependent manner on treatment with different concentrations of the LFPS-II and observed optimum production of 18 µM NO per 5 x 10^5 macrophages at 100 µg/mL of the LFPS-II. Proliferation of splenocytes and thymocytes is an index of immunostimulation. Splenocyte proliferation index was found maximum at 25 µg/mL of the LFPS-II, as compared to other concentrations. Hence, 25 µg/mL of the LFPS-II can be considered as efficient splenocyte stimulator. Again, 50 µg/mL of this same sample showed maximum effect on thymocyte proliferation.

This work has been published in ***Carbohydrate Polymers*, 2013**, *94*, 918-926.

Chapter-5: This chapter describes the isolation, purification, and structural characterization of β-glucan, isolated from alkaline extract of an edible mushroom, *Russula albonigra* (Krombh.) Fr. and also study of immunostimulation as well as antioxidant activities.

Structural analysis of β-glucan (PS) [6]

GLC analysis of the alditol acetates of this polysaccharide revealed the presence of glucose only. The PS showed specific rotation $[\alpha]_D^{31}$ –19.5 (*c* 0.1, 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, Kamerling, & Vliegenthart and it was found that glucose had D-configuration. The polysaccharide was methylated according to the method of Ciucanu & Kerek followed by hydrolysis and then converted to alditol acetate. The GLC–MS analysis of partially methylated alditol acetates 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 ratio of nearly 1:2:2:1, respectively. These results indicate the presence of nonreducing end, (1→3)-, (1→6)-, and (1→3,6)-linked D-glucopyranosyl residues in the β-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. The GLC analysis of the alditol acetates of the periodate-oxidised, reduced PS showed the presence of D-glucose along with glycerol 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 ratio of nearly 2:1. 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 product showed the presence of D-glucose and D-glycerol. The GLC-MS analysis of the of methylated

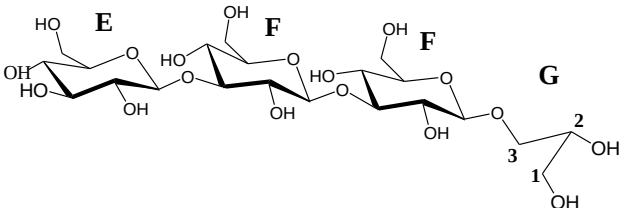
SDPS revealed the presence of 1,5-di-*O*-acetyl 2,3,4,6-tetra-*O*-methyl-D-glucitol and 1,3,5-tri-*O*-acetyl 2,4,6-tri-*O*-methyl-D-glucitol in a ratio of nearly 1:2. Partial hydrolysis of the β-glucan was carried out with 0.1 M TFA to know the backbone sequence of the β-glucan in the repeating unit. As a result of this hydrolysis, two fractions were obtained i.e. partially hydrolysed polysaccharide (F1) and partially hydrolysed oligosaccharide (F2). The Methylation analysis of F1 revealed the presence of 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol only indicating the presence of (1→6)-linked backbone of the PS and F2 revealed the presence of (1→3)-linked, and terminal glucopyranosyl moieties present as oligosaccharide side chain. All the above chemical investigation proved that the repeating unit of the PS had a backbone consisting of three (1→6)-β-D-glucopyranosyl residues, one of which was branched at *O*-3 position with the side chain consisting of two (1→3)-β-D-glucopyranosyl and a terminal β-D-glucopyranosyl residue.

The ^{1}H NMR (500 MHz) spectrum at 30 ºC showed four signals in the anomeric region at δ 4.74, 4.72, 4.52, and 4.50 in a ratio of nearly 1:1:2:2. They were designated as residues **A_I**, **A_{II}**, **B**, **C**, **D_I**, and **D_{II}** according to their decreasing proton chemical shifts. In the ^{13}C (125 MHz) spectrum at 30 ºC three anomeric signals appeared at δ 103, 102.7, and 102.5 in a ratio of nearly 1:3:2. Based on the result of the HSQC experiment, the anomeric carbon signal at δ 103.0 corresponded to **B,** whereas the signal at δ 102.7 corresponded to **A_I**, **A_{II}**, and **C** and the peak at δ 102.5 was correlated to **D_I** and **D_{II}** residues of the anomeric proton signals, respectively. All the ^{1}H and ^{13}C signals were assigned using DQF-COSY, TOCSY, HSQC experiments. Coupling constants were measured from DQF-COSY spectrum. The large $J_{H\text{-}2,H\text{-}3}$ and $J_{H\text{-}3,H\text{-}4}$ coupling constant values of 8~10 Hz in residues **A**-**D** support the presence of the glucopyranosyl configuration in the polysaccharide. Residues **A**-**D** were established as β-anomers from the coupling constant values $J_{H\text{-}1,H\text{-}2}$ ~8 Hz, and $J_{C\text{-}1,H\text{-}1}$ ~ 160 Hz. In residues **A** (**A_I** and **A_{II}**), the downfield shift of C-3 (δ 84.5) with respect to standard value of methyl glycosides indicated that they were (1→3)-linked β-D-Glc*p*. All the chemical shifts of residue **B** were nearly analogous with the standard values of methyl glycoside of β-D-glucose. This observation clearly indicated that the residue **B** was non-reducing end β-D-Glc*p*. In residue **C**, the chemical shift values of C-3 (δ 84.5) and C-6 (δ 68.7) showed

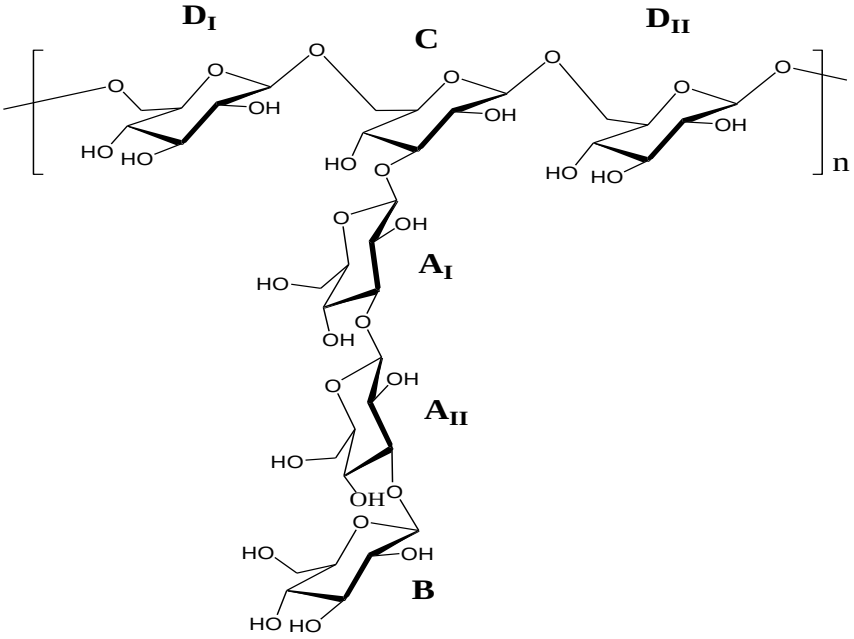
downfield shifts, indicating the presence of (1→3,6)-linked β-D-Glc*p*. Two **D** residues (**D_I** and **D_{II}**) were same in all chemical shift values except the values of C-6. The different downfield shifts of C-6 (δ 68.8 and 69.0) of two **D** residues supported the presence of (1→6)-linking in β-D-Glc*p* with different chemical environments. Among two **D** residues, one residue (**D_{II}**) was glycosidically attached to the rigid part (**C**) and other residue (**D_I**) was away from it. Between **D_I** and **D_{II}**, C-6 of **D_{II}** appeared slightly downfield in comparison to **D_I** residue due to the neighbouring effect of rigid part **C** of the backbone. Consequently, the C-6 value of the rigid residue **C** also resonated at fairly upfield compared to the C-6 of the **D_I** and **D_{II}** for the same reason. The linking at C-6 of the residues **C** and **D** were further confirmed from DEPT-135 spectrum.

The sequences of glucosyl moieties were determined from NOESY as well as ROESY experiments. A long range HMBC experiment was carried out to confirm the NOESY connectivities. From both NOESY and HMBC experiment, the inter-residual contacts along with some intra-residual contacts were observed. Thus, the HMBC and NOESY connectivities confirmed the repeating unit in the PS.

For further confirmation of the sequence of linkages in PS, the Smith degraded material (SDPS) was prepared and NMR experiment was carried out. The ^{13}C NMR (125 MHz) spectrum at 30 ˚C of SDPS showed two anomeric carbon signals at δ 102.5 and 102.7 in a ratio of nearly 2:1, corresponding to→3)-β-D-Glc*p*-(1→ (**F**) and β-D-Glc*p*-(1→ (**E**) residues respectively. The carbon signals C-1, C-2, and C-3 of the glycerol moiety were assigned as δ 68.10, 72.0, and 62.56 respectively. The nonreducing β-D-Glc*p*-(1→ (**E**) was generated from (1→3)-β-D-Glc*p* (**A_{II}**) due to complete oxidation of the β-D-Glc*p*-(1→ (**B**) and also one (1→3)-β-D-Glc*p* (**F**) was produced from the (1→3,6)-β-D-Glc*p* (**C**) due to oxidation followed by Smith degradation of the (1→6)-β-D-Glc*p* moiety (**D_I**) and the other (1→3)-β-D-Glc*p* (**F**) was retained from (1→3)-β-D-Glc*p* (**A_I**). The glycerol (**G**) moiety was generated from (1→6)-β-D-Glc*p* (**D_{II}**) after periodate oxidation followed by Smith degradation and be attached to (1→3)-β-D-Glc*p* moiety (**F**) as a gro part. Hence, Smith degradation resulted in the formation of an oligosaccharide unit from the parent polysaccharide and the structure of which was established as:



Therefore, the above result indicated that the (1→3)-linked β-D-glucose was present at the side chain, branching at *O*-3 of one backbone residue. This observation excluded the possibility of (1→3)-linked backbone. The ^{13}C spectrum was carried out with partially hydrolyzed polysaccharide (F1) and showed no C-3 signal for (1→3)-linked β-D-Glc*p* but a characteristic C-6 signal at δ 68.9 was observed. This result further proved that the glucan possessed (1→6)-linked backbone with (1→3)-linked moieties located at the branched point. This also excluded the possibility of alternatively (1→6) and (1→3)-linked moieties in the backbone. Hence, considering all the results of chemical investigations and NMR spectroscopic evidences, the structure of repeating unit of the β-glucan was established as:



Immunological studies of β-glucan (PS)

Macrophage activation by the PS was observed *in vitro*. Enhanced production of NO was observed in a dose-dependent manner on treatment with different concentrations of the PS and found optimum production of 22 µM NO per 5 x 10^5 macrophages at 100 µg/mL. Proliferation of splenocytes and thymocytes is an index of immunostimulation. At 50 µg/mL of the PS, splenocyte proliferation index was found maximum as compared to other concentrations. Hence, 50 µg/mL of the PS can be considered as efficient splenocyte stimulators. Again 25 µg/mL of this same sample showed maximum effect on thymocyte proliferation.

Antioxidant activities of β-glucan (PS)

The PS showed potent hydroxy radical scavenging activity which rose gradually with the increase of concentration. The IC_{50} value of hydroxy radical scavenging activity of the PS was found to be 265 µg/mL and also the PS was found to act as a notable scavenger of superoxide radicals. The IC_{50} value of superoxide radical scavenging activity of the PS was determined 130 µg/mL. In the presence of chelating agent, the complex formation is disrupted, resulting in the reduction of the red color of ferrozine. The PS demonstrated a marked capacity for iron binding ability, where the 50% chelation was found at a concentration of 300 µg/mL. In the reducing power assay, the more antioxidant compounds convert the oxidation form of iron (Fe^{+3}) in ferric chloride to ferrous (Fe^{+2}). So the yellow color of the test solution changed from green to blue as the reducing power of sample increases. At concentration of 500 µg/mL the PS showed reducing power of 0.5. Oxidation of β carotene and linoleic acid generate free radicals. Hence, β carotene is oxidized, and gradually losing its orange color which is then monitored spectrophotometrically. The PS had inhibition effect on β carotene bleaching. The PS showed 50% inhibition at a concentration of 180 µg/mL.

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