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

THESIS SUBMITTED TO VIDYASAGAR UNIVERSITY

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

(SCIENCE)

BY

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2016

“Dedicated to my beloved Parents”

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CERTIFICATE FROM THE SUPERVISOR

This is to certify that the research work presented in the thesis entitled **"Isolation, purification and characterization of bioactive polysaccharides from an edible mushroom, *Russula albonigra*"** has been carried out under my direct supervision and a bonafide research work of **Mr. Ashis Kumar Nandi**. This work is original and has not been submitted for any degree/diploma or any academic award anywhere before.

Date:

(Syed Sirajul Islam)
Research Supervisor

DECLARATION

I hereby declare that the research work embodied in this thesis has been carried out by me in the Department of Chemistry & Chemical Technology, Vidyasagar University, Midnapore-721 102, West Bengal, India, under the supervision of Professor Syed Sirajul Islam, Department of Chemistry & Chemical Technology, Vidyasagar University, Midnapore-721 102, West Bengal, India. I also affirm that this work is original and has not been submitted before in part or full for any degree/diploma or any other academic award to this or any other University or Institution.

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ACKNOWLEDGEMENTS

*I would like to express my sincere respect and gratitude to my supervisor **Dr. Syed Sirajul Islam**, Professor, Department of Chemistry & Chemical Technology, Vidyasagar University, Midnapore-721102, West Bengal, India, for his excellent guidance, constant encouragement and valuable suggestions throughout the whole course of my research work.*

I express my warm regards and deepest gratitude to all the faculty members of our Department, Prof. Braja Gopal Bag, Prof. Ajay Kumar Misra, Dr. Sudipta Dalai, Dr. Sumita Roy, Dr. Subal Chandra Manna, Dr. Maidul Hossain and Prof. Amiya Kumar Panda for motivating me during my research work.

I am thankful to all the staff members of this Department for their co-operation and support.

I record my special thanks to all senior and junior research scholars of our laboratory: Dilip da, Soumitra da, Indranil da, Sunil da, Kaushik da, Bivas da, Krishnendu da, Debsankar da, Sadhan da, Subhas da, Arnab da, Pradip da, Soumitra da (Jr.), Sukesh da, Kankan da, Ramsankar da, Chanchal da, Biswajit da, Sanjoy da, Saikat da, Kousik da, Eshita di, Ipsita da, Praloy da, Dilip da (Jr.), Prasenjit, Surajit, and Manabendra for their help and co-operation.

It's a great pleasure to express my sincere thanks to Dr. Tapas Kumar Maiti, Department of Biotechnology, I.I.T., Kharagpur-721 302, West Bengal, India, and Dr. Krishnendu Acharya, Professor, Molecular and Applied Mycology and Plant Pathology Laboratory, Department of Botany, University of Calcutta, 35, Ballygunge Circular Road, Kolkata-700019, West Bengal, India for their co-operation and helpful suggestions.

I am extremely thankful to Mr. Barun Majumder, Bose Institute, Kolkata-700 054, West Bengal, India, for preparing NMR spectra.

I express my profound regards to my grandfather (Late Binoy Krishna Nandi), my parents (Shri Kanailal Nandi and Smt. Ava Rani Nandi), and other family members for their inspiration and encouragement during the whole course of my work.

It is very difficult to mention every individual name and his/her contribution to my life. I thank all of them.

*I greatly acknowledge the Council of Scientific and Industrial Research (**CSIR**), Government of India for providing me financial assistance.*

I desire to express my sincere gratitude to Hon'ble Vice-Chancellor, Prof. Ranjan Chakrabarty and the Registrar, Dr. Jayanta Kishore Nandi, Vidyasagar University for giving me such a wonderful opportunity to accomplish my work.

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Russula albonigra (Krombh.) Fr.

Ectomycorrhizal Edible Mushroom

Scientific Classification

Kingdom:	**Fungi**
Division:	**Basidiomycota**
Class:	**Agaricomycetes**
Order:	**Russulales**
Family:	**Russulaceae**
Genus:	***Russula***
Species:	***Albonigra***

PREFACE

The research work embodied in the present thesis entitled "**Isolation, purification and characterization of bioactive polysaccharides from an edible mushroom, *Russula albonigra***" deals with the structural studies as well as some important biological activities of different polysaccharides isolated from the fruiting bodies of an ectomycorrhizal edible mushrooms *Russula albonigra* (Krombh.) Fr.

The thesis has been presented in five different chapters including abstract.

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

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

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

Chapter-4: It deals with the 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: This chapter represents the isolation and purification of β-glucan (PS), isolated from the 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.

Each copy of the publications has been attached at the end of the thesis.

ABBREVIATIONS

Ac	Acetyl
Ac_2O	Acetic anhydride
AcOH	Acetic acid
ADCC	Antibody dependent cell mediated cytotoxicity
AF	Antibody formation
AIDS	Acquired immune deficiency syndrome
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BRM	Biological response modifier
BSTFA	N,O-bis(trimethylsilyl)trifluroacetamide
BuOH	Butanol
CF_3COOH	Trifluoro acetic acid
CH_3CO_2H	Acetic acid
$CHCl_3$	Chloroform
CH_3I	Methyl iodide
CH_3OH	Methanol
cm	Centimetre
Con A	Concanavalin A
CTL	Cytotoxic T-Lymphocyte
°C	Degree centigrade
1D	1-Dimensional
2D	2-Dimensional
Da	Dalton
DEPT	Distortionless enhancement by polarization transfer
DEAE	Diethyl aminoethyl
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxy ribo nucleic acid
DQF-COSY	Double-quantum filtered correlation spectroscopy

D_2O	Deuterium oxide
EDTA	Ethylene diamine tetra acetic acid
EtOAc	Ethyl acetate
EtOH	Ethyl alcohol
FBS	Fetal bovine serum
$FeCl_3$	Feric chloride
g	Gram
Glc	Glucose
GLC	Gas-liquid chromatography
GLC-MS	Gas-liquid chromatography mass spectrometry
GPC	Gel permeation chromatography
h	Hour(s)
HBSS	Hank's balanced salt solution
HCL	Hydrochloric acid
HDP	Host defense potentiator
HCOOH	Formic acid
HIV	Human immunodeficiency virus
HMBC	Heteronuclear multiple bond correlation
HOD	Deuterated water
HPLC	High performance liquid chromatography
HSQC	Heteronuclear single quantum coherence
Hz	Hertz
H_2O	Water
H_2O_2	Hydogen peroxide
H_2SO_4	Sulfuric acid
IL-1	Interlukine-1
IL-2	Interlukine-2
J	Coupling constants
Kg	Kilogram
KOH	Potasium hydroxide

KH_2PO_4	Potasium hydrogen phosphate
LAL	Limulus amebocyte lysate
LFPS	LPS free polysaccharide
LPS	Lipopolysaccharide
M	Molar
MDA	Malondialdehyde
Me	Methyl
MIC	Minimum inhibitory concentration
mg	Milligram
MHz	Mega hertz
min	Minute(s)
mL	Mililiter
mM	Millimolar
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Mw	Molecular weight
m/z	Mass to charge ratio
$NaBH_4$	Sodium borohydrate
$NaIO_4$	Sodium metaperiodate
NBT	Nitro blue tetrazolium
NCCS	National Centre for Cell Science
NK	Natural killer cell
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NOE	Nuclear overhauser effect
NOESY	Nuclear overhauser enhancement spectroscopy
OS	Oligosaccharides
p	Pyranose
P_2O_5	Phosphorus pentoxide
PBS	Phosphate buffered saline

PC	Paper chromatography
PMAA	Partially methylated alditol acetate
ppm	Parts per million
PS	Polysaccharide
PSK	Protein bound polysaccharide
R	*Russula*
RBC	Red blood cell
RNA	Ribo nucleic acid
ROS	Reactive oxygen species
rpm	Rotation per minute
RPMI	Roswell Park Memorial Institute
SPI	Splenocyte proliferation index
TBA	Thiobarbituric acid
TCA	Trichloro acetic acid
TFA	Trifluoro acetic acid
TOCSY	Total correlation spectroscopy
TMS	Tetra methyl silane
TPI	Thymocyte proliferation index
UV	Ultraviolet
vis	Visible
v/v	Volume by volume ratio
α	Alfa
β	Beta
λ_{max}	Absoption maximum
μg	Microgram
μL	Microliter
μM	Micromolar

CONTENTS

Chapter-2: 33-56

Methodology

ABSTRACT

The present thesis entitled "**Isolation, purification and characterization of bioactive polysaccharides from an edible mushroom, *Russula albonigra***" is mainly based on the determination of the structure as well as some important biological activities of different polysaccharides isolated from the fruiting bodies of an ectomycorrhizal edible mushrooms *Russula albonigra* (Krombh.) Fr. The entire thesis is divided into five chapters.

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). 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 eluant. 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. The polysaccharide was methylated by the Ciucanu and Kerek

method, 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 (PS-I) isolated from hot aqueous extract of the fruiting bodies of an edible mushrooms *Russula albonigra* (Krombh.) Fr.

Structural analysis of PS-I

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**.

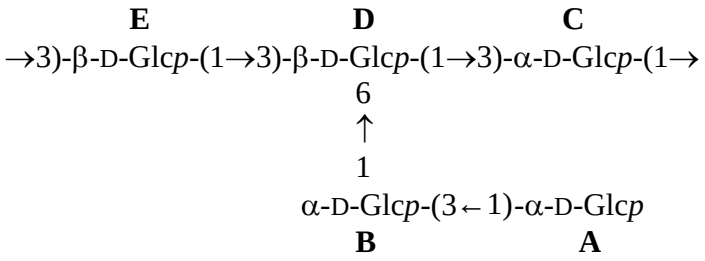
The sequences of glucosyl moieties were determined from ROESY (Fig.4, Table 2) as well as NOESY (not shown) experiments. In ROESY experiment, the inter-residual contacts **A**H-1/**B**H-3; **B**H-1/**D**H-6a, **D**H-6b; **C**H-1/**E**H-3; **D**H-1/**C**H-3 and **E**H-1/**D**H-3 along with some other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

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

B **D** **E** **D**
→3)-α-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→ ; →3)-β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→ ;
3 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 (PS-II) isolated from hot aqueous extract of the fruiting bodies of an edible mushrooms *Russula albonigra* (Krombh.) Fr.

Structural analysis of PS-II

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, $(1\rightarrow2)$-linked L-fucopyranosyl, $(1\rightarrow3)$-linked D-glucopyranosyl, $(1\rightarrow3,4)$-linked D-glucopyranosyl, $(1\rightarrow6)$-linked D-galactopyranosyl, and $(1\rightarrow2,6)$-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 $(1\rightarrow3)$-linked and $(1\rightarrow3,4)$-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**.

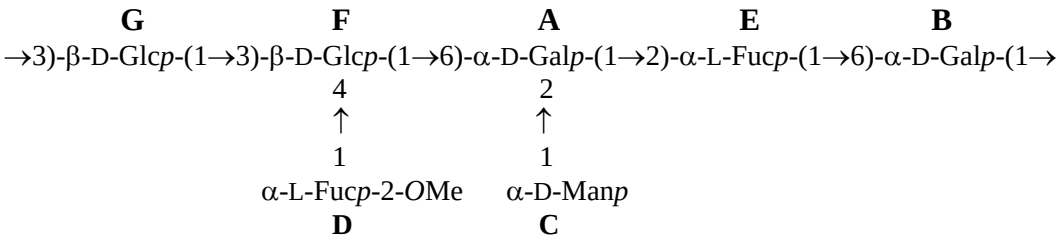
The sequences of glycosyl moieties were determined from ROESY as well as NOESY experiments. In ROESY experiment, the inter-residual contacts **A**H-1/**E**H-2; **B**H-1/**G**H-3;

CH-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 (PS), 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 -glucan (PS)

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

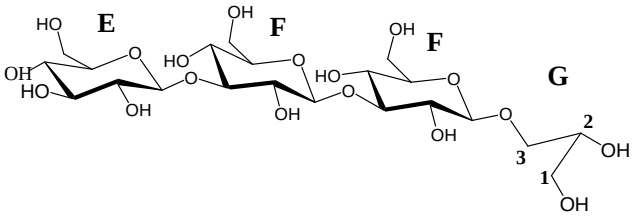
SDPS revealed the presence of 1,5-di-*O*-acetyl 2,3,4,6-tetra-*O*-methyl-D-glucitol and 1,3,5-tri-*O*-acetyl 2,4,6-tri-*O*-methyl-D-glucitol in a ratio of nearly 1:2. Partial hydrolysis of the β-glucan was carried out with 0.1 M TFA to know the backbone sequence of the β-glucan in the repeating unit. 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

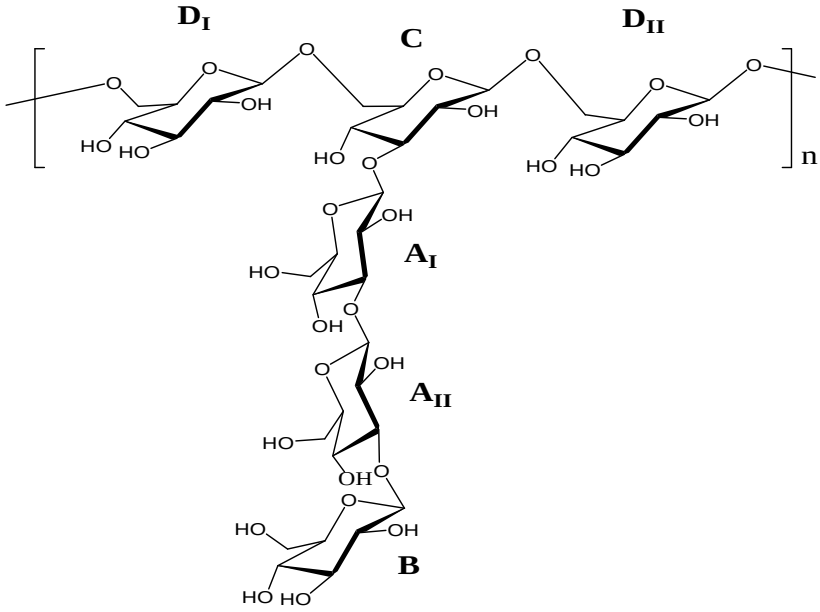
downfield shifts, indicating the presence of (1→3,6)-linked β-D-Glc*p*. Two **D** residues (**D_I** and **D_{II}**) were same in all chemical shift values except the values of C-6. The different downfield shifts of C-6 (δ 68.8 and 69.0) of two **D** residues supported the presence of (1→6)-linking in 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 -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 -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.

This work has been published in ***Carbohydrate Polymers*, 2014**, *99*, 774-782.

CHAPTER-1

General introduction to carbohydrates and bioactive polysaccharides

1.1. Carbohydrates

Carbohydrates are produced on the earth through photosynthesis and essential components of all living organisms. They play an important role in the structure of DNA and RNA and are also involved in cellular recognition process. These molecules play a vital role in the evolution of life and also provide the energy for life processes including growth and movement [Hiller, 1987]. A carbohydrate is an organic compound with the empirical formula $C_x(H_2O)_y$. But the class is too large to fit into such a simple formula. Hence, a more comprehensive definition of carbohydrate was proposed by Robyt [1998] as "Carbohydrates are polyhydroxy aldehydes or ketones or compounds that can be derived from them by oxidation, reduction or replacement of different functional groups."

❖ Classification of carbohydrates

Carbohydrates are classified into four groups: monosaccharides, disaccharides, oligosaccharides, and polysaccharides.

Monosaccharides are chiral polyhydroxy aldehyde or ketone to a smaller carbohydrate unit which cannot be hydrolysed. They are crystalline solids containing a single aldehyde or ketone functional groups that often exist in cyclic hemiacetal form. The simplest likely sugar is glyceraldehyde, a three carbon sugar that cannot form a cyclic hemiacetal. Some examples of monosaccharides are glucose, mannose, galactose, fructose, ribose, and arabinose.

Disaccharides are composed of two monosaccharide units bound together by a glycosidic linkage. Sucrose is the sweetest of the disaccharides. It is composed of glucose and fructose. Lactose, made up of galactose and glucose, is also a very important disaccharide present in the milk of the mammals.

Oligosaccharides are carbohydrates that are composed of several monosaccharide residues joined through glycosidic linkages. Carbohydrates consisting of three to ten monosaccharide residues with a defined structure are oligosaccharides. According to the number of units, they are called trisaccharides, tetrasaccharides, pentasaccharides etc.

Maltotriose is composed of three glucose units whereas raffinose is formed by glucose, galactose, and fructose units. Stachyose is the example of tetrasaccharide.

❖ Polysaccharides – the first biopolymer

The great bulk of the carbohydrates in nature are present as polysaccharides. Polysaccharides are complex and large macromolecule which was proposed as the first biopolymer formed on Earth [Tolstoguzov, 2004]. These are made up of monosaccharide joined together by glycosidic linkages. Their structures may be linear or they may contain various degrees of branching. Depending on the structure, these macromolecules possess distinct properties compared to their monosaccharide building blocks. They are amorphous, insoluble in water, and have no sweet taste [Varki et al., 2008]. The polysaccharides serve two principal functions: (1) used by both 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. Hence, polysaccharides are extremely important in organisms for the purpose of energy storage as well as structural integrity. They are produced by a variety of species including microbes, algae, plants, and animals and play important roles within the biology of life processes. In comparison to other biopolymers like proteins and nucleic acids, polysaccharides offer the highest capacity for carrying biological information because they have potential structural variability [Sharon and Lis, 1993].

❖ Classification of polysaccharides

- **Based on the type of the monomers, polysaccharides are divided into two categories:**

  (i) Homopolysaccharides: These are composed of only one type of monosaccharide and known as homopolysaccharides e.g. cellulose, starch, glycogen, chitosan etc.

  (ii) Heteropolysaccharides: Polysaccharides that are constituted of different type of monosaccharide units are known as heteropolysaccharides e.g. arabinoxylan, galactomannan, xyloglucan, glucomannan etc.

- **Based on structural features, polysaccharides are mainly classified into three categories**

(i) Cationic polysaccharides:

Natural: chitosan

Semi-Natural: cationic guar gum, cationic hydroxyethylcellulose (HEC)

(ii) Anionic polysaccharides:

Natural: pectin, alginic acid, hyaluronic acid, xanthan gum, gum arabic, gum karaya, gum tragacanth

Semi-Natural: cellulose gum, carboxymethyl-chitin

(iii) Nonionic polysaccharides:

Natural: starch, dextrin, guar gum

Semi-Natural: cellulose ethers (hydroxyethylcellulose, methylcellulose, nitrocellulose)

❖ Some common and essential polysaccharides

➢ Starch

Starch is a polymer consisting of glucopyranosyl residues joined by α-glycosidic linkages. They are water insoluble molecules and form a colloidal dispersion in hot water. Starch generally contains 20-25% amylose and 75-80% amylopectin [William, MacArdle, and Victor, 2006]. Amylose is a linear glucose polymer with α-(1→4) glycosidic linkages [**Figure 1**], whereas amylopectin is a branched glucan composed of (1→4)-α-D-Glc*p* and (1→4,6)-α-D-Glc*p* residues. Plants store glucose as amylose or amylopectin. Rice, wheat, potato, sorghum and maize (corn) are the major sources of starch in human diet.

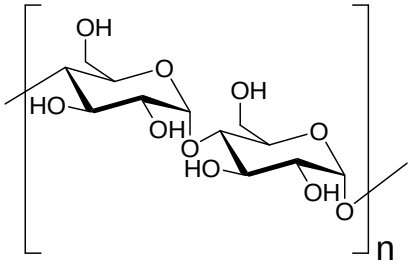


Figure 1. α-amylose, the linear component of starch

➢ Glycogen

Glucose is stored as glycogen in animal tissues by the process of glycogenesis. When glucose cannot be stored as glycogen or used immediately for energy, it is converted to fat. It is a homopolysaccharide having similar structure to amylopectin with more extensive branching. It is stored in liver and muscles. Glycogen is sometimes referred to as *animal starch*. It is composed of a branched chain of glucose residues. It is a polymer of α-(1→4) glycosidic linkages, with α-(1→6)-linked branches. It is insoluble in water and turns red when mixed with iodine. Glycogen plays an important role in the glucose cycle and found in the form of granules in the cytoplasm of several types of cells. It acts like an energy reserve that can be quickly used for sudden need for glucose. The amount of glycogen stored in the body especially within the muscles and liver depends on metabolic rate and eating habits [Ingermann and Virgin, 1987; Miwa and Suzuki, 2002]. Small amounts of glycogen are found in the kidneys, and even smaller amounts in certain glial cells in the brain and white blood cells. The uterus also stores glycogen during pregnancy to nourish the embryo [Neil et al., 2006].

➢ Cellulose

Cellulose is the major component of the primary cell wall of green plants [Crawford, 1981; Updegraff, 1969] which is composed of β-(1→4)-D-glucose [**Figure 2**]. It is an unbranched polymer with about ten thousand glucose units per chain. Hydroxyl groups projecting out from each chain form hydrogen bonds with neighboring chains and create a rigid cross-linking between the chains which make cellulose as strong support material. It is the most abundant organic substance in the living world and it has been estimated that more than half of the total organic carbon on the planet comes from cellulose.

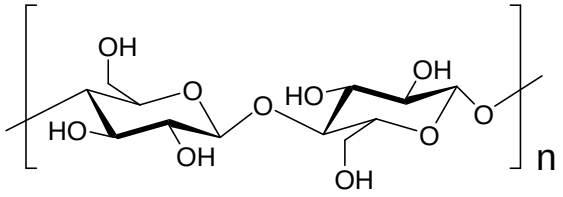


Figure 2. Cellulose, β-(1→4) linked glucose polymer

➢ Chitosan

Chitosan is one of the most abundant naturally occurring polysaccharide in the world. Chitin is a homopolysaccharide composed of β-(1→4)-linked N-acetyl-D-glucosamine residues [Rianaudo, 2006]. Removal of N-acetyl groups from chitin yields chitosan [**Figure 3**] which has enhanced solubility. It is obtained from the outer skeleton of shellfish, including crab, lobster, and shrimp. Chitosan is used to control obesity, cholesterol and Crohn's disease. It is also used in the treatment of patients of kidney failure, loss of strength and appetite, and trouble sleeping (insomnia). In pharmaceutical industry, chitosan is used as filler in tablets as a carrier in controlled drug delivery.

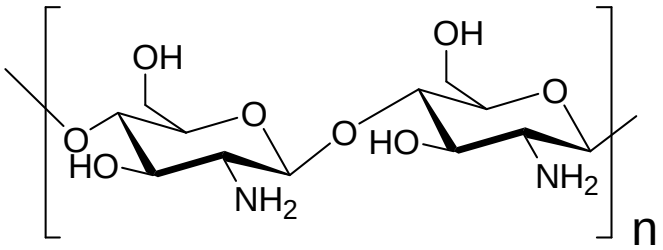


Figure 3. Chitosan, β-(1→4)-linked N-acetyl-D-glucosamine

➢ Inulin

Inulin is the reserve polysaccharide of many plants. It consists of thirty (1→2)-β-fructose units and has a molecular weight of about 5,000. Fructose is present as furanose ring in inulin. These are present in many vegetables and fruits, including onions, garlic, bananas, asparagus, and chicory etc. These are also called fructans as it consists of fructose units that typically have a terminal glucose unit. Inulin is nondigestible by human intestinal enzymes, but they are totally fermented by colonic microflora.

➢ Pectin

Pectin is a complex set of heterogeneous polysaccharides containing galacturonic acid or its ester in the backbone [**Figure 4**]. These are important part of human diet but do not play a significant role to nutrition. They are present in most primary cell walls and in the non-woody parts of terrestrial plants. Fruits like apples, quince, plums, gooseberries, oranges and other citrus fruits contain much pectin, while soft fruits like cherries, grapes and strawberries contain small amount of pectin. Pectins are traditionally used as gelling

agent, thickening agent for the production of jams and jellies. It is also used in fillings, medicines, sweets, as a stabilizer in fruit juices and milk drinks, and as a source of dietary fiber. Pectins have anti-inflammatory activity and other pharmaceutical activities [Attele, Wu, and Yuan, 1999]. They can reduce glucose levels in normal and hyperglycemic mice, inhibit gastric lesions and also protect animals from the lethal effects of ionizing radiation [Suzuki and Hiking, 1989].

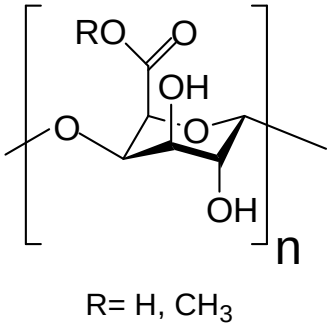


Figure 4. Pectins contain α-(1→4)-linked galacturonic acid or its ester in the backbone

➢ Heparin

Heparin is an acidic mammalian polysaccharide consisting of highly sulfated (1→4)-linked hexosamine and uronic acid residues [**Figure 5**]. It is commonly extracted from animal tissues such as bovine lung and porcine intestine. It has a variety of biological functions including blood anticoagulation, mitogenesis, cell migration and anti-inflammation promotion of cell adhesion [Laremore et al., 2009].

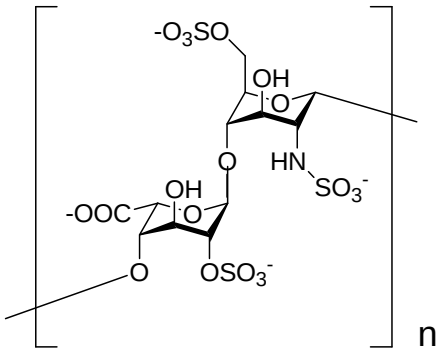


Figure 5. Heparin, sulfated (1→4)-linked hexosamine and uronic acid

➢ Hyaluronic acid

Hyaluronic acid (HA) is the simplest mucopolysaccharide. It is a linear, high molecular weight polysaccharide [**Figure 6**] commonly found in soft connective tissues of animals. HA is comprised of disaccharide repeating units of glucuronic acid and N-acetyl glucosamine residues. HA is mostly useful in a number of medical applications due to its biocompatibility and rheological properties [Doughty and Glavin, 2009].

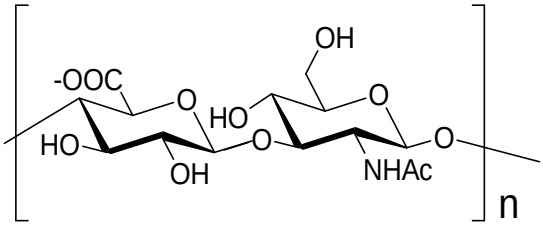
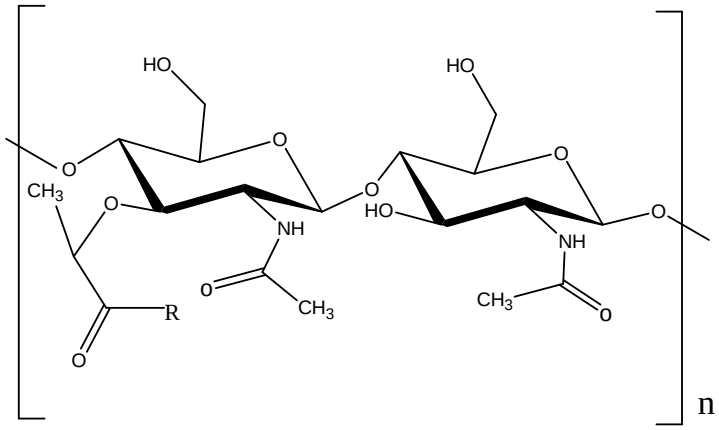


Figure 6. Hyaluronic acid, consisting of disaccharide repeating unit of glucuronic acid and N-acetyl glucosamine

➢ Murein

Murein (peptidoglycan) is the structural component of the cell wall of all bacterial species like *Escherichia coli* etc. It consists of β-(1→4)-linked *N*-acetyl glucosamine (GlcNAc) and *N*-acetyl-D-muraminic acid (MurNAc) [**Figure 7**]; the latter differing from GlcNAc where the 3-position is substituted with an *O*-lactic acid group. The peptides are attached by an amide linkage to the lactic acid of MurNAc and they are unusual because they contain rare D-amino acids. Molecular modeling suggested that the glycan strands are rigid structures, whereas the peptides are flexible [Vollmer and Bertsche, 2008].



R= Different peptide side chains

Figure 7. Murein (peptidoglycan), consisting of β-(1→4)-linked *N*-acetyl glucosamine (GlcNAc) and *N*-acetyl-D-muraminic acid (MurNAc) with different peptide side chains

➢ Arabinoxylan

Arabinoxylans are the copolymers of two pentose sugars arabinose and xylose and usually classified as pentosans. It consists of a xylan backbone with L-arabinofuranose attached randomly by α-(1→2) and/or α-(1→3) linkages to the xylose units throughout the chain [Dervilly-Pinel et al., 2004]. Another arabinoxylan reported by the present research group contains a backbone chain of (1→4)-α-D-xylopyranosyl residue, substituted at C-2 with one unit of two adjacently linked (1→3)-α-L-arabinofuranosyl residues and the other one was terminated by β-L-arabinofuranosyl residue [Das et al., 2013]. These are located both in primary and secondary cell walls of plants including woods and cereal grains [McCartney et al., 2005]. They exhibit antioxidant activity owing to their bound phenolic acids [Rao and Muralikrishna, 2006].

➢ Galactomannan

Galactomannans are polysaccharides consisting of the mannopyranose backbone with β-(1→4) linkages to which α-(1→6) linked galactopyranose units are attached as side group [Mathur and Mathur, 2005]. They are present in several vegetable gums that are used to increase the viscosity of food products. The approximate ratios of mannose to

galactose for the following gums are Fenugreek gum (1:1) [Chatterjee et al., 1982], Guar gum (2:1), Tara gum (3:1), and Locust bean gum or Carob gum (4:1).

1.2. Mushrooms

1.2.1. Definition and description

Mushroom is "a macro fungus with distinctive fruiting body which can be hypogeous or epigeous, large enough to be seen with the necked eye and to be picked up by hand" [Chang and Miles, 1992]. Fungi are extraordinary organism, which are neither plants nor animals. Generally, fleshy species of the group of fungi are called mushrooms [Lakhanpal and Rana, 2005]. The mushroom cell is encapsulated by an extracellular matrix called cell wall which protects it from osmotic pressure, environmental stress and determines the shape of the cell. The cell wall has been described as a rigid layer of glycoprotein and polysaccharide which is dynamic enough to survive with cell growth.

The mushroom fruiting body may be typically looking like umbrella or of various other shape, size and color. Commonly, it consists of a cap or pileus and a stalk or stipe but others have additional structures like scale, ring or skirt, gills, tubes, volva etc as shown in **Figure 8**.

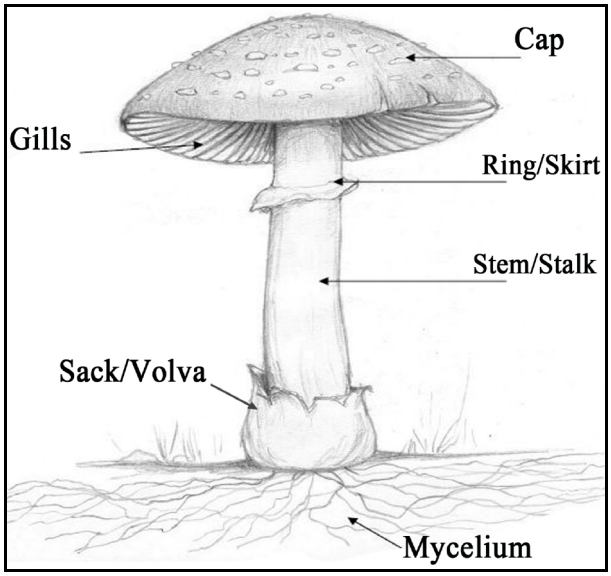


Figure 8. Different parts of Mushroom [Mushroom structure: Teacher's notes, www.fungi4school.org]

1.2.2. Ectomycorrhizal Mushroom

Mycorrhiza is a special type of fungus that lives within and outer surface of the roots of plants, especially trees and accordingly they are known as endomycorrhiza and ectomycorrhiza. The mutuality association of plant roots and fungi is beneficial to both organisms, and they live together through symbiotic mechanism. Mycorrhiza confers many attributes to plants such as growth stimulation due to increased nutrient uptake, and bio-control of root diseases. Their role in making plants resistant to draught and frost is worth mentioning. Ectomycorrhizal fungi assist the plants in extracting nutrients like phosphorus and other minerals and in return receive photosynthetically fixed sugars from the host plant for their growth and development [Chakraborty et al., 2004; Lilleskov et al., 2009; Mehrotra, 1991]. About 90% of all trees depend on micorrhizal fungus. *Russula albonigra* (Krombh.) Fr. [**Figure 9**] , an ectomycorrhizal and basidiomycetes fungus, family Russulaceae grows in symbiotic relationship with the roots of Sal (Shorea robusta) and other coniferous trees in the forest during rainy and autumn season. It was collected from the Sal forests of Bisnupur, Bankura, West Bengal, India by the research group of Dr. Krishnendu Acharya, Professor, Department of Botany, University of Calcutta, West Bengal. The voucher specimens had been deposited with the accession code AMFH-143 in the Mycological Herbarium of the University of Calcutta, Kolkata and identified by Dr. Acharya who is also a mycologist.

Figure 9. Photograph of the fruit bodies of an ectomycorrhizal edible mushroom, *Russula albonigra* [Accession code AMFH-143, Mycological Herbarium of the University of Calcutta, Kolkata, and West Bengal, India].

1.2.3. Chemical composition and nutritional value

Mushrooms are nutritionally renewable natural gift for humankind. Mushrooms possess ~ 90% water by weight. The remaining 10% consists of 10-40% protein, 2-8% fat, 3-28% carbohydrate, 3-32% fiber and 8-10% ash with potassium, calcium, phosphorous, magnesium, and iron, zinc and copper [Breene, 1990]. Most mushrooms contain some vitamins, particularly niacin, thiamine, riboflavin, and vitamin C. Besides actual nutrients, mushrooms contain a wide variety of bioactive molecules including terpenoids, steroids, phenols, nucleotides, glycoproteins and polysaccharides. Hence, mushrooms are good sources of dietary supplements, functional foods, phyto-chemicals, and nutraceuticals [Chang and Buswell, 1996; Zeisel, 1999]. These dietary supplements are used for the enhancement of health, fitness, and prevention of various human diseases.

From mycological point of view, the edible part of the fungus, mainly the fruit body is called basidiocarp, which is the outcome of the modification of secondary and tertiary mycelium. It is reported that mushroom are being used widely in many countries for food and fodder [Cheung, 2008; Ghorai et al., 2009; Sands, 2013]. Mushrooms have excellent flavor and taste, with high nutritive values [Wang et al., 2014]. Studies on a number of species of mushrooms have revealed that in addition to the flavoring properties, the protein part of some mushrooms is equal to animal muscle protein in nutritive value [Fitzpatrick et al., 1946; Lintzel, 1941]. Chemical analyses have shown that the composition of amino and fatty acids, vitamins, minerals present in the commercially available edible mushrooms is comparable to meats and higher than fruits and vegetables. It contains large amount of well-balanced essential amino acids [Mattaila et al., 2001].

1.2.4. Medicinal properties

Although, mushrooms are used as tasty, edible, and nutritional food worldwide [Lucas et al.,1957] they are also used in folk medicine and considered to be one of the most useful antitumor agents for clinical uses [Wasser and weis, 1999b; Mizuno et al., 1996]. Currently, mushroom derived substances having antitumor and immunomodulating properties are used as dietary supplements or drugs [Brochers et al., 1999; Mizuno et al.,

1999]. Different parts of the mushroom are being used for curing of blood sugar, high blood pressure, and beauty treatment [Wasser and weis, 1999a].

1.3. Mushroom polysaccharides

Biologically active polysaccharides are present in fruit bodies, cultured mycelium, and culture broth of mushrooms. A number of bioactive polysaccharides have been identified in many mushroom species with antitumor and immunomodulating properties [Tzianabos, 2000; Mizuno, 1999]. Mushrooms are potential source of different polysaccharides like chitin, hemicelluloses, glucans and heteroglycans. Mushroom polysaccharides are present generally as glucans with different types of glycosidic linkages, such as (1→6)-β-D-glucan [Bhanja et al., 2012], (1→3)-β-D-glucan [Ohno et al., 1993; Chakraborty et al., 2006], (1→6)-, (1→3,6)-β-D-glucans [Maity et al., 2013], (1→3)-, (1→3,6)-β-D-glucan [Bhanja et al., 2012; Yoshioka et al., 1985], (1→3)-α-D-glucan [Wang et al., 2007; Yoshida et al., 1996], (1→6)-α-D-glucan [Rout et al., 2004], (1→3)-, (1→6)-branched α,β-D-glucan [Rout et al., 2005] and some are true heteroglycans [Mandal et al., 2011; Bhunia et al., 2010]. In some mushroom species, active polysaccharides bound with proteins or peptides are known as polysaccharide-protein or polysaccharide-peptide complex [Cui and Chisti, 2003].

1.3.1. Structures and biological activities

A broad range of biologically active polysaccharides of different chemical structures from mushrooms have been investigated. Studies on therapeutic activities of mushroom polysaccharides include antitumor, immunomodulating, anti-inflammatory, antiviral, antioxidative, cytotoxic, and antidiabetic effects [Smiderle et al., 2008; Hobbs, 2000; Zhang, Cui and Cheung, 2007]. The most significant molecule isolated from mushrooms so far is β-D-glucan which consist of D-glucose monomers, joined together by β-glycosidic linkages. The structures of the several biologically active linear and branched β-D-glucans have been reported. There are series of investigations on β-D-glucan which revealed that they possess antitumor and anticancer activities [Morikawa et al., 1985; Mansell et al., 1975]. It has been established that the most active forms of β-glucans

contain (1→3)-, (1→6)-, and (1→3,6) linkages. (1→3)-, (1→6)-, and (1→3,6)-β-D-glucans are able to enhance and stimulate the immune system of humans and thus called biological response modifiers (BRMs) [Miura et al., 1996]. Glucans having α- or both α- and β- linkages are also isolated from different mushrooms [Rout et al., 2004; Zhang and Cheung, 2002; Mizuno et al., 1990; Wang et al., 2007]. (1→3)-α-D-glucan derivatives have shown important medicinal properties [Ghoneum et al., 1995; Kiho et al., 1994]. Linear low molecular weight (1→4)-α-D-glucan is used as an immunomudulator and anti-cancer agent [Mizuno et al., 1995; Matsushita et al., 1998]. There are also some reports on antitumor and immunoenhancing cytotoxic polysaccharides of heteroglycans [Gao et al., 1996; Mandal et al., 2011], β-glucan-protein complexes [Kawagishi et al., 1990], α-manno-β-glucan [Mizuno et al., 1995], α-glucan-protein complexes and heteroglycan-protein complexes [Zhuang et al., 1993].

The activity of mushroom polysaccharides depends greatly on their molecular structure, molecular weight, size, branching pattern, structural modification, conformation, and solubility. It is true that structural features such as β-(1→3) linkages in the main chain of the glucan and additional β-(1→6) branch points are needed for antitumor action. High molecular weight glucans appear to be more effective than those of low molecular weight [Mizuno et al., 1999]. The immune functions of mushroom polysaccharides apparently depend on their conformational complexity. It has been found that higher degree of structural complexity is associated with more potent immunomodulatory and anti-cancer effects. A triple-helical conformation of (1→3)-β-D-glucans is known to be important for their immune-stimulating activity. Polysaccharides that form triple-helical conformation have clinical applications for the treatment of cancers like human breast cancer (MCF-7), human promyelocytic leukemia (HL-60), and human liver cancer (HpG2) [Zhang, Cui and Cheung, 2007]. Solubility in water is one of the more important characteristics of mushroom polysaccharides. In mushrooms, β-glucans are present either in their water soluble or insoluble form.

It has been found that structural modifications sometime improve the medicinal properties [Mizuno et al., 1999] as well as water solubility of the mushroom polysaccharides. The procedures used for modification of mushroom polysaccharides are

Smith degradation, formolysis, and carboxymethylation. A water insoluble, alkali soluble linear (1→3)-α-D-glucan obtained from *Amanita muscaria* and *Agrocybe aegerita* had little antitumor effect, while carboxymethylated products showed potent antitumor activity [Yoshida et al., 1996].

1.3.2. Some important antitumor and immunomodulating mushroom polysaccharides

Mushroom polysaccharides have been drawn the attention of chemist and immunobiologists on account of their potent immunostimulating and antitumor properties [Wasser and weis, 1999b; Wasser, 2002]. Polysaccharides are used presently in the treatment of cancer of the digestive organs, lung and breast, as well as cancer of the stomach and cervical cancer respectively [Ooi and Liu, 2000; Wasser, 2002]. Several mushroom polysaccharides are widely used and commercialized worldwide as anti cancer agents for therapeutic purposes. **Lentinan** (from *Lentinus edodes*, **Japan**), **Schizophyllan** (from *Schizophyllum commune),* **Krestin** (from turkey tail mushroom *Trametes versicolor*), **Agarican** (from *Agaricus blazei*, **USA**), and **Grifron-D** (from *Grifola frondosa*, **Japan**) have been commercialized and used clinically as anti-tumor agents.

Lentinan, produced from Shiitake mushroom, *Lentinus edodes,* is a (1→3)-, (1→6)-β-D-glucan [Chihara et al., 1969]. It is now used as an antitumor drug and dietary supplement [Hobbs, 2000]. It is nontoxic to tumor cells, but inhibits tumour growth by stimulating the immune system [Chihara et al., 1978]. Lentinan has been successfully used in prolonging the overall survival of cancer patients, especially those with gastric and colorectal carcinomas [Furue and Kitoh, 1981; Taguchi et al., 1985].

Schizophyllan is the polysaccharide derived from the mushroom *Schizophyllum commune*. The polysaccharide Schizophyllan shows antitumor activity against both the solid and ascite forms of Sarcoma 180, as well as against the solid form of Sarcoma 37, Erlich Sarcoma, Yoshida Sarcoma and Lewis lung carcinoma [Hobbs, 1995]. Early clinical studies with schizophyllan in combination with conventional chemotherapy (tegafur or mitomycin C and 5-fluorouracil) in a study of 367 patients with gastric cancer

showed significant increase in median survival [Furue, 1985]. Studies of schizophyllan in combination with radiotherapy showed significant prolong of survival of stage II cervical cancer patients [Okamura et al., 1986, 1989].

Krestin (PSK) is a unique protein bound polysaccharide obtained from the mushroom *Trametes versicolor*, which has been used as a chemoimmunotherapy agent in the treatment of cancer and also exhibits a marked effect against different types of tumors in Japan and other Asian countries. It contains 75% glucan and 25% protein. It has been shown to have no substantial effect on immune responses of the host under normal conditions [Ehrke et al., 1983; Tsukagosihi et al., 1984]. It can restore the immune potential to the normal level after the host was depressed by tumor burden or anti-cancer chemotherapeutic agents [Dong et al., 1996; 1997]. PSK has great potential as an adjuvant cancer therapy agent, with positive results seen in the adjuvant treatment of gastric, esophageal, colorectal, breast cancers [Iguchi et al., 2001]. It prevents liver cancer, chronic active hepatitis [Ying et al., 1987], lung cancer [Tsang et al., 2003], and also useful for hepatitis B [Amagase, 1987; Lin and Huang, 1987; Mizuno, 1995]. PSK has also been shown to possess antiviral activities against ectromelia virus and cytomegalovirus infections [Tsukagoshi et al., 1984] and cell-free infection of human immunodeficiency virus (HIV) [Tochikura et al., 1987].

Agarican, the polysaccharide isolated from ***Agaricus blazei*** was shown to be a stimulant of immune system, promoting body's natural defense mechanism to fight a variety of infectious agents including cancer. The immunostimulating activity and antitumor action of *Agaricus blazei* extracts were investigated against Sarcoma 180 and fibrosarcoma tumor-bearing mice [Kawagishi et al., 1989, 1990, Ebina et al., 1998]. Seven polysaccharide fractions obtained from *A. blazei* fruit bodies were demonstrated to have antitumor activity [Fujimiya et al., 1998; Mizuno et al., 2002]. A new antitumor polysaccharide, (1→2)-, (1→3)-β-D-glucomannan [Mizuno et al., 1990] active against Sarcoma 180 was recently separated from liquid cultured mycelium of *Agaricus blazei*.

Studies have shown that (1→3)-, (1→6)-β-D-glucan (**Grifron-D, GD**) derived from another mushroom *Grifola frondosa* (also known as Maitake) have strong antitumor activity [Nishida et al. 1988; Zhuang et al., 1994]. GD has been shown to have a cytotoxic

affect on human prostate cancer cells (PC9) in vitro, possibly acting through oxidative stress, and causing 95% cell death by an apoptosis [Fulleroton and Samadi, 2000].

Besides these, a number of antitumor polysaccharides were isolated from other mushrooms and reported. Such as, the β-glucan isolated from ***Pleurotus ostreatus*** has a strong antitutumor activity [Yoshioka et al., 1985].

1.3.3. Mechanism of biological action

The biochemical mechanisms that mediate the biological activity of polysaccharides are still not clearly understood. Mushroom polysaccharides do not attack cancer cell directly but they activate immune systems through stimulation of Natural Killer cells (NK-cell), T-cell, B-cell and macrophage-dependent immune cells in the host. Stimulating this aspect of the immune system, it exhibits carcinostatic activities. Polysaccharides are regarded as biological response modifiers, BRM [Wasser and Weis, 1999a]. BRM has been defined as those agents that modify the host's biological response by stimulation of the immune system, which may result in various therapeutic effects. Several β-D-Glucans [Wasser and Weis, 1999b] and α-D-Glucans [Whistler et al., 1976] are widely used as antitumor and immunomodulating agents. A possible pathway of the biological action of β-D-Glucan has been presented in **Figure 10** [Mizuno, 2002].

Lentinan appears to act as a host defense potentiator (HDP) which is able to restore the responsiveness of host cells to lymphocytokynes, hormones, and other biologically active substances by stimulating maturation, differentiation or proliferation of cells involved in host defense mechanism [Chihara et al., 1987; Chihara, 1992]. HDP is functionally different from BRM. Lentinan is thus able to increase host resistance against various kinds of infectious diseases including AIDS. Lentinan is known to be able to restore the suppressed activity of helper T cells in the tumor-bearing host to their normal state, leading to complete restoration of humoral immune responses [Ooi and Liu, 1999]. Lentinan has also been shown to inhibit suppressor T cells activity *in vivo* and to increase the ratio of activated T cells and cytotoxic T cells in the spleen when administered to gastric cancer patients undergoing chemotherapy. The biological action of Lentinan has been demonstrated by Chihara et al. [1970] and presented in **Figure 11**.

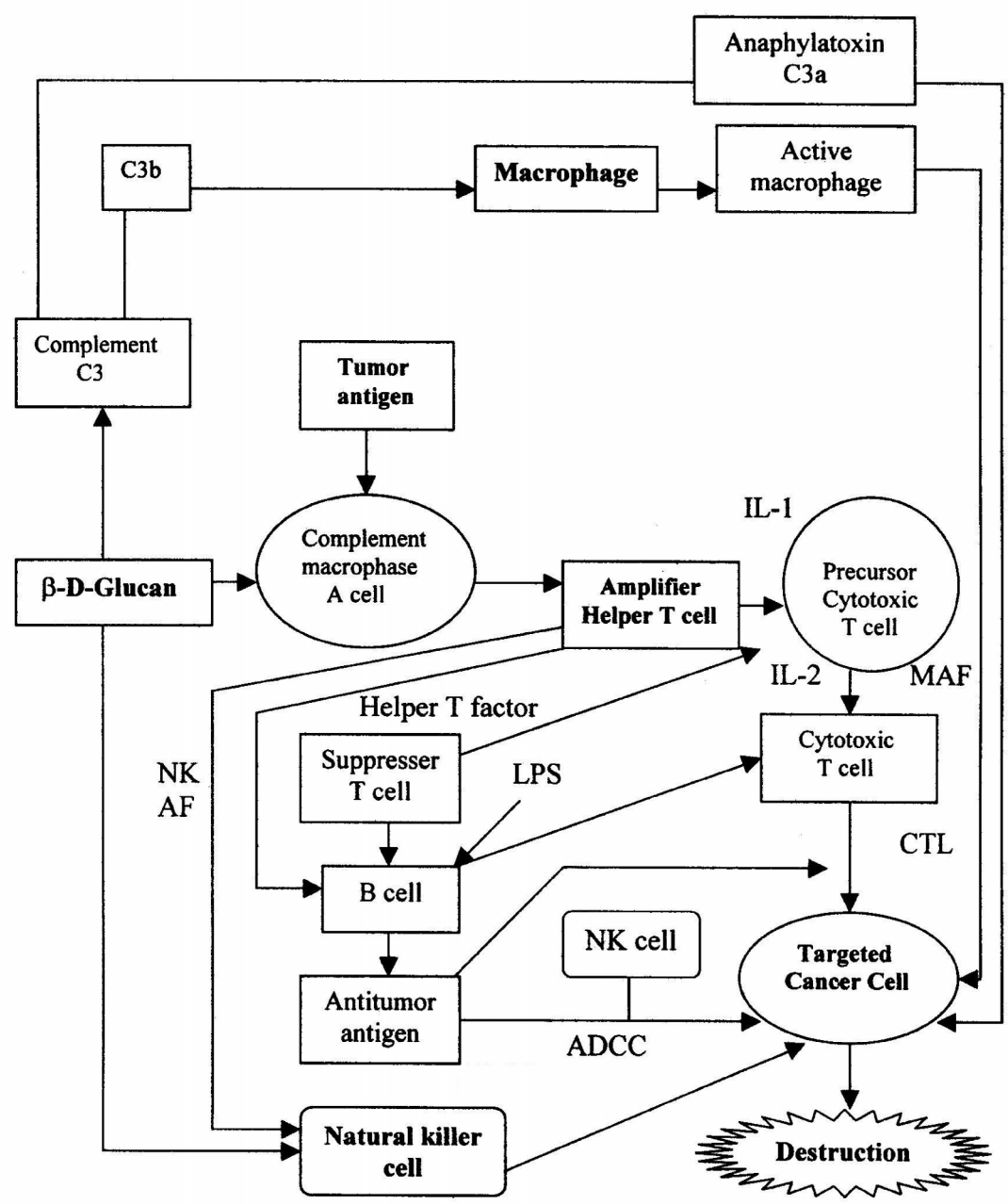


Figure 10. Possible immune mechanism: β-D-glucan as biological response modifier (BRM) to target cancer cells [Mizuno, 2002].

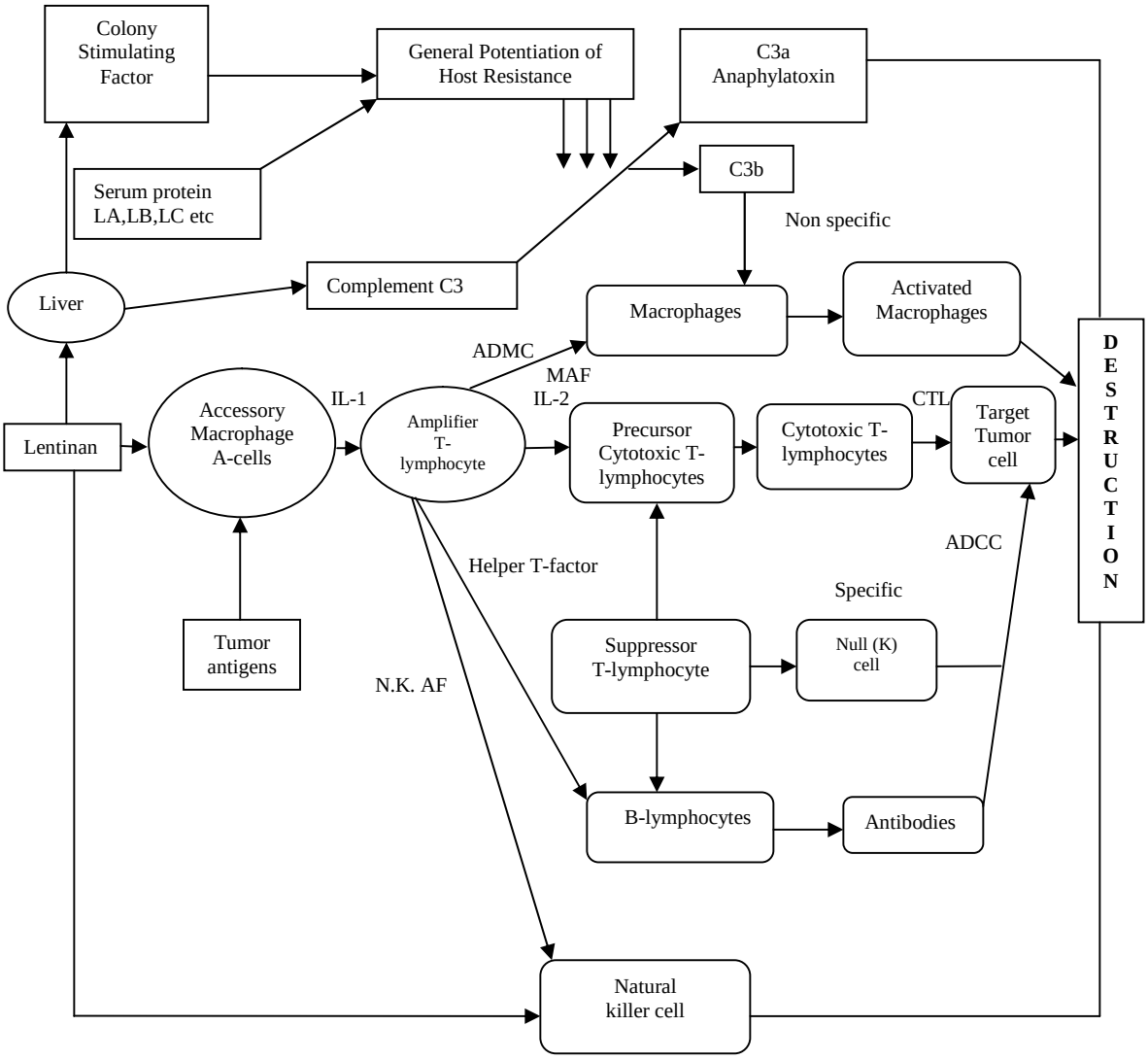


Figure 11. Possible pathways of lentinan action [Chihara et al., 1970; Chihara, 1992]. NK: Natural Killer cell; AF: Antibody Formation; LPS: Liver Protein Serum; ADCC: Antibody Dependent Cell mediated Cytotoxicity; CTL: Cytotoxic T-Lymphocyte; MAF: Macrophage Activating Factor; IL-1: Interlukine 1; IL-2: Interlukine 2

1.4. Antioxidant properties

Oxidation is crucial phenomenon to many living organisms for the creation of energy to stimulate biological processes. Oxidative stress is induced by a wide range of factors such as UV radiation, pathogen invasion, and herbicide action. Generation of reactive oxygen species (ROS) is characteristic of oxidative stress. Free radicals are formed as part of body's normal metabolic process. The oxidative properties of oxygen play a vital role in diverse biological functions such as utilization of nutrients, electron transport to produce ATP, and removal of xenobiotics [Hemnani and Parihar, 1998; Blokhina, Virolainen, and Fagerstedt, 2003]. However, oxygen derived free radicals such as superoxide radical ($O_2^{\cdot}$), hydroxy radicals ($OH^{\cdot}$), and hydrogen peroxide (H_2O_2) etc. are generated during the oxidative metabolism. Neutralization of this radical activity by naturally occurring substances mainly by supplementation of food having antioxidant property is becoming one of the most acceptable modes of modern therapy [Rai and Acharya, 2012].

The oxidant status in humans reflects the dynamic balance between the anti-oxidant defense and pro-oxidant conditions and this has been suggested as a useful device in estimating the threat of oxidative damage [Tiwari, 2004]. The imbalance between pro-oxidant and anti-oxidant due to 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 [Kozarski et al., 2011; Acharya, Giri, and Biswas, 2011]. Antioxidants act as a major defense against radical mediated toxicity by protecting the damages caused by free radicals such as ROS [Manzi et al, 1999]. Antioxidant supplements and food containing antioxidants have been used to help the human body reduce oxidative damage. Recently, phytochemicals in food materials and their effects on health, especially the suppression of active oxygen species by natural antioxidants from teas, spices and herbs have been extensively studied [Ho et al., 1994]. Several Chinese herbs exhibited significant antioxidant activity [Kim et al., 1994; Su, 1992]. Several common and commercial mushrooms like *V. Volvacea, A. biporous, L. edodes, P. cystiodyosus,* and *P. ostreatus* showed the antioxidant properties [yang, Lin, and Mau,

2002]. The polysaccharides from mushrooms and plant also showed antioxidant properties [Maity et al., 2011; Patra et al., 2012; Patra et al., 2013]. The antioxidant properties of polysaccharides include its scavenging capacities against hydroxy, superoxide radicals and hydrogen peroxide, and metal chelating ability as well as reducing power. Mushrooms contain antioxidants such as ascorbic acid, tocopherols, phenolic compounds and carotenoids. Hence, mushrooms and its polysaccharides could be used as a natural antioxidant material and also be recommended as an excellent food for consumption.

1.5. Conclusions

Carbohydrates are the essential components in nature which played a key role in establishment and evolution of the life. All living organism like fungi, bacteria etc produce polysaccharides. Mushroom polysaccharides exhibit immunostimulating and antitumor properties. The commercially available pharmaceutical products like lentinan, schizophyllan, grifolan, and PSK (polysaccharide–protein complex) have shown potential clinical results. Mushroom polysaccharides activate immune systems as well as exhibit potent antioxidant activities. Hence, they have a great role for future application as drugs in immune and cancer therapy.

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CHAPTER -2

METHODOLOGY

2.1. Isolation and purification of polysaccharides from the fruit bodies of the edible mushroom, *Russula albonigra*

2.1.1. Polysaccharides (PS-I and PS-II) from aqueous part

Fresh fruit bodies of the mushroom *Russula albonigra* (Krombh.) Fr. (500 g) were washed, crushed and boiled in distilled water for 12 h. The aqueous extract was kept overnight at 4 °C and filtered through linen cloth. The filtrate was centrifuged at 8000 rpm (using a Heraeus Biofuge stratus centrifuge) for 45 min at 4 °C. The supernatant was precipitated in 1:5 (v/v) EtOH, kept overnight at 4 °C. The precipitated polysaccharide was collected through centrifugation and then dissolved in a minimum volume of distilled water and dialyzed through a dialysis tubing cellulose membrane (D9652, Sigma-Aldrich, retaining Mw >12,000 Da) against distilled water for 6 h. The material retained inside the cellulose bag was then centrifuged as mentioned above. The residue was rejected and the filtrate (water soluble part) was freeze dried to obtain crude polysaccharide. The crude polysaccharide was further purified successively using pet ether, benzene, $CHCL_3$, and CH_3OH through soxhlet extraction. The material was again dissolved in water, dialyzed and freeze dried. It was further purified by passing through Sepharose 6B column to obtain pure polysaccharide. Schematic diagram of isolation and purification of the polysaccharides of aqueous part of the mushroom fruit bodies are shown in **Figure 1**.

❖ **Flow diagram of isolation and purification of the polysaccharides**

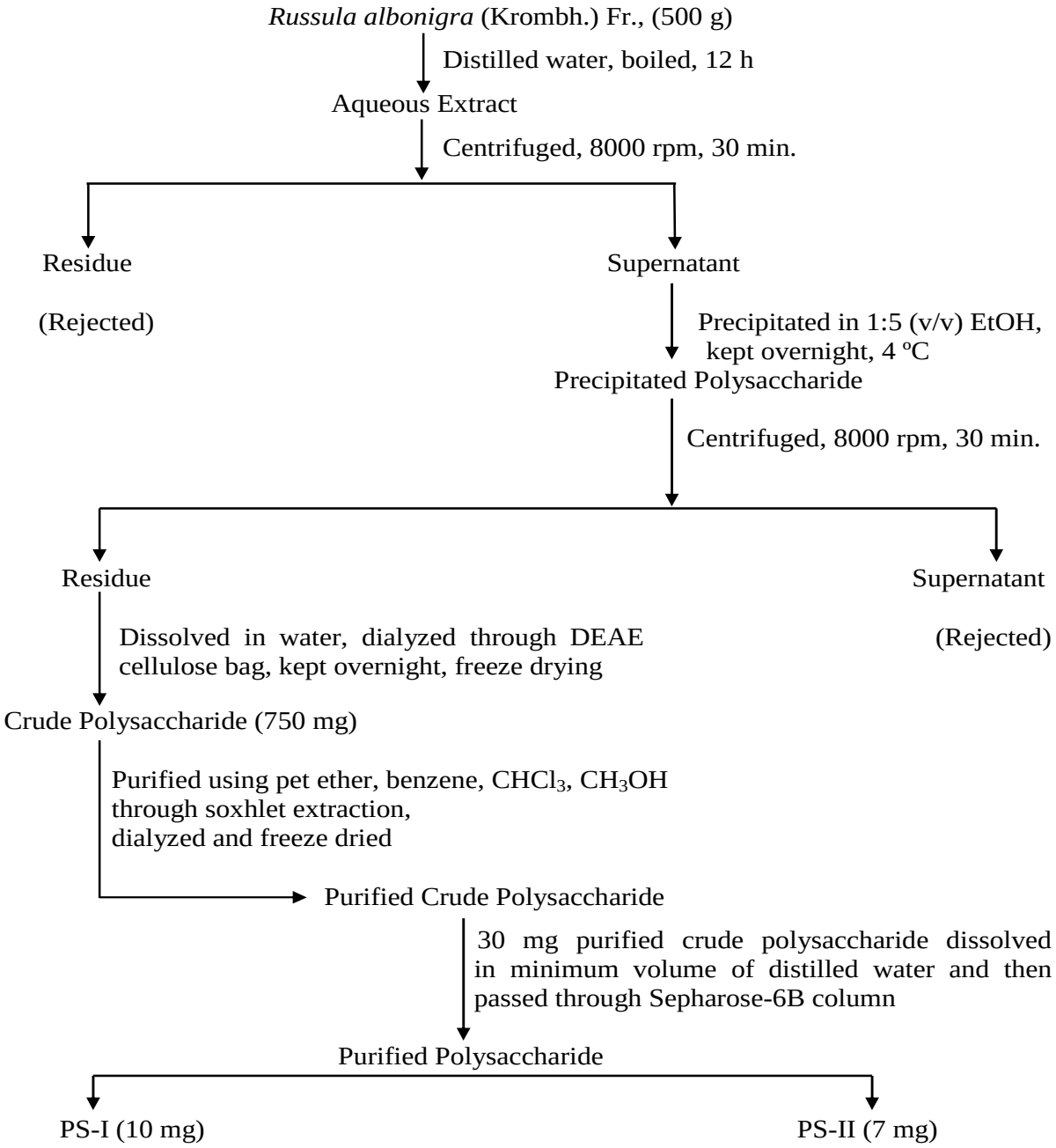

Figure 1. Schematic diagram of isolation and purification of the polysaccharides from aqueous extract of the mushroom fruit body, *Russula albonigra.*

2.1.2. Polysaccharide (PS) from alkaline part

Fresh fruit bodies of the mushroom *R. albonigra* were gently washed with distilled water and then boiled with 4% NaOH solution for 1h. The alkaline extract was kept overnight at 4 °C and filtered through linen cloth. The isolation and purification steps of polysaccharide from alkaline part of the mushroom fruit bodies are shown in **Figure 2**.

❖ **Flow diagram of isolation and purification of the polysaccharide**

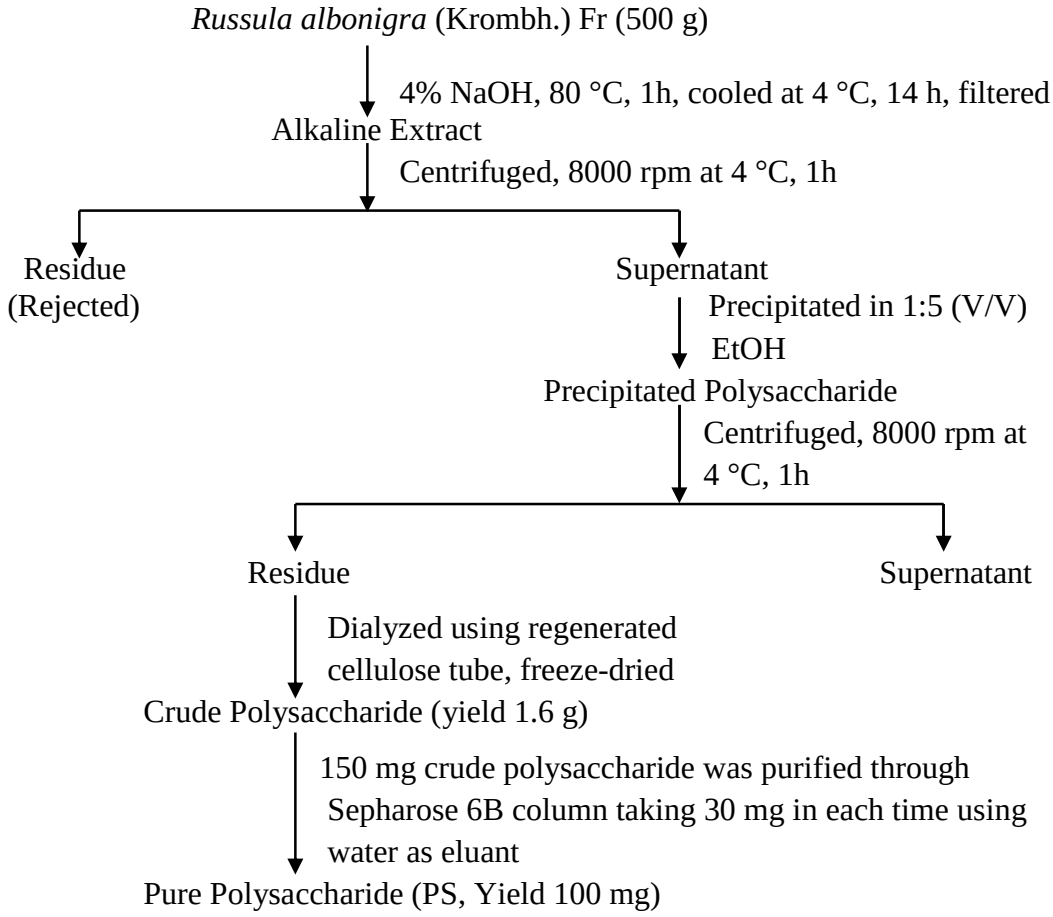

Figure 2. Schematic diagram of isolation and purification of the polysaccharide from alkaline extract of mushroom fruit body, *Russula albonigra*.

2.1.3. Purification of polysaccharides

Purification of a polysaccharide is essential for determination of the structure of its repeating unit. The technique of Gel-permeation chromatography (GPC) is extensively used to separate a mixture of polysaccharides. In this technique molecules are separated on the basis of their size relative to the pores of the packing materials. This technique is also known as size exclusion chromatography (SEC). Different bio gels such as Sephadex G-50, G-75, G-100 and Sepharose-4B, 6B etc are generally used to separate polysaccharides of different molecular weight.

In the present thesis the crude polysaccharide was purified through Sepharose 6B column (fractionation range 10,000-10,00,000 Da) in water as eluant using a Redifrac fraction collector. Eluent was collected in test tubes and monitored spectrophotometrically at 490 nm with the phenol-sulfuric acid method [York et al., 1985] using Shimadzu UV-vis spectrophotometer, model 1601. A chromatogram of sample distribution was obtained by plotting the test tube number against absorbance. The purification procedure was carried out in several lots. Fractions were collected and freeze dried.

2.2. Determination of physical properties

2.2.1. Optical rotation measurement

The pure polysaccharide (5 mg) was dissolved in 5 mL distilled water and then optical rotation was measured on a Jasco Polarimeter (Model P-1020) at room temperature.

2.2.2. Molecular weight determination

The molecular weights of the polysaccharides were determined by gel-permeation chromatographic technique. Standard dextrans [Hara et al., 1982] T-200, T-70, and T-40 were passed through a Sepharose 6B column using water as an eluant, and then the elution volumes were plotted against the logarithms of their respective molecular weights. The elution volume of the polysaccharide was then plotted in the same graph, and molecular weight of the polysaccharide was determined.

2.3. Structural analysis of polysaccharides

It is most important to purify the polysaccharide as much as possible for determination of structure of the polysaccharide. Different techniques like chromatography, ultra centrifugation, dialysis, precipitation and re-precipitation with different solvent systems are adopted for this purpose. The structural analysis of the polysaccharides (PS) depends on two methods: (i) chemical method that includes total acid hydrolysis, methylation, periodate oxidation, and Smith degradation studies, (ii) spectroscopic method comprising of 1D (^{1}H, ^{13}C, DEPT-135) and 2D (TOCSY, DQF-COSY, NOESY, ROESY, HSQC, HMBC) NMR analyses. A schematic diagram of the methodology adopted for structure determination of polysaccharides has been presented in **Figure 3**.

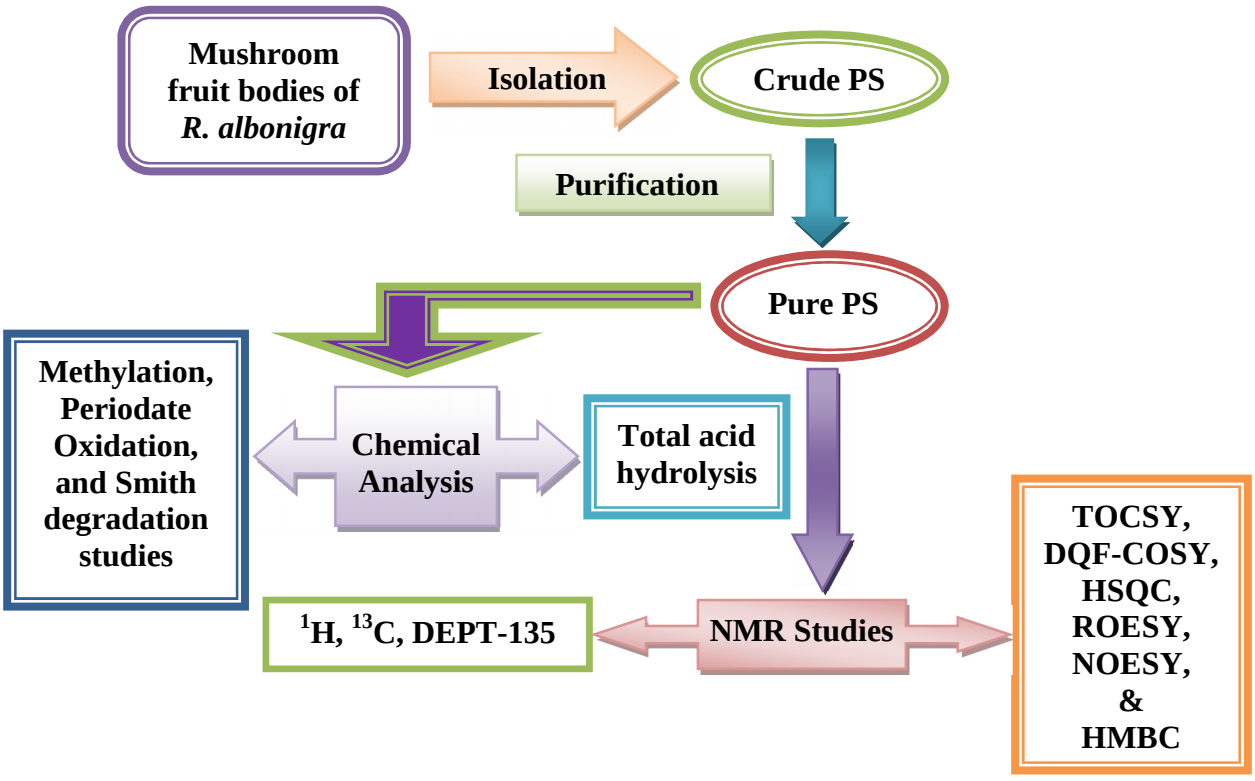

Figure 3. Schematic diagram of structural analysis of polysaccharides.

2.4. Chemical analysis

2.4.1. Monosaccharide analysis

Every polysaccharide is composed of different monosaccharide constituents. So, monosaccharide analysis is essential to determine the complete structure of the polysaccharide. Total acid hydrolysis of the polysaccharide was carried out to determine the monosaccharide constituents present in the polysaccharide. Polysaccharide (3 mg) was hydrolyzed with 2 M CF_3COOH (2 mL) in a round-bottomed flask at 100 ºC for 18 h in a boiling water bath. The excess acid was completely removed by co-distillation with water. Then, the hydrolyzed product was reduced with $NaBH_4$ (9 mg) followed by acidification with dilute CH_3COOH, and then excess boric acid was removed by co-distillation with pure CH_3OH. The reduced sugars (alditol) were acetylated with 1:1 pyridine-Ac_2O in a boiling water bath for 1 h to give alditol acetates [**Figure 4**]. The excess pyridine-Ac_2O was removed by repeated co-distillation with toluene. The alditol acetates were extracted with chloroform and then analyzed by Gas-liquid Chromatography (GLC), Hewlett-Packard, model 5730 A, with flame ionization detector and glass columns (1.8 m x 6 mm) packed with 3% ECNSS-M (A) on Gas Chrom Q (100-120 mesh) and 1% OV-225 (B) on Gas Chrom Q (100-120 mesh). All GLC analyses were performed at 170 ºC.

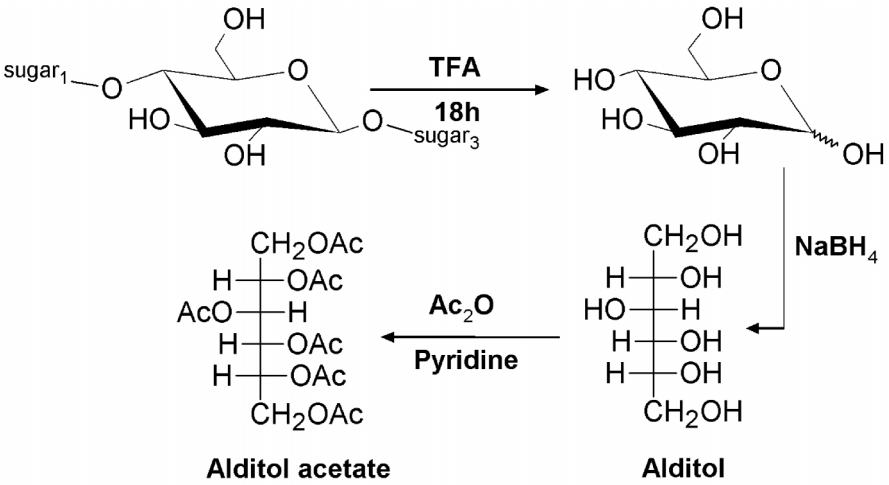

Figure 4. Schematic diagram of preparation of alditol acetates.

2.4.2. Paper chromatographic studies

Paper chromatography is an analytical method for separating and identifying complex mixtures of similar compounds. This is a method for testing the purity of compounds and identifying the substances, where substances are distributed between a stationary phase and a mobile phase. The stationary phase is usually a piece of high quality filter paper. The mobile phase is a developing solution that travels up the stationary phase, carrying the samples with it. Components of the sample will separate readily according to how strongly they absorb on the stationary phase versus how readily they dissolve in the mobile phase. During the present thesis work, sugar mixture was separated by descending paper chromatography. In this type of paper chromatography, development of paper is done by allowing the solvent to travel down the paper and the mobile phase is present in the upper portion. Paper chromatographic studies [Martin and Synge, 1941; Partridge, 1946] were performed on Whatmann Nos. 1 (for small quantities) and 3 mm (for large quantities up to 30 mg) sheets. Solvent systems were used (X) BuOH-HOAc-H_2O (v/v/v, 4:1:5, upper phase) [Boggs et al., 1950] and (Y) EtOAc-pyridine-H_2O (v/v/v, 8:2:1) [Hamilton and Thompson, 1957]. The spray reagents for development of chromatograms were used as: silver nitrate in acetone (1.2%), methanol in sodium hydroxide solution, and 5% sodium thiosulphate solution [Hoffman et al., 1972].

2.4.3. Determination of absolute configuration

The absolute sugar configuration was determined by the method of Gerwig, Kamerling, and Vliegenthart [1978]. The polysaccharide (1.0 mg) was hydrolyzed with CF_3COOH, and then the acid was removed. A solution of 250 μL of 0.625 (M) HCl in R-(-)-2-butanol was added and heated at 80 °C for 16 h. Then the reactants were evaporated and TMS-derivatives were prepared with *N*,*O*-bis(trimethylsilyl)trifluroacetamide (BSTFA). The products were analyzed by GLC using a capillary column SPB-1 (30m × 0.26mm), a temperature program (3 °C/min) from 150 to 210 °C. The 2,3,4,6-tetra-*O*-TMS-(+)-2-butylglycosides obtained were identified by comparison with those prepared from the D and L enantiomers of different monosaccharides.

2.4.4. Linkage analysis

Linkage analysis is one of the most important steps during structural investigation. The different mode of linkage of monosaccharide units in a polysaccharide is determined by methylation analysis which is finally confirmed with the help of periodate oxidation study.

2.4.4.1. Methylation analysis

Methylation experiment is a powerful tool for the determination of the structure of polysaccharide in carbohydrate chemistry. The different mode of linkage of each monosaccharide unit in a polysaccharide is determined by methylation experiment. Methylation analysis includes conversion of all free hydroxyl groups into methoxy groups followed by hydrolysis. Acid hydrolysis of the resulting poly-methyl-ethers only cleaves the inter-glycosidic linkages, keeping the methyl ether bonds intact. The hydrolyzed monomers are reduced and acetylated to produce volatile partially methylated alditol acetates (PMAA) as shown in **Figure 5**. The substitution pattern of the *O*-acetyl group of the PMAA reflects the linkage patterns of the corresponding sugars in the original polymer. There are several methods for methylation, but the most popular one is the method adopted by Ciucanu and Kerek [1984] which was adopted in the present investigations and reported in this thesis. According to this method the polysaccharides (4.0 mg) were treated with finely powdered sodium hydroxide (NaOH) in DMSO (1mL) to de-protonate the hydroxyl groups yielding polyanion (alkoxide ions) followed by addition of methyl iodide (1 mL) in stirring condition. The methylated polysaccharide was isolated by making a partition between $CHCl_3$ and water (5:2, v/v). The organic layer containing product was washed with water for several times and dried. The methylated biopolymer was hydrolyzed with 90% HCOOH (1 mL) at 100 ºC for 1 h, and excess HCOOH was evaporated by co-distillation with distilled water. The hydrolyzed product was then reduced with $NaBH_4$, acetylated with pyridine-Ac_2O (1:1) and the excess was removed by repeated co-distillation with toluene. The alditol acetates of the methylated sugars were extracted with chloroform and analyzed by Gas-liquid Chromatography-Mass Spectroscopy (GLC-MS). GLC-MS analysis was performed on Shimadzu GLC-MS

Model QP-2010 Plus automatic system, using ZB-5MS capillary column (30 m x 0.25 mm). The program was isothermal at 150 ºC; hold time 5 min, with a temperature gradient of 2 ºC min^{-1} up to a final temperature of 200 ºC.

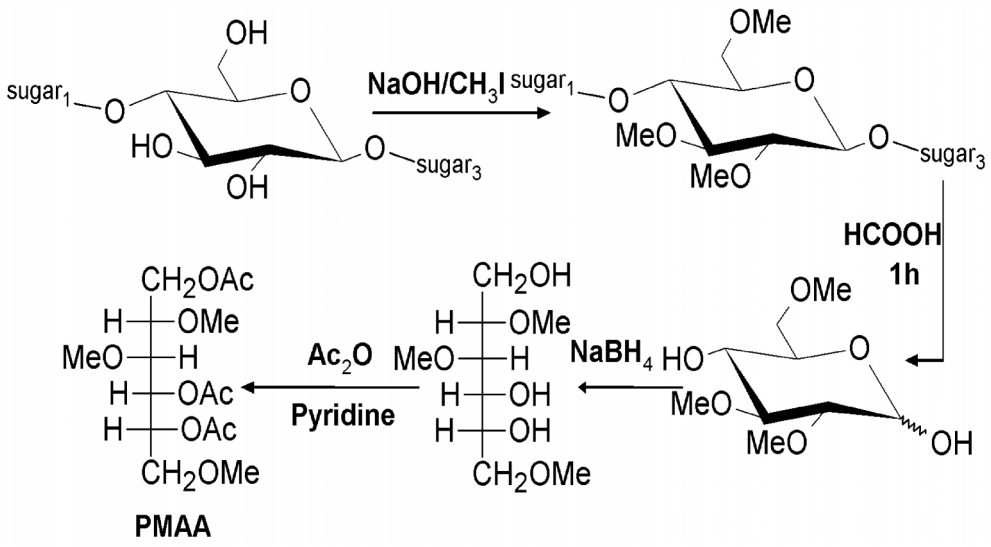

Figure 5. Schematic diagram of preparation of partially methylated alditol acetates (PMAA) of polysaccharides.

2.4.4.2. Periodate oxidation study

The linkage information obtained from the methylation analysis was further confirmed by periodate oxidation study. Polysaccharides have the potential to react with oxidizing agents such as periodic acid or its salts due to the presence of free hydroxyl groups. 1,2-diols react with periodic acid or its salt to form two aldehydes groups due to the cleavage of the carbon-carbon sigma bond. Non-reducing end sugar residues and (1→6)-linked hexopyranose residues have three adjacent hydroxyl groups. In these cases double cleavage will occur forming one molar equivalent of formic acid and two molar equivalent of periodate is consumed as shown in **Figure 6**. (1→2)- and (1→4)-linked hexopyranose units consume one equivalent of periodate per mole yielding a dialdehyde.

Whereas (1→3)-linked hexopyranose residues will not be affected by this reaction because of absence of vicinal OH groups.

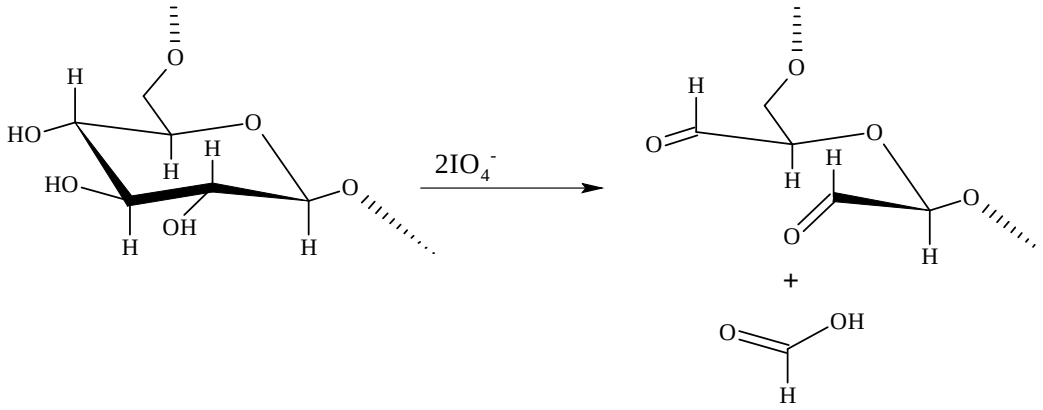

Figure 6. Schematic diagram of periodate oxidation of (1→6)-linked hexopyranoside.

In this experiment, polysaccharide (5 mg) was oxidized with 0.1 M sodium metaperiodate (2 mL) at room temparature in the dark during 48 hours and the excess periodate was consumed by adding 1,2-ethane diol. The solution was dialyzed against distilled water for 1 h followed by reduction with $NaBH_4$ for 15 h and neutralized with acetic acid. [Goldstein et al., 1965; Hay et al., 1965]. The periodate oxidized-reduced product was divided into two portions. One portion was hydrolyzed with 2 M CF_3COOH for 18 h to prepare alditol acetates and subjected to GLC analysis. Another portion was methylated by the method of Ciucanu and Kerek [1984] and the alditol acetate of this methylated product (PMAA) was analyzed by GLC–MS.

2.4.4.3. Smith degradation

Oligosaccharides (OS) are simply characterized by NMR spectroscopy than polysaccharides. Polysaccharides are able to be degraded to oligosaccharides by acidic hydrolysis that can be very specific for certain kinds of linkages. Smith degradation is another method to degrade PS to OS or modified PS. Smith degradation is used to simplify the elucidation of the repeating unit by discriminating removal of some of the residues. This procedure can be applied when several overlapping of NMR signals occurs

in the structural studies of repeating unit. The Smith degradation involves sequential treatment of PS with $NaIO_4$ (sodium metaperiodate), sodium borohydride, and dilute trifluoroacetic acid. The oxidation yields a product in which vicinal hydroxyl groups have been oxidised to aldehydes by cleavage of carbon-carbon bonds. Residues without any vicinal hydroxyl groups remain unaffected. The reduction of aldehyde yields a polyalcohol. Mild acidic hydrolysis of these yields OS or modified PS that contain sugar residues and fragments of modified sugar residues.

In the present thesis the polysaccharide (25 mg) was oxidized with 0.1 M sodium metaperiodate (20 mL) at 25 °C in the dark during 72 h. The oxidation was stopped by the addition of 1,2-ethanediol, and the solution was dialyzed against distilled H_2O. Thereafter, $NaBH_4$ was added and kept at room temperature for 15 h, with intermittent stirring. The mixture was neutralized with 50% AcOH and again dialyzed against distilled water, and freeze dried and then subjected to mild hydrolysis with 0.5 M TFA for 15 h at 25 °C. TFA was removed by repeated evaporation with water at 37 °C. Finally, it was purified by passing through a Sephadex G-25 column. A part of this material was subjected to methylation analysis and the remainder was used for ^{13}C NMR studies [Rout et al., 2005].

2.4.4.4. Sequence analysis: Partial acid hydrolysis

Since polysaccharides have exceptionally large molecular size and wide distribution of molecular weight, complete sequencing of complex polysaccharides is basically very difficult. In order to obtain information about the sequence of a complex polysaccharide, the polymer is commonly degraded into different fragments in a controlled manner. The resulting mixture of fragments is fractionated and easily characterized. Partial degradation of polysaccharides by acid hydrolysis is based on the fact that some glycosidic linkages are more labile to acids than the others. If a polysaccharide contains only a limited number of acid labile glycosidic linkages, a partial hydrolysis may afford a mixture of monosaccharides, oligosaccharides or polysaccharide. Detailed characterization of these products obtained after partial hydrolysis will provide meaningful information about the sequence of the polysaccharides [Dong et al., 2002].

In the present thesis the polysaccharide (30 mg) was dissolved in 0.1 M TFA (6 mL) was hydrolyzed at 100 °C for 1 h. Acid was removed by repeated evaporation of water at 37 °C. The residue was dissolved in water (4 mL), to which three volumes of ethanol were added. The precipitate was washed with ethanol and then freeze-dried and used for methylation and ^{13}C NMR analysis. The supernatant was dried by evaporation, and the residue was dissolved in water, and reduced with $NaBH_4$ at 25 ºC for 2 h. After neutralization with 1 M AcOH, it was desalted by passing through a Sephadex G-25 column. The carbohydrate containing eluate was collected, freeze-dried and subjected to methylation analysis.

2.5. Nuclear magnetic resonance (NMR) spectroscopy

Nuclear Magnetic Resonance (NMR) spectroscopy is the most powerful and non-destructive technique for the determination of structure of the polysaccharides. It is used for identification of exact monosaccharide composition, elucidation of α or β anomeric configurations, establishment of linkage patterns, and sequence of the sugar units in the repeating unit of the polysaccharide. Different types of 1D (^1H, ^{13}C, and DEPT-135) and 2D (DQF-COSY, TOCSY, NOESY, ROESY, HSQC and HMBC) NMR techniques have been applied for complete assignment of the structure of the polysaccharides and shown in the following diagram [**Figure 7**].

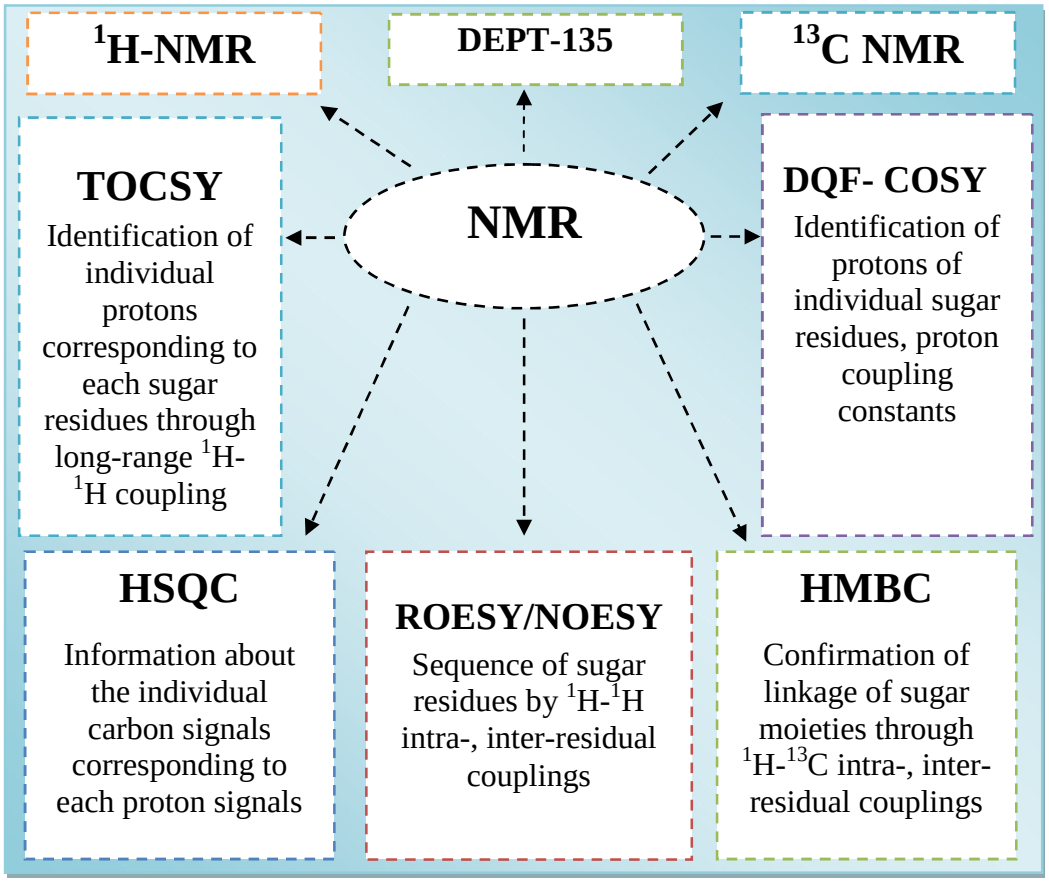

Figure 7. Schematic diagram of determination of structure of a polysaccharide by NMR spectroscopic methods.

➢ Preparation of NMR sample and instrumentation

Polysaccharides contain a very small amount of water and it has also hydroxyl (OH) proton. In ^{1}H NMR a major problem is that the OH protons of sugar units and the proton signals from residual water appear in the region of the ring protons, and therefore creates a definite interference for peak identification. Hence, proper sample preparation is very important before running an NMR spectrum. In the present thesis all NMR experiments were performed in D_2O. The samples were prepared free from water by keeping over P_2O_5 in vacuum for several days, and deuterium exchanged by repeated lyophilization with D_2O (99.96%. atom ^{2}H, Aldrich). Then ^{1}H NMR and ^{13}C NMR experiments were performed with a Bruker Avance DPX-500 instrument. The signal for residual HOD was

suppressed at δ 4.70 at 30 °C using presaturation method. The 2D-(DQF-COSY) NMR experiment was carried out using standard pulse sequence at 30 °C. The NOESY and ROESY mixing delay were 300 ms whereas delay time in the HMBC experiment was 80 ms. The ^{13}C NMR experiments of the polysaccharide were carried out taking acetone as the internal standard, fixing the methyl carbon signal at δ 31.05 by using D_2O as the solvent.

➢ One-dimensional NMR

Two types of 1D NMR spectroscopy, ^{1}H NMR and ^{13}C NMR are commonly used. The polysaccharide has chemical shifts in the range of δ 1.0-6.0 that can be determined by the ^{1}H-NMR spectroscopy. The anomeric proton resonances are generally found in the range of δ 4.4-5.5. The remaining ring proton resonances are found in the range of δ 3.0-4.2. In case of deoxy sugars the methyl protons appear in the region of δ 1.1-1.3. The anomeric protons from each monosaccharides give recognizable signals depending on their α or β anomeric configurations. Normally the α-anomer resonates downfield compared to the β-anomer in D-pyranoses. The vicinal coupling constant between the anomeric H-1 and the H-2 ($J_{1\text{-}2}$) indicates the relative orientation of two protons. If both of them are in an axial configuration in pyranose structures, a large coupling constant (7-8 Hz) is observed, whereas if they are equatorial-axial, this is smaller ($J_{1\text{-}2}$ ~ 4 Hz) [Jansson et al., 1987]. Different sugars are identified from their characteristic coupling constants.

Although ^{13}C NMR signals are much weaker than ^{1}H, it has major advantages over the ^{1}H-NMR spectroscopy because in the former the signals are spread out over a wide range. In the ^{13}C NMR spectra, anomeric carbons signals appear in the region of δ 90-110 whereas the other carbons in the region of δ 60-90. In case of deoxy and methoxy sugars the methyl carbons appear in the region of δ 15-20 and δ 55-61, respectively. The α-anomeric carbon signals appear generally in the range of δ 95-103 whereas most of the β-anomeric carbons appear in the region of δ 101-105. Unsubstituted ring carbons usually appear in the region of δ 65-75 [Agarwal, 1992]. If there is any linkage at any carbon, the signal for that carbon will show a downfield shift by δ 4-10, and the carbon next to that

one will appear in a little upfield region (by δ 0.7-4.7) [Agarwal, 1992; Gruter et al., 1993]. Signals for carbonyl carbons are generally observed between δ 165-185.

Sometimes the ^{1}H J_{1-2} values are not sufficient to determine the anomeric configurations of the sugars. If the glycosyl residue has the *manno*-configuration, the distinction between the two anomeric forms is very difficult since the coupling constant values for α (J_{1-2}~ 1.8 Hz) and β (J_{1-2} ~ 1.5 Hz) are too close to be differentiated. The one bond ^{13}C-^{1}H coupling constants are useful for determination of the anomeric configuration of sugar residues [Bock et al., 1984]. For D sugars a $^{1}J_{C1,H1}$ ~ 170 Hz indicates an α-anomeric sugar configuration whereas $^{1}J_{C1,H1}$ ~ 160 Hz indicates a β-anomeric sugar configuration [Bock and Pedersen, 1974]. This is reversed for the L sugars. C-1, H-1 coupling constants were measured from proton coupled ^{13}C NMR experiment.

➢ Two-dimensional NMR

Two-dimensional NMR experiments are especially used to analyze complex spectra, which are difficult to predict by conventional methods. The connectivity between the nuclei in a residue and the cross connectivity between the nuclei of different residues can be obtained from 2D NMR spectroscopy. In the present thesis different 2D NMR experiments such as DQF-COSY, TOCSY, NOESY, ROESY, HSQC and HMBC have been used.

❖ DQF-COSY (Double Quantum Filtered Correlation Spectroscopy)

DQF-COSY spectrum gives us the information about the protons of an individual sugar residue through a three-bond coupling. The strategy of assigning a COSY spectrum is to find one unmistakable characteristic signal from which to begin the tracing of a spin system or network. An anomeric proton is often chosen as the starting point because it is connected with the other proton atoms of that residue. Coupling constants can also be measured from DQF-COSY spectrum.

❖ TOCSY (Total Correlation Spectroscopy)

TOCSY spectrum correlates the protons that are in the same spin system and yields both long range and short range correlations. It is valuable for establishing the scalar connectivity if the proton signals are within a spin system, especially when the multiplets overlap, or there is extensive second order coupling. It is useful in the identification of individual monosaccharide residue. TOCSY is a very essential and powerful tool to confirm the assignments of the ^{1}H NMR spectrum. The TOCSY experiment was recorded at mixing time of 300 ms, and complete assignment required several TOCSY experiments having mixing times ranging from 60 to 300 ms.

❖ NOESY (Nuclear Overhauser Enhancement Spectroscopy)

NOESY spectrum provides the information through space rather than through bond couplings. NOE connectivies are normally observed between the anomeric protons of a particular sugar residue to protons of another sugar residue that is glycosidically linked to the former. NOESY experiments provide the information on linkages and sequence of sugar residues in a polysaccharide. The NOESY mixing delay was 300 ms.

❖ ROESY (Rotating Frame Overhauser Enhancement Spectroscopy)

ROESY spectrum is also used to determine the glycosydic linkage position as well as the sequence information of the polysaccharide. Here the signals arisen from protons of two different sugar residues, which are close in space but not closely connected by chemical bonds. A ROESY spectrum yields through space correlations via the Rotational nuclear overhauser effect (ROE). ROESY is especially useful for cases where NOESY signals are weak because they are near the transition between negative and positive. ROESY cross peaks are always negative. The ROESY experiment also yields cross peaks arisen from chemical exchange.

❖ HSQC (Heteronuclear Single Quantum Coherence)

HSQC spectrum represents a direct correlation between a carbon and a proton of all signals in the spectra. All the ^{13}C chemical shifts of the sugar residues are here assigned

from the individual proton signals assigned from DQF-COSY and TOCSY spectrum as they are directly correlated.

❖ HMBC (Hetero Multiple Bond Coherence Spectroscopy)

HMBC experiment gives long range coupling between proton and carbon (two or three bonds away) with high sensitivity. Once all the ^{13}C signals are assigned, especially the anomeric and glycosidically linked carbons, unambiguous glycosidic linkages and sequences of the sugar residues can be established through the long range ^{13}C-^{1}H correlations. An HMBC experiments establish the multiple-bond correlation through the glycosidic bonds, and these together with NOESY/ROESY experiments provide necessary information of linkages and sequence of the repeating units of a polysaccharide.

2.6. Immunological Studies

2.6.1. Preparation of LPS free polysaccharide (LFPS) for immunological studies

Prior to immunoactivation studies, LPS which may contaminate during isolation and purification process was removed from polysaccharide in order to discard the contribution of LPS in immunostimulation. The polysaccharide was passed through polymyxin-B agarose matrix (Sigma 160 and Aldrich, USA) packed in 2 mL column (1 cm×2 cm), with 0.5 mL/min flow rate. It was equilibrated with 10 mM phosphate buffer, pH 7.4. The bacterial lipopolysaccharides (LPS) were bound to the matrix and the unbound LPS free polysaccharide were eluted and collected for immunoenhanching studies.

2.6.2. Limulus amebocyte lysate (LAL) test

Limulus amebocyte lysate (LAL) test was carried out *in vitro* for detection of bacterial endotoxin. The test was performed using gel clot technique [Liu et al., 2009]. Limulus amebocyte lysate (LAL) (G2125, sensitivity: 0.125 EU/mL) was purchased from Quantum Biotech, Mumbai, India. The control standard endotoxin (CSE) (code E0125) and water (code W1004) for the bacterial endotoxin test (BET) were provided by

Quantum Biotech, Mumbai, India. Four tubes were taken, each containing 0.1 mL of LAL reagent. In two tubes, 0.1 mL LFPS aqueous solution were added, meanwhile 0.1 mL BET water and 0.1 mL CSE were added to the rest two tubes as negative control and positive control, respectively. All tubes were incubated for 1 h in a water bath at 37 °C. After the test tube was inverted 180° slowly, it is positive (+) if the gel in tube is not deformed and does not slip from the wall and a negative (-) test is characterized in the absence of a gel or by the formation of a viscous mass which does not hold when the tube is inverted. Test is invalid when positive control is (-) or negative control is (+).

2.6.3. Test for macrophage activity by nitric oxide assay

Polysaccharides function as immunostimulator by activating the macrophages. The macrophages activation induced by the polysaccharides was studied by nitric oxide (NO) production in culture supernatant *in vitro*. RAW 264.7 growing in Dulbecco's modified Eagle's medium (DMEM) was seeded in 96 well flat bottom tissue culture plate at 5 x 10^5 cells/mL concentration (180 µL).Cells were left overnight for attachment and treatment with different concentrations (12.5, 25, 50, 100 or 200 µg/mL) of glucan were given. After 48 hrs of treatment culture supernatant of each well were collected and NO content was estimated using Griess Reagent [Green et al., 1982] at 540 nm. (1:1 of 0.1% in 1-napthylethylenediamine in 5% phosphoric acid and 1% sulfanilamide in 5% phosphoric acid). The amount of NO produced was normalized to the cell numbers at the end of the treatment in order to confirm that iNOS expression was responsible for polysaccharide induced NO production. Lipopolysaccharide (LPS), L6511 of *Salmonella enterica* serotype typhimurium (sigma, St. Louis, USA) was used as positive control.

2.6.4. Splenocyte and thymocyte proliferation assay

Splenocyte and thymocyte proliferations are the measurement of immunoactivation. A single cell suspension of spleen and thymus was prepared from Swiss Albino mice under aseptic conditions by homogenization in Hank's balanced salt solution (HBSS). The present study was approved by the ethics committee of Institutional Animal Ethics Committee, IIT Kharagpur, India. The suspension was centrifuged to obtain cell pellet.

The contaminating RBC was removed by hemolytic Gey's solution. After washing two times in HBSS the cells were resuspended in complete RPMI (Roswell Park Memorial Institute) with serum and antibiotics added. RPMI and foetal bovine serum (FBS) has been obtained from Gibco whereas antibiotics were obtained from Himedia. Cell concentration was adjusted to 1×10^6 cells/mL and viability of splenocytes and thymocytes (as tested by trypan blue dye exclusion) was always over 90%. The cells (180 µL) were plated in 96 well flat bottom tissue culture plates and incubated with 20 µL of various concentrations of the LFPS (12.5, 25, 50, 100 or 200 µg/mL). PBS (10mM, Phosphate Buffer Saline, pH-7.4) was taken as negative control whereas LPS (4 µg/mL, Sigma) and Concavalin A (Con A, 10 µg/mL, Himedia) served as positive controls. All cultures were set up in triplicate for 72 h at 37 °C in a humidified atmosphere of 5% CO_2. Proliferation of splenocytes indicated as Splenocyte Proliferation Index (SPI) and Thymocytes written as Thymocyte Proliferation Index (TPI) were checked by standard MTT assay method [Ohno et al., 1993]. The data are reported as the mean ± standard deviation of different observations and compared against PBS control [Maiti et al., 2008; Mallick et al., 2010; Shah et al., 2007]. To establish the immunoenhancing activity of polysaccharide, HeLa cancer cell line which was not a part of the immune system was chosen as control and treated with different concentrations (12.5 to 200 µg/mL) of polysaccharide and proliferation was measured.

2.7. Antioxidant properties

2.7.1. Hydroxy radical scavenging activity

Hydroxy radicals are the most injurious reactive oxygen species (ROS) which generated during the oxidative metabolism. Now a day, neutralization of these radicals is essential phenomenon. The reaction mixture (1mL) consisted of KH_2PO_4 - KOH buffer (20 mM, pH 7.4), 2-deoxy-D-ribose (2.8 mM), variable concentration (100 - 400 µg/mL) of PS, $FeCl_3$ (100 mM), EDTA (104 µM), ascorbate (100 µM) and H_2O_2 (1 mM). It was incubated at 37°C for 1 h. 2 mL thiobarbituric acid (TBA) and trichloroacetic acid (TCA) solution (100 mL contained 375mg TBA, 15mg TCA; 2 mL concentrated HCL added to 98 ml of TBA-TCA solution) was added and incubated at boiling water bath for 15 min.

After cooling, absorbance was measured at 535 nm [Acharya, Chatterjee, and Ghosh, 2011]. Butylated hydroxytoluene (BHT) was used as positive control.

2.7.2. Superoxide radical scavenging activity

Superoxide radicals are another harmful reactive oxygen species. The method by Martinez, Marcelo, Marco, & Moacyr [2001] for determination of the superoxide anion was followed with modification in the riboflavin-light-nitrobluetetrazolium (NBT) system. Each 3 mL reaction mixture contained 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, various concentrations (100 - 400 µg/mL) of PS, 100 µM EDTA, 75 µM NBT and 2 µM riboflavin. Reaction started by illuminating sample with light and the increased absorbance was measured at 560 nm after 10 min of illumination. Identical tubes with the reaction mixture were kept in the dark and served as blank. Butylated hydroxyanisole (BHA) was used as a positive control.

2.7.3. Chelating ability of ferrous ions

Chelating ability was determined according to the method of Dinis, Madeira, & Almeida [1994]. Reaction mixture (4 mL) contained different concentrations of PS (100-400 µg/mL) mixed with 3.7 mL of water and 0.1 mL of 2 mM ferrous chloride. The reaction was initiated by the addition of 0.2 mL of 5 mM ferrozine. After 10 min incubation at room temperature, the absorbance was determined at 562 nm against a blank. EDTA was used as positive control. The percentage of inhibition of ferrozine- Fe^{2+} complex formation is given by this formula:

$$\% \text{ inhibition} = \{(A_0-A_1)/ A_0\} \times 100.$$

Where, A_0 was the absorbance of the control and A_1 the absorbance in the presence of mushroom fractions.

2.7.4. Determination of reducing power assay

Reducing power was determined according to the method of Oyaizu [1986]. Various concentrations of polysaccharide (200-600 µg/mL) were mixed with 2.5 mL sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide (1%). The mixture was incubated for 20 min and then 2.5 mL of trichloroacetic acid (10%) was added. 2.5 mL of this solution was mixed with 2.5 mL distilled water and 0.5 mL $FeCl_3$ (0.1%) and incubated for 15 min. The absorbance was measured at 700 nm against buffer. Ascorbic acid was used as standard. IC_{50} value is the effective concentration at which the absorbance was 0.5 for reducing power.

2.7.5. ß carotene bleaching assay

Inhibition of β carotene bleaching was determined according to the method of Dapkevicius, Venskutonis, Van Beek, & Linssen [1998]. Reaction mixture consisted of 0.5 mg β carotene in 1ml HPLC grade chloroform, 25 µL linoleic acid and 200 mg Tween 40. Chloroform was completely evaporated. Then 50 mL distilled water saturated with O_2 was added with vigorous shaking. Aliquots (4 mL) of this emulsion were transferred into different tubes containing different concentrations of PS (100 - 400 µg/mL) and absorbance was read at 490 nm against water. The tubes were placed at 50˚C for 2 hrs and again absorbance was taken. Butylated hydroxyanisole (BHA) was used as positive control.

2.8. Conclusions

The methodologies that have been adopted to determine the structure of polysaccharides and their immunological (splenocytes, thymocytes, and macrophages activation) as well as some antioxidant studies have been discussed in this chapter. The structure of the repeating unit of the polysaccharides is determined using two types of methods: (1) Chemical method that includes total acid hydrolysis, paper chromatographic, methylation, periodate oxidation, Smith degradation, and partial acid hydrolysis studies (2) Spectroscopic method comprising of 1D and 2D NMR experiments.

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CHAPTER-3

Structural and immunological studies of α,β-glucan (PS-I) isolated from the aqueous extract of the edible mushroom, *Russula albonigra*

3.1. Introduction and earlier work

The *Russula albonigra* (Krombh.) Fr., an ectomycorrhizal [Chakraborty et al., 2004; Lilleskov et al., 2009; Mehrotra, 1991] basidiomycetes [Shimono, Kato, and Takamatsu, 2004] fungus, family Russulaceae [Buyck et al., 2008] grows in symbiotic relationship with the roots of Sal (Shorea robusta) and other coniferous trees in the forest during rainy and autumn season throughout the world. Ectomycorrhizal fungi assist the plants in extracting nutrients like phosphorus and other minerals and in return receive photosynthetically fixed sugars from the host plant for their growth and development. *Russula albonigra* (Krombh.) Fr. is edible and non-toxic [Pradhan et al., 2010] one and turns into black when handled or aged [Smith and Lebel, 2001]. Several species of the genus *Russula* like *R. aeruginea* Lindblad. Fr., *R. alutacea* (Pers.:Fr.) Fr., *R. cyanoxantha* (Schaeff. ex Secr.) Fr., *R. decolorans* (Fr.) Fr., and *R. fragilis* (Pers.:Fr.) Fr are also edible [Chandra, 1989] and their comparable taxonomic studies along with this mushroom have been reported [Arora, 1986; Smith and Lebel, 2001].

Mushrooms have not only been used as a food or food flavoring materials, but also recognized as an important source of biologically active compounds on account of their immunomodulatory and antitumor properties [Borchers et al., 1999; Wasser and Weis, 1999], especially, polysaccharides and polysaccharide-protein complexes which exhibit anticancer activities [Sun and Liu, 2009]. Several (1→3)-β-D-glucan [Ohno et al., 1993; Chakraborty et al., 2006], (1→3)-, (1→6)-β-D-glucan [Mizuno et al., 1990], (1→6)-β-D-glucan [Kiho et al., 1992; Ukaya, Ito, and Hisamatsu, 2000], (1→4)-, (1→6)-α-D-glucan [Fujimiya et al., 1998], and (1→3)-α-, (1→6)-β-D-glucan [Ohno et al., 1993] are widely used an immunoenhancing and antitumor materials. A new (1→3)-, (1→6)-branched α,β-D-glucan [Ojha et al., 2010; Rout et al., 2005; Roy et al., 2009], (1→6)-, (1→3,6)-β-D-glucan [Maji et al., 2012] and water insoluble (1→3)-, (1→3,6)-β-D-glucan [Bhanja et al. 2012] were also reported by our group.

Two water soluble polysaccharides (PS-I and PS-II) have been isolated from the fruit bodies of the edible fungus, *Russula albonigra* (Krombh.) Fr. and the detailed structural

investigation and study of immunoenhancing properties of PS-I was only carried out and reported in this chapter.

3.2. Present work

3.2.1. Isolation and purification of polysaccharides from *R. albonigra*

Fresh mushroom fruit bodies (500 g) were washed thoroughly with distilled water and boiled with distilled water for 12 h followed by centrifugation, precipitation in EtOH, and freeze drying to yield 750 mg of crude polysaccharide. The water soluble crude polysaccharide (30 mg) was purified by gel permeation chromatography on column (90 × 2.1 cm) of Sepharose 6B yielded two polysaccharides fraction [**Figure 1**]. Fraction I (test tubes, 20-32) and fraction II (test tubes, 36-45) were collected and freeze dried, yielding purified polysaccharide PS-I (10 mg) and PS-II (7 mg), respectively. The same procedure was repeated several times to yield more pure polysaccharide. Now, the PS-I was further purified by passing through Sepharose 6B column to yield pure polysaccharide for the study of different experimental analysis.

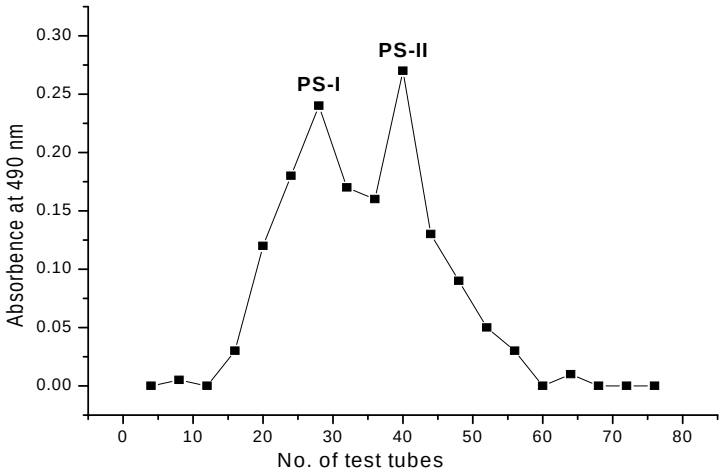


Figure 1. The gel permeation chromatogram of the polysaccharides (PS-I and PS-II) isolated from hot aqueous extract of fruit bodies of the edible mushroom, *Russula albonigra*.

3.2.2. Optical rotation and molecular weight of PS-I

The PS-I showed specific rotation $[\alpha]_D^{31}$ –13.9 (*c* 0.6, water). The molecular weight [Hara et al., 1982] of PS-I was estimated as ~1.94 × 10^5 Da from a calibration curve prepared with standard dextrans [**Figure 2**].

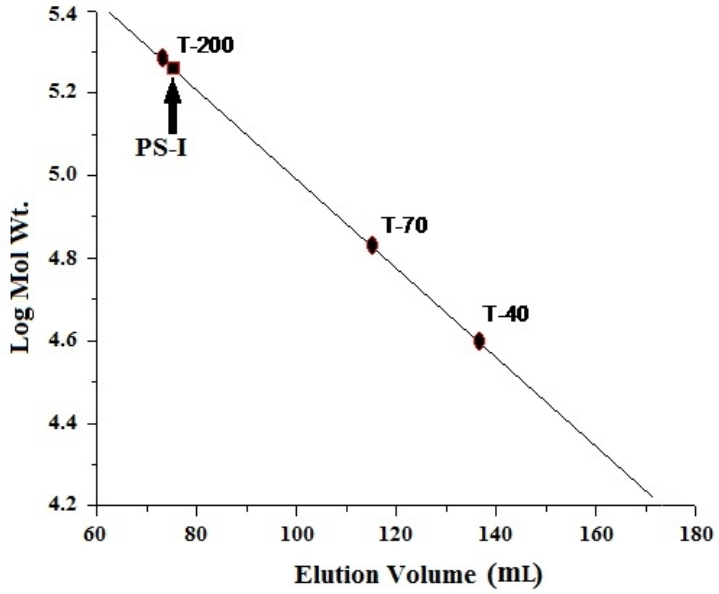


Figure 2. Determination of molecular weight of the PS-I isolated from hot aqueous extract of fruit bodies of the edible mushroom, *Russula albonigra*.

3.2.3. Structural analysis of PS-I

3.2.3.1. Chemical analysis of PS-I

PS-I was hydrolyzed with 2M trifluoroacetic acid and then alditol acetate [Lindhall, 1970] was 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, Kamerling, and Vliegenthart [1978]. The mode of linkages of the PS-I was determined by the methylation analysis using the Ciucanu and Kerek method [1984] 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 $(1\rightarrow3,6)$, $(1\rightarrow3)$-linked, and terminal glucopyranosyl residues in a molar ratio of nearly 1:3:1[**Figure 3, Table 1**]. GLC analysis of alditol acetates of the periodate-oxidized [Goldstein et al., 1965; Hay, Lewis, and Smith, 1965], $NaBH_4$-reduced, methylated [Abdel-Akher and Smith, 1950] PS-I 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 3:1 [**Figure 4, Table 2**]. These results clearly indicated that the terminal glucopyranosyl residues were consumed during oxidation whereas, $(1\rightarrow3,6)$-linked and $(1\rightarrow3)$-linked glucopyranosyl residues remain unaffected which further confirmed the mode of linkages present in the PS-I.

Table 1

GLC-MS data for methylated polysaccharide (PS-I) of the edible mushroom, *Russula albonigra*

Methylated sugars	**Linkage types**	**Molar ratio**	**Major Mass fragments (m/z)**
2,3,4,6-Me_4-Glc*p*	Glc*p*-(1→	1	43, 45, 59, 71, 87, 101, 117, 129, 145, 161, 205
2,4,6-Me_3-Glc*p*	→3)-Glc*p*-(1→	3	43, 45, 58, 71, 74, 87, 99, 101, 117, 129, 143, 161, 203, 233
2,4-Me_2-Glc*p*	→3,6)-Glc*p*-(1→	1	43, 58, 74, 87, 101, 117, 129, 143, 159, 173, 189, 201, 233

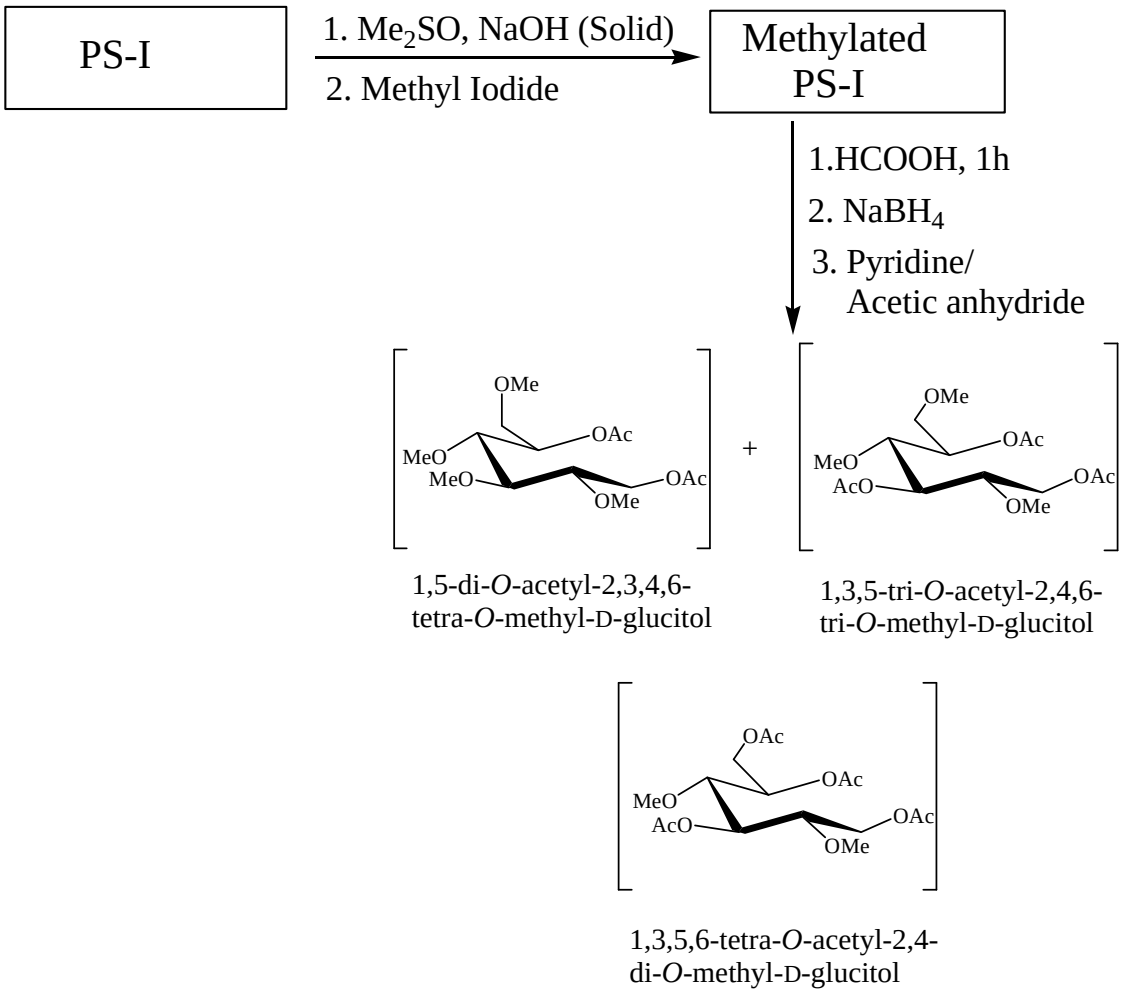


Figure 3. Schematic presentation of methylation experiment of PS-I isolated from the edible mushroom, *Russula albonigra*.

Table 2

GLC-MS analysis of periodate oxidized methylated polysaccharide (PS-I) of the edible mushroom, *Russula albonigra*.

Methylated sugars	Linkage types	Molar ratio	Major Mass fragments (m/z)
2,4,6-Me_3-Glc*p*	→3)-Glc*p*-(1→	3	43, 45, 58, 71, 87, 99, 101, 117, 129, 143, 161, 201, 233
2,4-Me_2-Glc*p*	→3,6)-Glc*p*-(1→	1	43, 58, 87, 99, 101, 117, 129, 139, 159, 189, 233

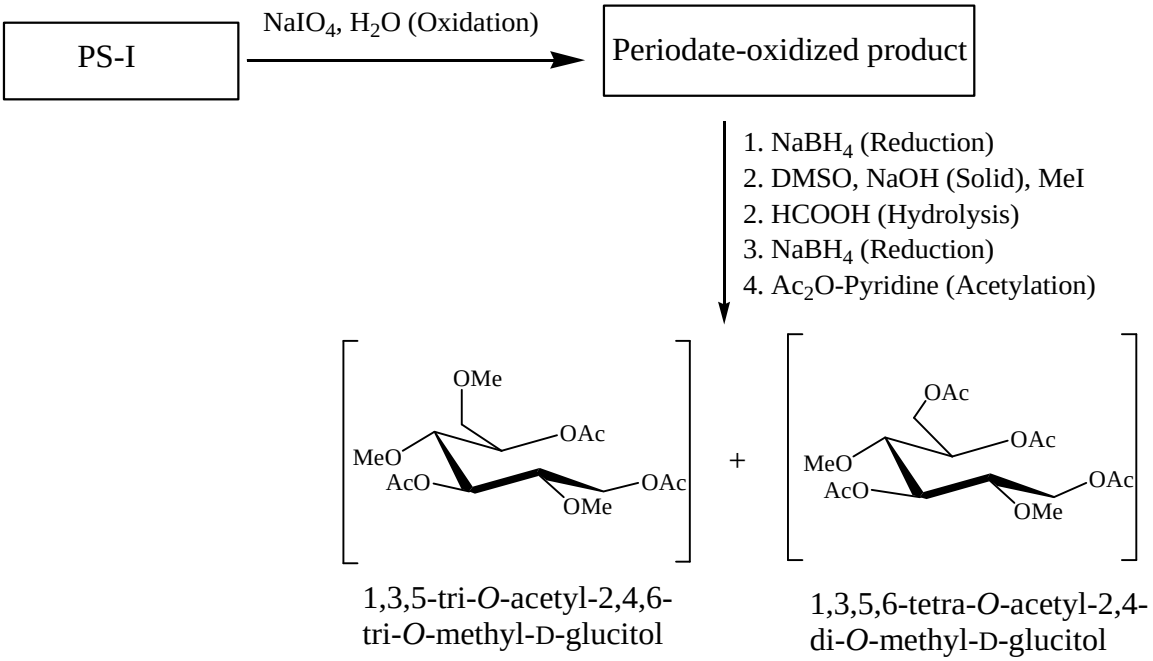


Figure 4. Schematic presentation of periodate oxidation reactions of PS-I isolated from the edible mushroom, *Russula albonigra*.

3.2.3.2. 1D and 2D NMR analysis of PS-I

The ^{1}H NMR (500 MHz) spectrum [**Figure 5, Table 3**] 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 [**Figure 6a, Table 3**] and DEPT-135 [**Figure 6b, Table 3**] 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 [**Figure 7**], 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.

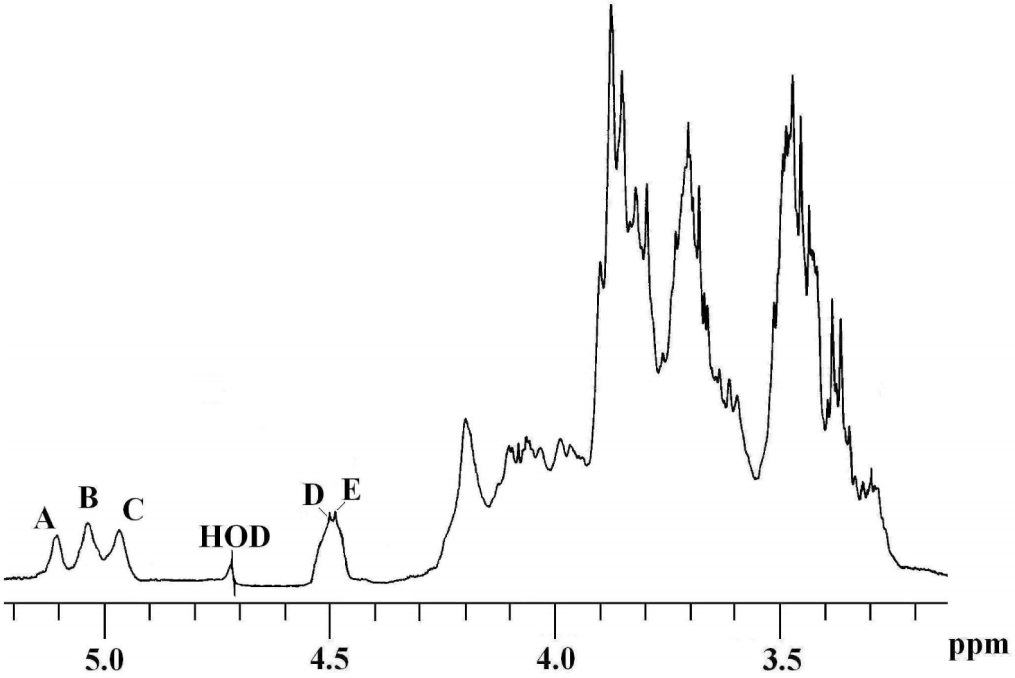


Figure 5. ^{1}H NMR spectrum (500 MHz, D_2O, 30 ºC) of the PS-I isolated from the edible mushroom, *Russula albonigra*.

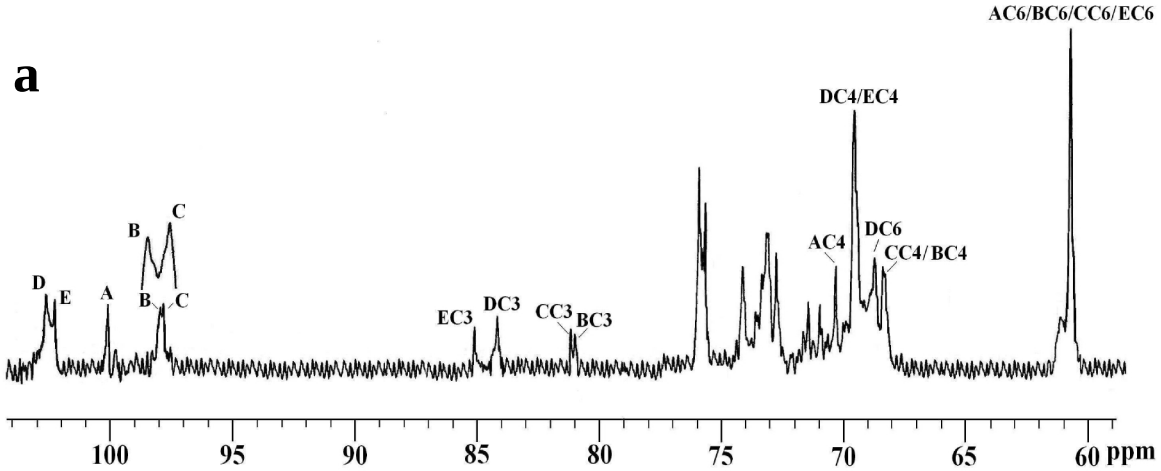


Figure 6. (a) ^{13}C NMR spectrum (125 MHz, D_2O, 30 ºC) of the PS-I isolated from the edible mushroom, *Russula albonigra*.

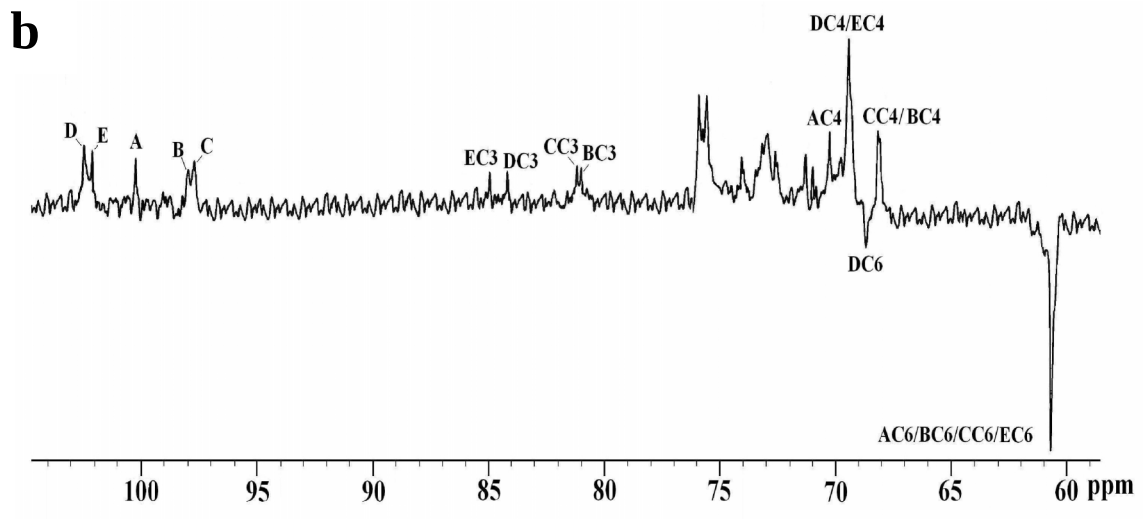


Figure 6. (b) DEPT-135 spectrum (D_2O, 30 ºC) of the PS-I isolated from the edible mushroom, *Russula albonigra*.

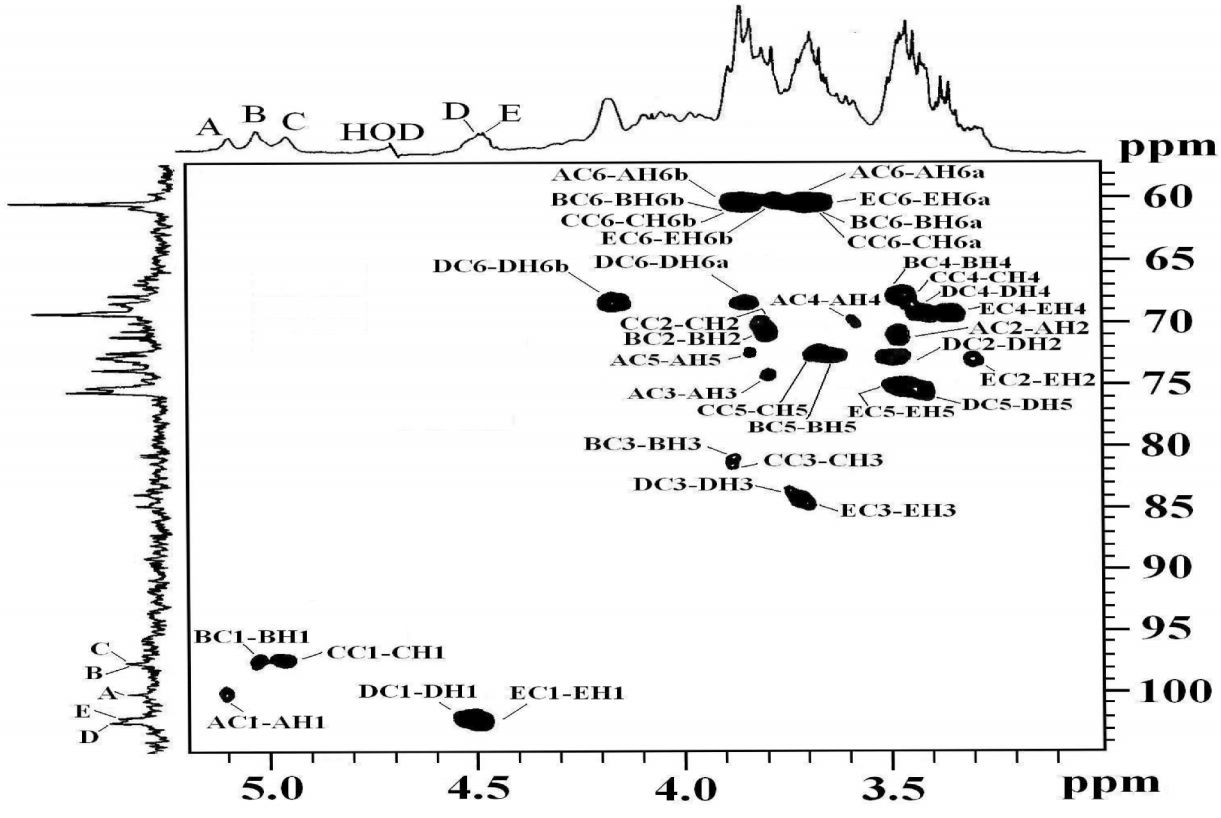


Figure 7. HSQC spectrum (D_2O, 30 ºC) of the PS-I isolated from the edible mushroom, *Russula albonigra*.

Table 3

The $^{1}H^{a}$ and $^{13}C^{b}$ NMR chemical shifts for the polysaccharide (PS-I) isolated from the edible mushroom, *Russula albonigra* in D_2O at 30 ^{o}C.

Glycosyl residue	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6a,H-6b/C-6
α-D-Glcp (1→	5.10	3.52	3.80	3.62	3.85	3.72[c], 3.91[d]
A	100.2	71.49	74.18	70.3	72.7	60.8
→3)-α-D-Glc*p*-(1→	5.04	3.83	3.88	3.49	3.68	3.70[c], 3.86[d]
B	98.0	71.0	81.0	68.3	72.8	60.8
→3)-α-D-Glc*p*-(1→	4.97	3.83	3.87	3.49	3.68	3.70[c], 3.86[d]
C	97.8	71.0	81.2	68.3	72.8	60.8
→3,6)-β-D-Glc*p*-(1→	4.51	3.50	3.74	3.45	3.48	3.87[c], 4.20[d]
D	102.4	73.1	84.3	69.6	76.0	68.8
→3)-β-D-Glc*p*-(1→	4.49	3.30	3.73	3.39	3.44	3.68[c], 3.80[d]
E	102.2	73.2	85.0	69.6	75.6	60.8

[a] Values of the ^{1}H chemical shifts were recorded with respect to the HOD signal fixed at δ 4.70 ppm at 30 ^{o}C.

[b] Values of the ^{13}C chemical shifts were recorded with reference to acetone as the internal standard and fixed at δ 31.05 ppm at 30 ^{o}C. [c, d] Interchangeable.

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 [Agrawal, 1992; Rinaudo and Vincendon, 1982]. 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 [Bhanja et al. 2012; Yoshioka et al., 1985] 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 [**Figure 6b**]. 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**.

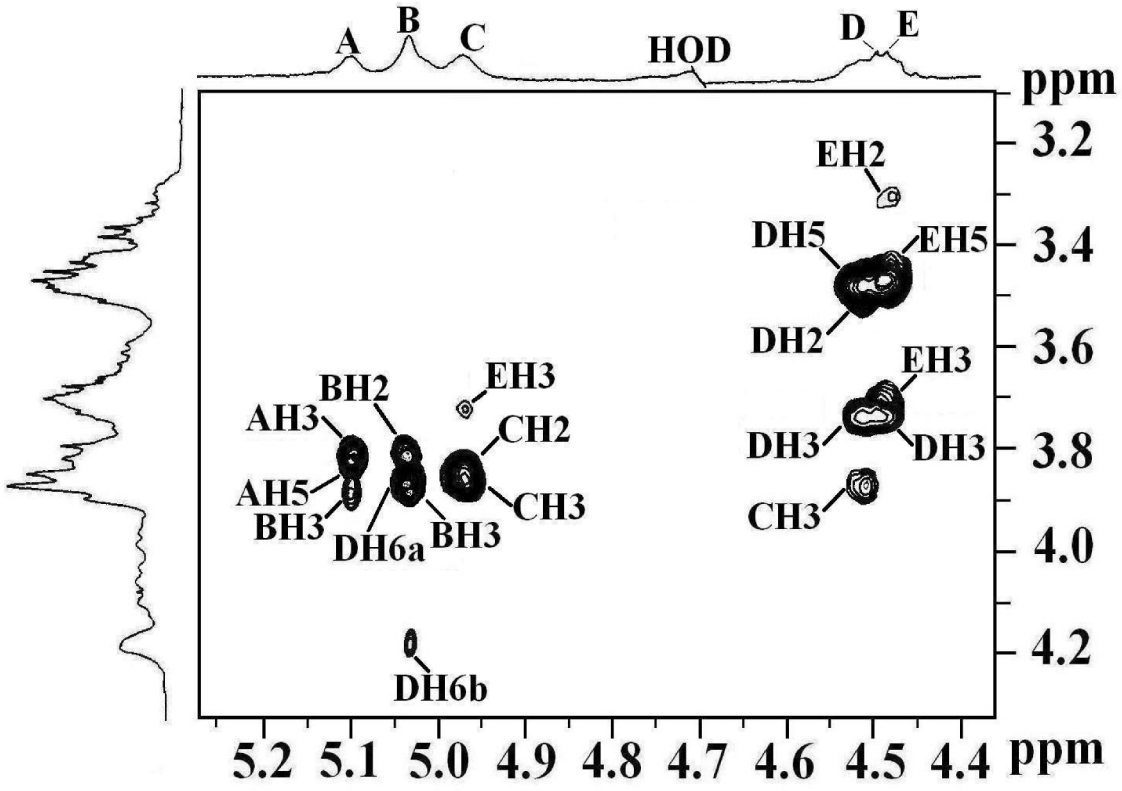


Figure 8. The part of ROESY spectrum of the PS-I isolated from the edible mushroom, *Russula albonigra*. The ROESY mixing time was 300 ms.

Table 4

ROE data for the polysaccharide (PS-I) isolated from the edible mushroom, *Russula albonigra* in D_2O at 30 ^oC.

Glycosyl residue	Anomeric proton	ROE contact protons	
	¢	¢	Residue, atom
α-D-Glc*p*-(1→ **A**	5.10	3.80 3.85 3.88	**A**H-3 **A**H-5 **B**H-3
→3)-α-D-Glc*p*-(1→ **B**	5.04	3.83 3.88 3.87 4.20	**B**H-2 **B**H-3 **D**H-6a **D**H-6b
→3)-α-D-Glc*p*-(1→ **C**	4.97	3.83 3.87 3.73	**C**H-2 **C**H-3 **E**H-3
→3,6)-β-D-Glc*p*-(1→ **D**	4.51	3.50 3.74 3.48 3.87	**D**H-2 **D**H-3 **D**H-5 **C**H-3
→3)-β-D-Glc*p*-(1→ **E**	4.49	3.30 3.73 3.47 3.74	**E**H-2 **E**H-3 **E**H-5 **D**H-3

The sequences of glucosyl moieties were determined from ROESY [**Figure 8, Table 4**] as well as NOESY 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→;

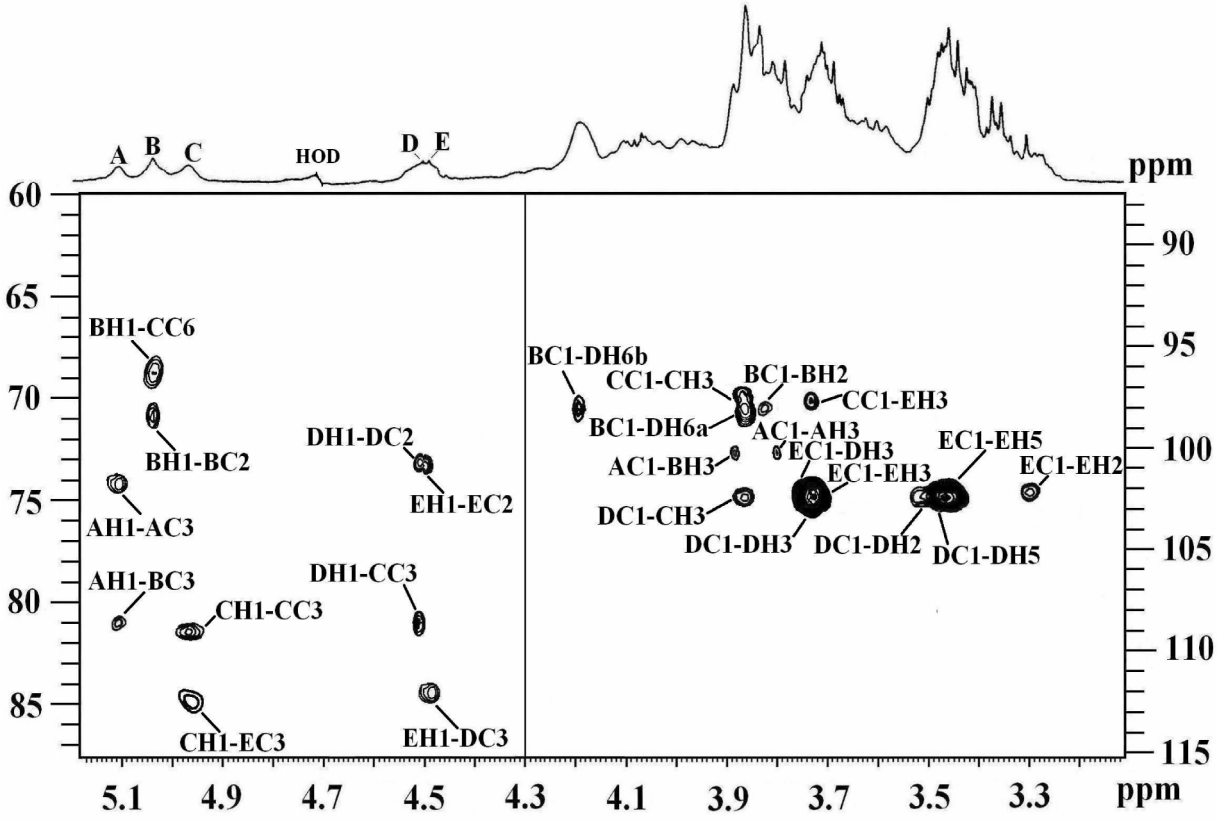


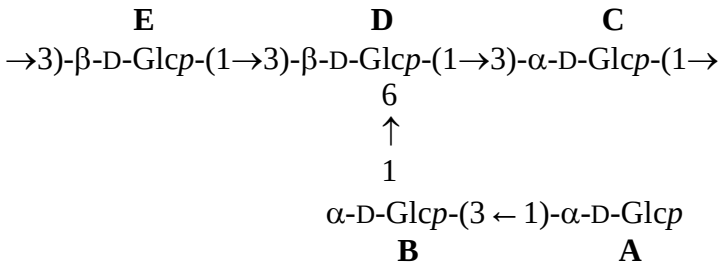
Figure 9. The part of HMBC spectrum of the PS-I isolated from the edible mushroom, *Russula albonigra*. The delay time in the HMBC experiment was 80 ms.

Table 5

The significant $^{3}J_{H,C}$ connectivities observed in an HMBC spectrum for the anomeric protons/carbons of the sugar residues of the polysaccharide (PS-I) isolated from the edible mushroom, *Russula albonigra* in D_2O at 30 ^{o}C.

Residues	Sugar linkage	H-1/C-1	Observed connectivities		
		¢H/¢C	¢H/¢C	Residue	Atom
A	α-D-Glc*p*-(1→	5.10 100.2	81.0 74.18 3.88 3.80	**B** **A** **B** **A**	C-3 C-3 H-3 H-3
B	→3)-α-D-Glc*p*-(1→	5.04 98.0	68.8 71.0 3.87 4.20 3.83	**D** **B** **D** **D** **B**	C-6 C-2 H-6a H-6b H-2
C	→3)-α-D-Glc*p*-(1→	4.97 97.8	85.0 81.2 3.73 3.87	**E** **C** **E** **C**	C-3 C-3 H-3 H-3
D	→3,6)-β-D-Glc*p*-(1→	4.51 102.4	81.2 73.1 3.87 3.50 3.74 3.48	**C** **D** **C** **D** **D** **D**	C-3 C-2 H-3 H-2 H-3 H-5
E	→3)-β-D-Glc*p*-(1→	4.49 102.2	84.3 73.2 3.74 3.30 3.73 3.47	**D** **E** **D** **E** **E** **E**	C-3 C-2 H-3 H-2 H-3 H-5

A long range HMBC experiment was carried out to confirm the ROESY connectivities. In HMBC experiment [**Figure 9, Table 5**], 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:



3.2.4. Immunostimulating properties of PS-I

Various immunological studies were also investigated with the PS-I which was very essential for its clinical application field. Macrophage activation of the PS-I was observed *in vitro*. On treatment with different concentrations of the PS-I an enhanced production of NO was observed in a dose-dependent manner with optimum production of 24.5 µM NO per 5 x 10^5 macrophages at 100 µg/mL of the PS-I [**Figure 10a, red bar**]. In order to establish that iNOS expression was responsible for NO production, the amount of NO produced was normalized to the cell numbers at the end of the treatment. After 48 h of treatment, the number of cells in the control increased from 1x10^5 to 2x10^5 cells/mL, whereas the number of cells increased from 1x10^5 to 2x1.4x10^5 cells/mL, 2x1.7x10^5 cells/mL, 2x1.8x10^5 cells/mL, 2x1.8x10^5 cells/mL on treatment with 12.5, 25, 50, 100 and 200 µg/mL of PS-I, respectively. The NO production was normalized with respect to

the increase of cell number as shown in **Figure 10a, blue bar**. But, actually the nitric oxide induction was measured to be 14 μM, 15 μM, 24 μM, 24.5 μM and 23 μM on treatment of 12.5, 25, 50, 100 and 200 μg/mL of PS-I, respectively. Thus, fold increase of nitric oxide induction in treatments of 12.5, 25, 50, 100 and 200 μg/mL) of PS-I will be 14/6 (2.3), 15/7(2.14), 24/10(2.4), 24.5/11(2.22) and 23/11(2.09), respectively. Proliferation index of macrophages on treatment with different concentration of PS-I was shown in **Figure 10b**.

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. The splenocytes and thymocytes activation tests were carried out in mouse cell culture medium with the glucan by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method [Chihara, 1978]. Proliferation of splenocytes and thymocytes is an indicator of immunostimulation. The splenocyte and thymocyte proliferation index as compared to Phosphate Buffered Saline (PBS) control if closer to 1 or below indicates low stimulatory effect on immune system. The PS-I was found to stimulate splenocytes and thymocytes as shown in **Figure 10c and 10d**, respectively and the asterisks on the columns indicate the statistically significant differences compared to PBS control. At 50 μg/mL of the PS-I, both splenocyte and thymocytes proliferation indices were found maximum as compared to other concentrations. Hence, 50 μg/mL of the PS-I can be considered as efficient splenocyte and thymocytes stimulators. To estlablish the immunoenhancing activity of PS-I, HeLa cancer cells which is not a part of the immune system was used as control cell line and treated with different concentrations (12.5 to 200 μg/mL) of PS-I. It was observed that the treatment of PS-I did not show any proliferation [**Figure 10e**] after 48 h of treatment and it was ruled out that PS-I can stimulate cell proliferation regardless of cell types.

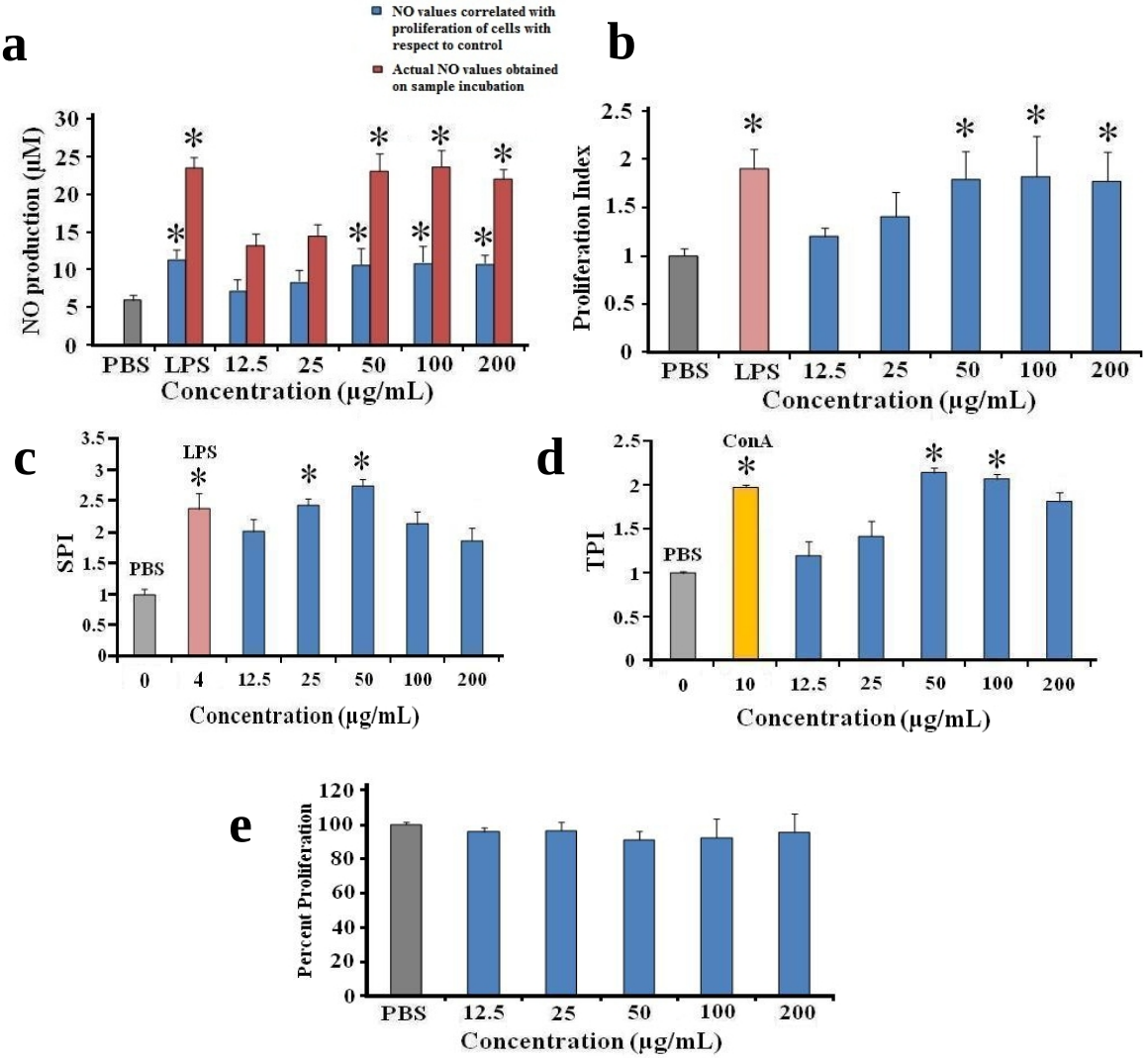


Figure 10. In vitro activation of raw macrophage stimulated with (a) different concentrations of PS-I in terms of Nitric oxide (NO) obtained after correlating with proliferation of cells and actual NO production obtained on sample incubation and (b) proliferation index of macrophages on treatment with (12.5 to 200 µg/mL) of PS-I.

Effect of different concentrations of PS-I on (c) splenocyte, (d) thymocyte proliferation and (e) percent proliferation of non-immune (HeLa cells) cells after the treatment (Significant compared to the PBS control).

3.3. Conclusions

A α,β-glucan (PS-I) was isolated from the hot aqueous extract of the fruit bodies of an ectomycorrhizal edible mushroom *Russula albonigra* (Krombh.) Fr. The repeating unit of the glucan consists of a backbone chain of two (1→3)-β-D-glucopyranosyl and one (1→3)-α-D-glucopyranosyl residues, one (1→3)-β-D-glucopyranosyl is branched at *O*-6 position with (1→3)-α-D-glucopyranosyl and terminated with a α-D-glucopyranosyl residue. PS-I showed *in vitro* macrophage, splenocyte and thymocyte activations and also treated as an immunoactive active material in future.

3.4. References

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CHAPTER-4

Structural elucidation of an immunoenhancing heteroglycan (PS-II) isolated from the aqueous extract of the edible mushroom, *Russula albonigra*

4.1. Introduction and earlier work

Edible mushrooms have been used as a delicious food or food flavoring materials. The important biologically active compounds like polysaccharides and polysaccharide-protein complexes from mushroom are being investigated widely for their antitumor and immunomodulatory [Borchers et al., 1999; Sarangi et al., 2006; Wasser and Weis, 1999] as well as anticancer activities [Ooi and Liu, 2000; Sun and Liu, 2009]. Mushroom polysaccharides do not attack cancer cells directly but exhibit antitumor activity through the activation of T cells, B cells, NK cells and macrophage-dependent immune systems in living organism [Wasser, 2002]. *Russula albonigra* (Krombh.) Fr., an ectomycorrhizal [Chakraborty et al., 2004; Lilleskov et al., 2009] edible and non-toxic [Pradhan et al., 2010] fungus, grows in symbiotic relationship with the roots of Sal (Shorea robusta) and other coniferous trees in the forest during rainy and autumn season. Two water-soluble fractions (PS-I and PS-II) were isolated from the fruit bodies of the edible mushroom, *Russula albonigra* (Krombh.) Fr. The first fraction, PS-I was identified as glucan which showed excellent immunoenhancing properties and reported [Nandi et al., 2012]. The second fraction, PS-II was characterized as heteroglycan which contained α-L-fucose as an important bioactive compound. The carbohydrate moiety α-L-fucose is essential for novel treatment approachs in human breast cancer [Jay et al., 2011] and also involved during *in vitro* fertilization [Jennifer et al., 2010] for improvement of infertility treatments. Several heteropolysaccharides with α-L-fucose from different mushrooms like *Lentinus edodes* [Carbonero et al., 2008], *Hericium erinaceus* [Zhang et al., 2012], *Agaricus blazei* Murill [Liu and Sun, 2011], *Fomitella fraxinea* (Imaz.) [Cho et al., 1998], *Agaricus bisporus* [Ruthes et al., 2013], *Phellinus baumii* Pilát [Ge et al., 2009], *Lentinus squarrosulus* (Mont.) Singer [Bhunia et al., 2010], *Calocybe indica* var. APK2 [Mandal et al., 2011] have been reported. Moreover, PS-II showed *in vitro* macrophage activation by NO production in RAW 264.7 cell line as well as splenocyte and thymocyte proliferations for which it can be used as immunostimulating material in future. The detailed structural

investigation and study of immunostimulating properties of PS-II were carried out in the present investigation and reported in this chapter.

4.2. Present work

4.2.1. Isolation and purification of polysaccharides from *R. albonigra*

Five hundred grams of fresh mushroom fruit bodies were washed thoroughly with distilled water, boiled with water for 12 h followed by centrifugation, precipitation in EtOH, and finally freeze dried to yield 750 mg of crude polysaccharide. Fractionation of water soluble crude polysaccharide (30 mg) through Sepharose 6B column yielded two polysaccharides [**Figure 1**]. Fraction I (test tubes, 20-32) and fraction II (test tubes, 36-45) were collected and freeze dried, yielding purified polysaccharide PS-I (10 mg) and PS-II (7 mg), respectively. The same procedure was repeated several lots to yield more pure polysaccharide. Here, the PS-II was further purified by passing through Sepharose 6B column to yield pure polysaccharide for detailed experimental analysis.

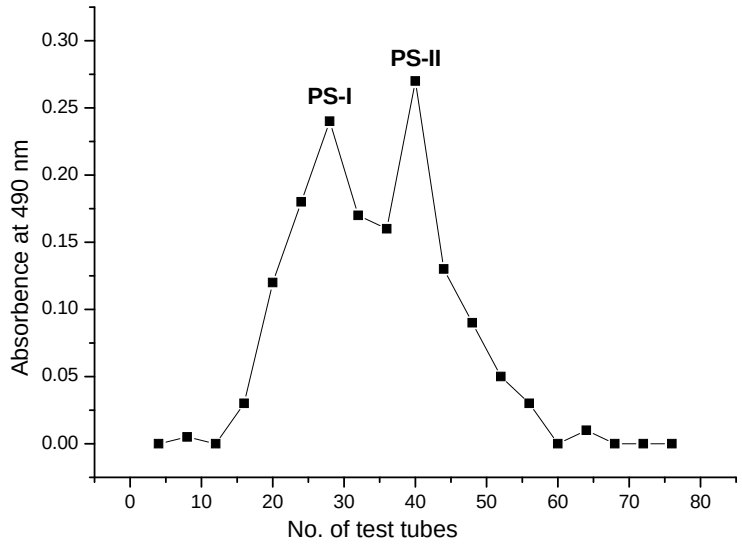


Figure 1. The gel permeation chromatogram of the polysaccharides isolated from hot aqueous extract of fruit bodies of the edible mushroom, *Russula albonigra*.

4.2.2. Optical rotation and molecular weight of PS-II

The pure PS-II showed a specific rotation $[\alpha]_D^{28}$ +34.5 (*c* 0.05, water). The molecular weight [Hara et al., 1982] of PS-II was estimated as ~1.45 × 10^4 Da from a calibration curve prepared with standard dextrans [**Figure 2**].

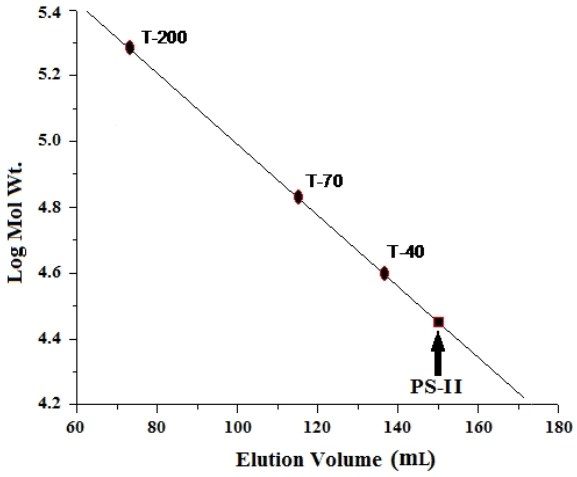


Figure 2. Determination of molecular weight of the PS-II isolated from hot aqueous extract of fruit bodies of the edible mushroom, *Russula albonigra*.

4.2.3. Structural analysis of PS-II

4.2.3.1. Chemical analysis of PS-II

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 [1978] 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 and Kerek [1984] 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 [**Figure 3, Table 1**]. These linkages were further confirmed by periodate oxidation experiment. GLC analysis of alditol acetates of periodate-oxidized [Goldstein et al., 1965; Hay et al., 1965], 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 [Abdel-Akher and Smith, 1950] 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 [**Figure 4, Table 2**]. 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.

Table 1

GLC-MS analysis of methylated polysaccharide (PS-II) isolated from the edible mushroom, *Russula albonigra*.

Methylated sugars	Linkage type	Molar ratio	Major Mass Fragments (m/z)
3,4-Me_2-Gal	→2,6)-α-D-Gal*p*-(1→	1	43,71,87,99,129,159,173,189,233
2,3,4-Me_3-Gal	→6)-α-D-Gal*p*-(1→	1	43,71,87,101,117,129,161,173,189,233
2,3,4,6-Me_4-Man	α-D-Man*p*-(1→	1	43,59,71,87,101,117,129,145,161,205
2,3,4-Me_3-Fuc	α-L-Fuc*p*-(1→	1	43,72,89,101,115,117,131,161,175
3,4-Me_2-Fuc	→2)-α-L-Fuc*p*-(1→	1	43,59,71,89,99,115,129,131,173,189
2,6-Me_2-Glc	→3,4)-β-D-Glc*p*-(1→	1	43,58,74,87,101,117,129,143,159,173, 189,233
2,4,6-Me_3-Glc	→3)-β-D-Glc*p*-(1→	1	43,74,87,101,117,129,143,161,173,203, 217,233

Table 2

GLC-MS analysis of methylated polysaccharide (PS-II) after oxidation of sodium periodate isolated from the edible mushroom, *Russula albonigra*.

Methylated sugars	Linkage type	Molar ratio	Major Mass Fragments (m/z)
2,6-Me_2-Glc	→3,4)-β-D-Glc*p*-(1→	1	43,58,74,87,101,117,129,143,159,173, 189,201,233
2,4,6-Me_3-Glc	→3)-β-D-Glc*p*-(1→	1	43,58,74,87,101,117,129,143,161,173, 203,217,233

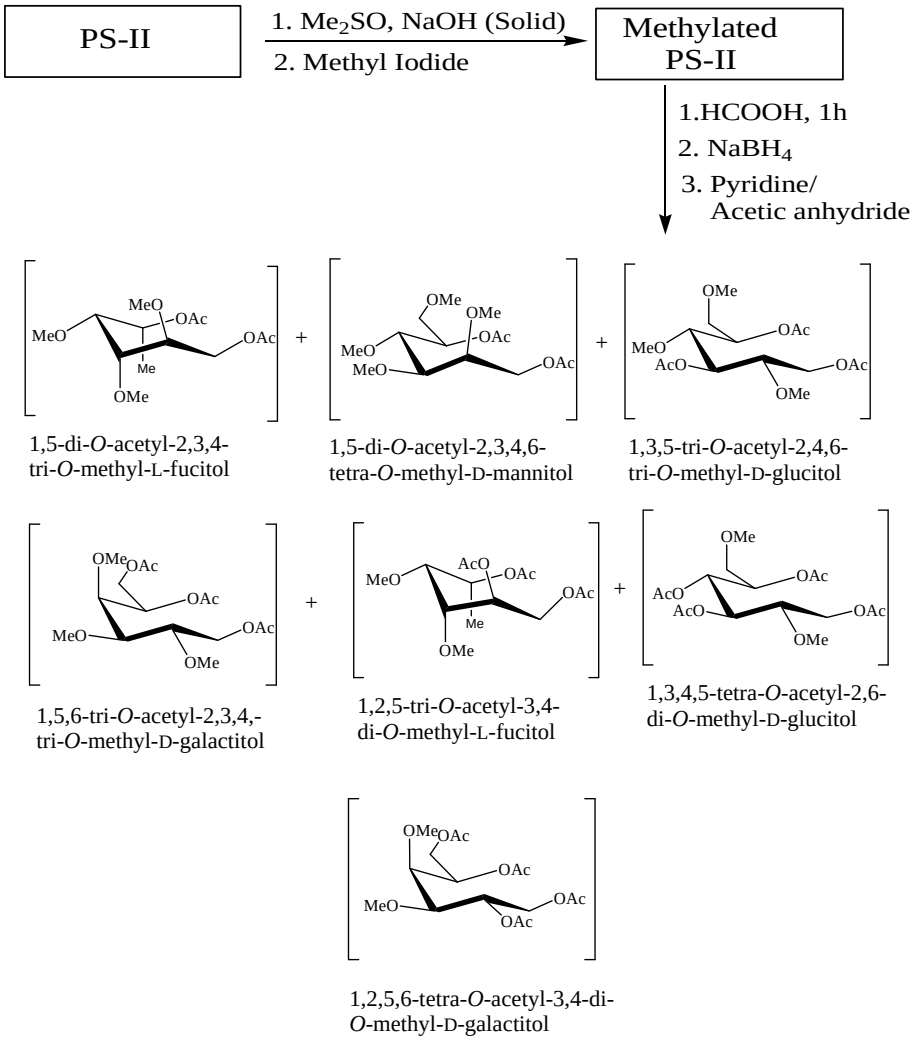


Figure 3. Schematic presentation of methylation experiment of the PS-II isolated from the edible mushroom, *Russula albonigra*.

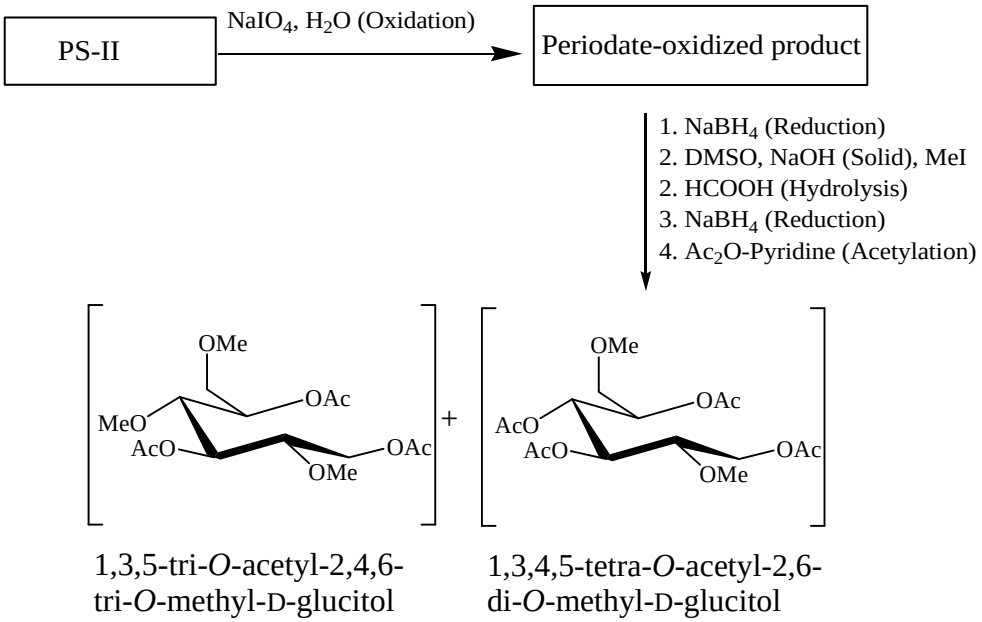


Figure 4. **S**chematic presentation of periodate oxidation reactions of the PS-II isolated from the edible mushroom, *Russula albonigra*.

4.2.3.2. 1D and 2D NMR analysis of PS-II

The ^{1}H NMR (500 MHz) spectrum [**Figure 5a, Table 3**] 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 [**Figure 5b, Table 3**] and DEPT-135 [**Figure 5c**] 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 [**Figure 5d**], 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 [**Figure 5d and 5e**] experiments. Coupling constants were measured from DQF-COSY spectrum.

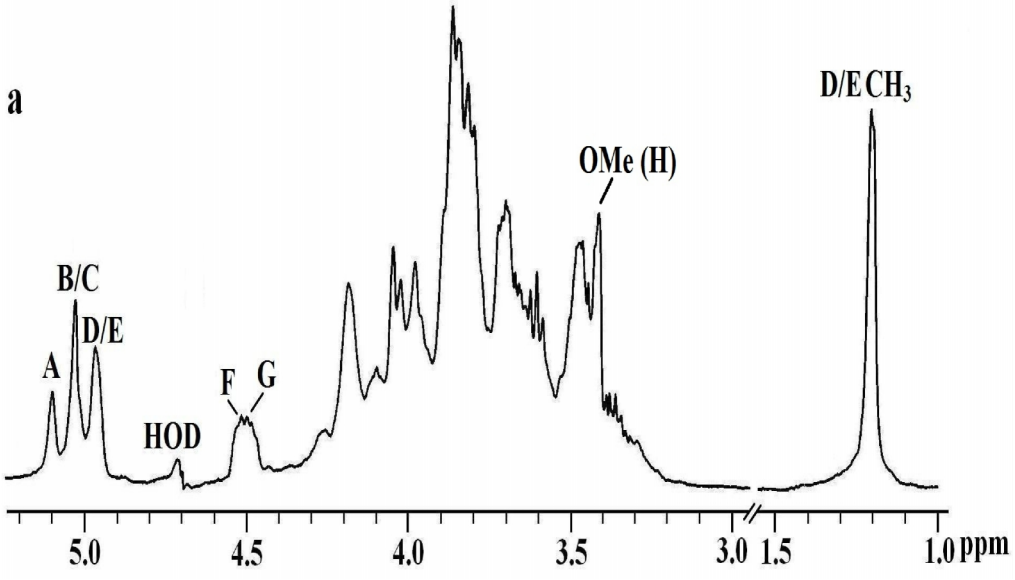


Figure 5. (a) ^{1}H NMR spectrum (500 MHz, D_2O, 30 ºC) of the PS-II isolated from the edible mushroom, *Russula albonigra*.

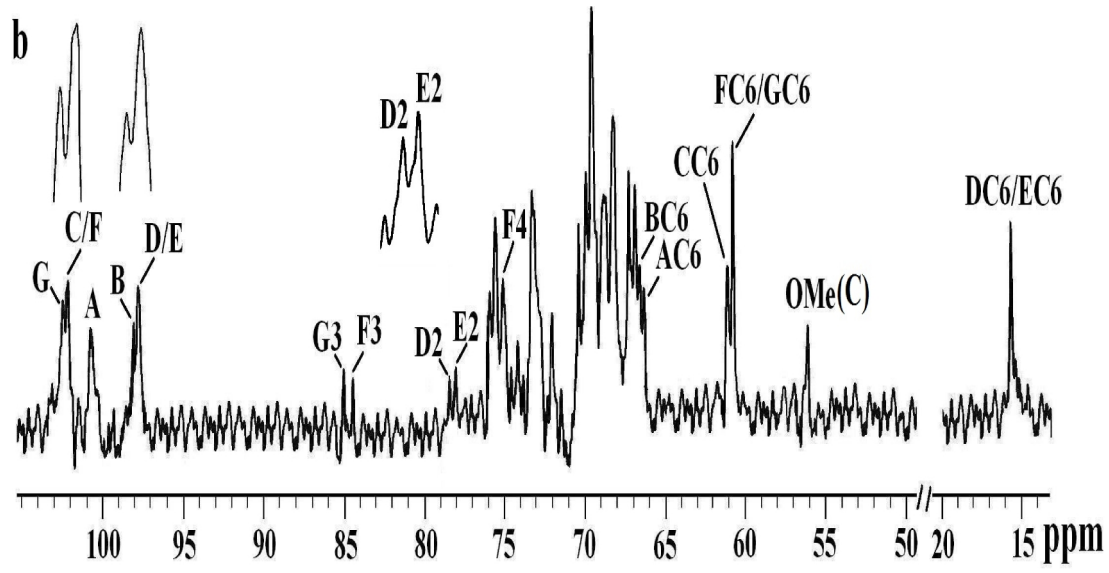


Figure 5. (b) ^{13}C NMR spectrum (125 MHz, D_2O, 30 ºC) of the PS-II isolated from the edible mushroom, *Russula albonigra*.

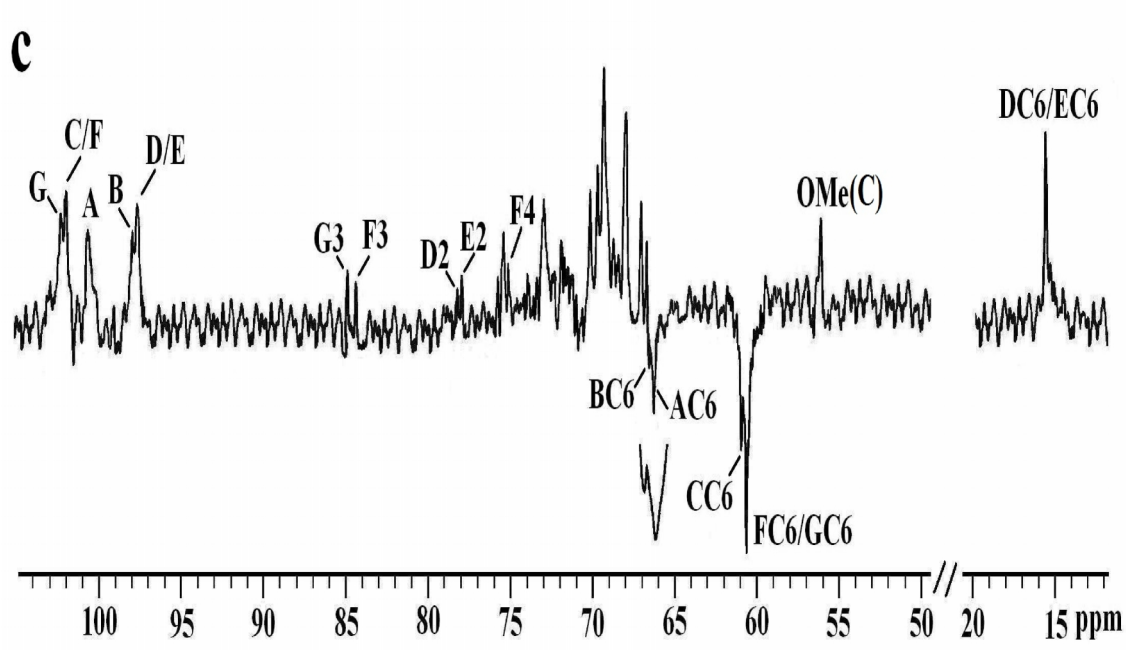


Figure 5. (c) DEPT-135 spectrum (D_2O, 30 ºC) of the PS-II isolated from the edible mushroom, *Russula albonigra*.

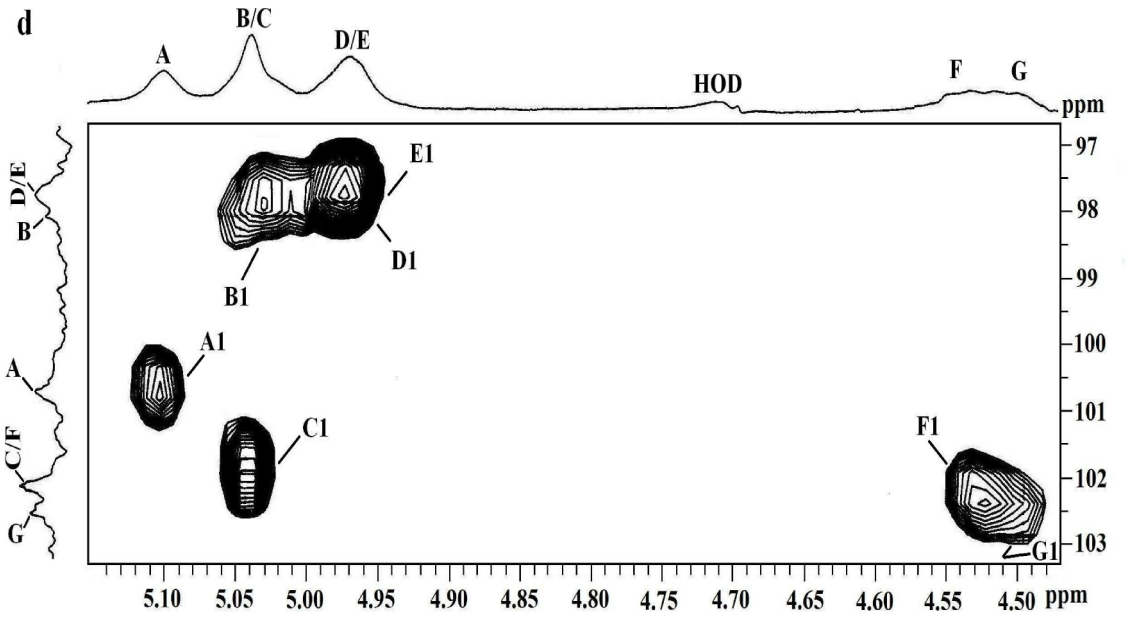


Figure 5. (d) The HSQC spectrum (D_2O, 30 ºC) of anomeric part of the PS-II isolated from the edible mushroom, *Russula albonigra*.

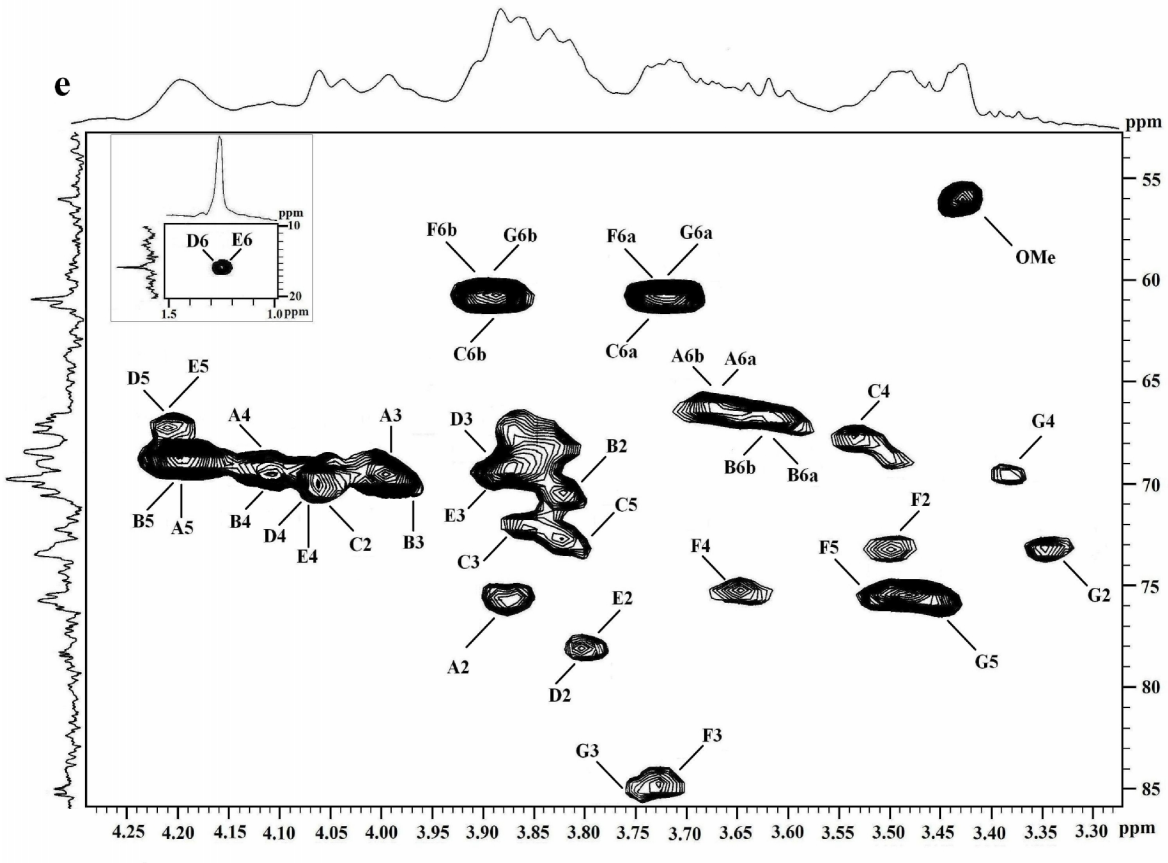


Figure 5. (e) The HSQC spectrum (D_2O, 30 ºC) other than anomeric part of the PS-II isolated from the edible mushroom, *Russula albonigra*.

Table 3

The $^{1}H^{a}$ and $^{13}C^{b}$ NMR chemical shifts for the polysaccharide (PS-II) isolated from the edible mushroom, *Russula albonigra* in D_2O at 30 ^{o}C.

Glycosyl residue	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6a,H-6b/C-6	-*O*Me
→2,6)-α-D-Gal*p*-(1→	5.10	3.87	4.00	4.12	4.20	3.67[c], 3.67[d]	
A	100.8	75.6	69.8	69.5	69.0	66.5	
→6)-α-D-Gal*p*-(1→	5.04	3.82	3.97	4.12	4.20	3.64[c], 3.64[d]	
B	98.0	70.5	70.0	69.5	69.0	66.7	
α-D-Man*p*-(1→	5.04	4.05	3.86	3.54	3.82	3.71[c], 3.88[d]	
C	102.2	70.4	72.0	67.7	73.0	61.2	
α-L-Fuc*p*-2*O*Me (1→	4.97	3.80	3.88	4.06	4.21	1.24	3.43
D	97.8	78.2	69.5	70.2	67.1	15.6	56.0
→2)-α-L-Fuc*p*-(1→	4.97	3.80	3.88	4.06	4.21	1.24	
E	97.8	78.0	69.5	70.2	67.1	15.6	
→3,4)-β-D-Glc*p*-(1→	4.52	3.50	3.73	3.65	3.48	3.70[c], 3.90[d]	
F	102.2	73.2	84.5	75.2	75.6	60.8	
→3)-β-D-Glc*p*-(1→	4.50	3.35	3.74	3.39	3.46	3.70[c], 3.90[d]	
G	102.5	73.2	85.0	69.6	76.0	60.8	

[a] Values of the ^{1}H chemical shifts were recorded with respect to the HOD signal fixed at δ 4.70 ppm at 30 ^{o}C.

[b] Values of the ^{13}C chemical shifts were recorded with reference to acetone as the internal standard and fixed at δ 31.05 ppm at 30 ^{o}C.

[c, d] Interchangeable

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 [Agarwal, 1992; Rinaudo and Vincendon, 1982] 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

[**Figure 5c**]. 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 [**Figure 7, Table 5**]. On the other hand, the downfield shift of C-2 (δ 78.0) with respect to standard values of methyl glycosides [Agarwal, 1992; Rinaudo and Vincendon, 1982] indicated that the residue **E** was also linked at C-2 position with residue **A** which further confirmed by the ROESY [**Figure 6, Table 4**]as well as HMBC experiment [**Figure 7, Table 5**]. 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 [Agarwal, 1992; Rinaudo and Vincendon, 1982] 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**.

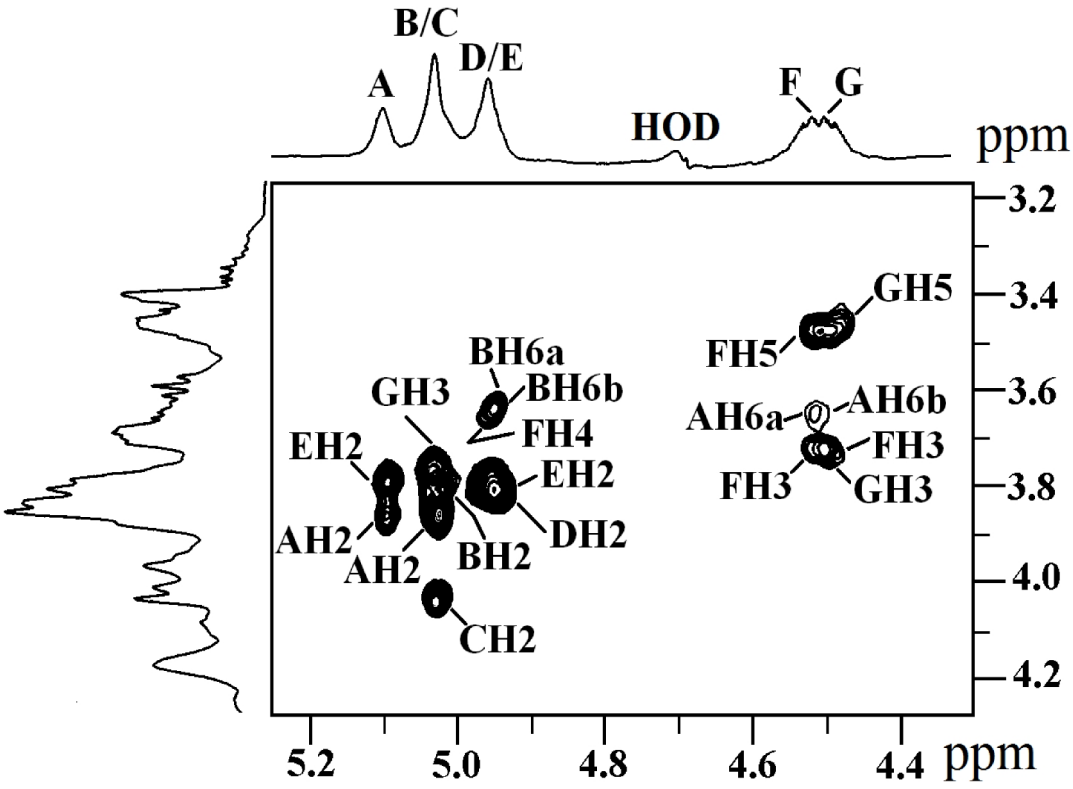


Figure 6. The part of ROESY spectrum of the PS-II isolated from the edible mushroom, *Russula albonigra*. The ROESY mixing time was 300 ms.

The sequences of glycosyl moieties were determined from ROESY [**Figure 6, Table 4**] 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**

Table 4

ROE data for the polysaccharide (PS-II) isolated from the edible mushroom, *Russula albonigra* in D_2O at 30 ^oC.

Glycosyl residue	Anomeric proton	ROE contact protons	
	¢	¢	Residue, atom
→2,6)-α-D-Gal*p*-(1→ **A**	5.10	3.87 3.80	**A**H-2 **E**H-2
→6)-α-D-Gal*p*-(1→ **B**	5.04	3.82 3.74	**B**H-2 **G**H-3
α-D-Man*p*-(1→ **C**	5.04	4.05 3.87	**C**H-2 **A**H-2
α-L-Fuc*p*-2*O*Me (1→ **D**	4.97	3.80 3.65	**D**H-2 **F**H-4
→2)-α-L-Fuc*p*-(1→ **E**	4.97	3.80 3.64	**E**H-2 **B**H-6a/6b
→3,4)-β-D-Glc*p*-(1→ **F**	4.52	3.73 3.48 3.67	**F**H-3 **F**H-5 **A**H-6a/6b
→3)-β-D-Glc*p*-(1→ **G**	4.50	3.74 3.46 3.73	**G**H-3 **G**H-5 **F**H-3

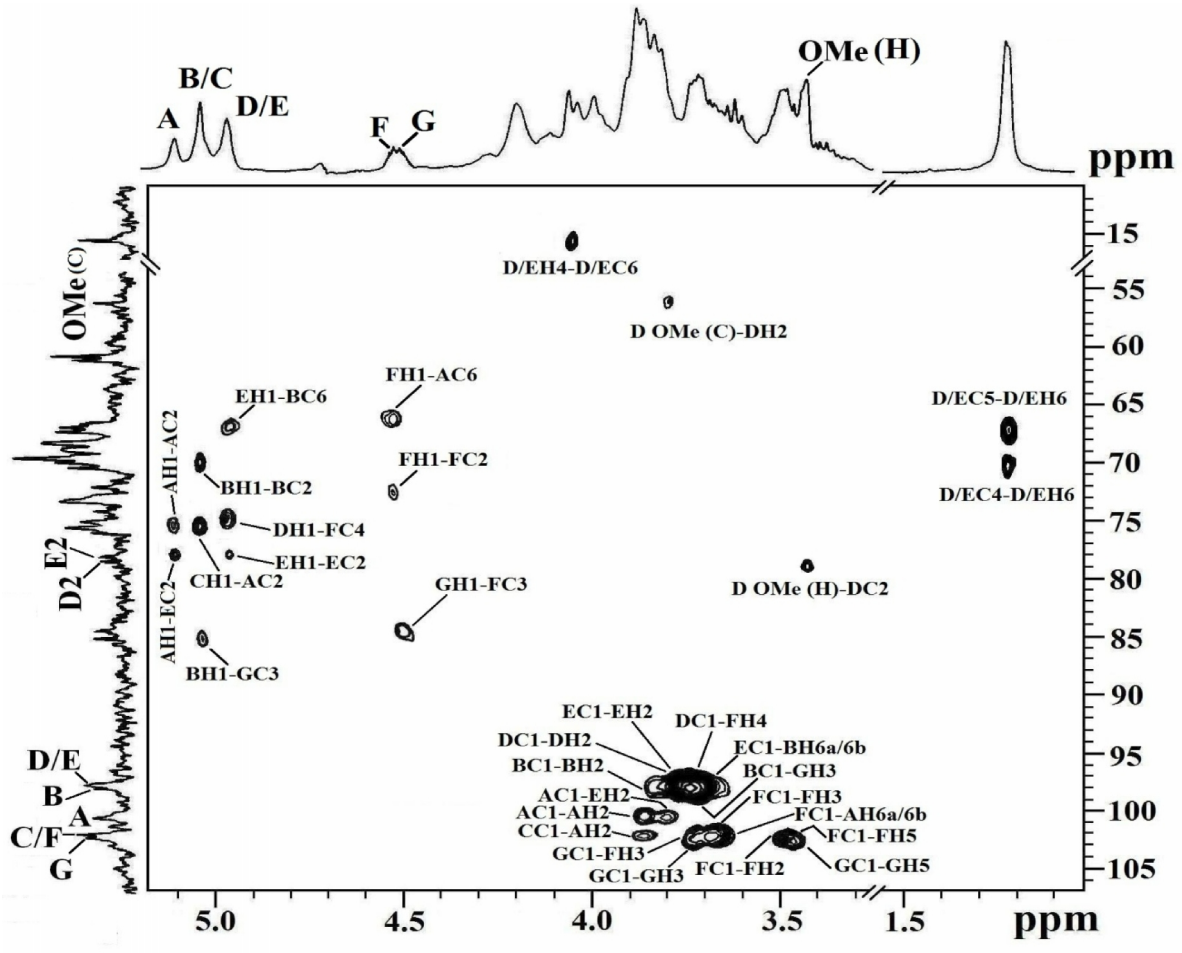


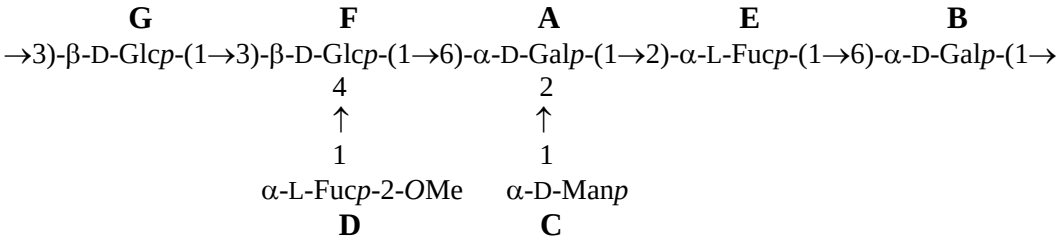
Figure 7. The part of HMBC spectrum of the PS-II isolated from the edible mushroom, *Russula albonigra*. The delay time in the HMBC experiment was 80 ms.

Table 5

The significant $^{3}J_{H,C}$ connectivities observed in an HMBC spectrum for the anomeric protons/carbons of the sugar residues of the polysaccharide (PS-II) isolated from the edible mushroom, *Russula albonigra* in D_2O at 30 ^{o}C.

Residues	**Sugar linkage**	**H-1/C-1**	**Observed connectivities**		
		¢H/¢C	**¢H/¢C**	**Residue**	**Atom**
A	→2,6)-α-D-Gal*p*-(1→	5.10	78.0	**E**	C-2
			75.6	**A**	C-2
		100.8	3.80	**E**	H-2
			3.87	**A**	H-2
B	→6)-α-D-Gal*p*-(1→	5.04	85.0	**G**	C-3
			70.5	**B**	C-2
		98.0	3.74	**G**	H-3
			3.82	**B**	H-2
C	α-D-Man*p*-(1→	5.04	75.6	**A**	C-2
		102.2	3.87	**A**	H-2
D	α-L-Fuc*p* -2*O*Me (1→	4.97	75.2	**F**	C-4
		97.8	3.65	**F**	H-4
			3.80	**D**	H-2
E	→2)-α-L-Fuc*p*-(1→	4.97	66.7	**B**	C-6
			78.0	**E**	C-2
		97.8	3.64	**B**	H-6a/6b
			3.80	**E**	H-2
F	→3,4)-β-D-Glc*p*-(1→	4.52	66.5	**A**	C-6
			73.2	**F**	C-2
		102.2	3.67	**A**	H-6a/6b
			3.50	**F**	H-2
			3.73	**F**	H-3
			3.48	**F**	H-5
G	→3)-β-D-Glc*p*-(1→	4.50	84.5	**F**	C-3
		102.5	3.73	**F**	H-3
			3.74	**G**	H-3
			3.46	**G**	H-5
D	α-L-Fuc*p* -2*O*Me(1→	**-*O*CH$_3$ ¢H/¢C**			
		3.43	78.2	**D**	C-2
		56.0	3.80	**D**	H-2

A long range HMBC experiment was carried out to confirm the ROESY connectivities. In HMBC experiment [**Figure 7, Table 5**], 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. this is shown as:



4.2.4. Immunostimulating properties of PS-II

Before the immunological studies, polysaccharide should be free from bacterial endotoxin which is very crucial step for the determination of accurate value of different biological experiment. A negative (-) LAL (Limulus amebocyte lysate) test indicated that LFPS-II which was obtained after passing the PS-II through polymixin-B matrix, was free from bacterial endotoxin. Immunological studies were also investigated with the LFPS-II. Macrophage activation by LFPS-II was observed *in vitro*. Upon treatment with different concentrations of the LFPS-II, enhanced production of NO was observed in a dose-dependent manner with optimum production of 18 µM NO per 5 x 10^5 macrophages at 100 µg/mL of the LFPS-II [**Figure 8a**]. Lentinan obtained from *Lentinus edodes* (Berk.) Sing., inhibit the tumor growth by stimulating the immune system [Suzuki et al., 1994] through activation of macrophages, T-helper, NK, and other cells.

Splenocytes are the cells present in the spleen that include T cells, B cells, and dendritic cells that stimulate the immune response in living organism. Thymocytes are

hematopoietic cells in thymus which generate T cells. The splenocytes and thymocytes activation tests were carried out in Swiss Albino mice cell culture medium with the LFPS-II by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method [Ohno et al., 1993]. Proliferation of splenocytes and thymocytes is an indicator of immunostimulation. The LFPS-II was found to stimulate splenocytes and thymocytes as shown in **Figure 8b and 8c**, respectively and the asterisks on the columns indicate the statistically significant differences compared to PBS control. At 25 µg/mL of the LFPS-II, splenocyte proliferation index was found to be maximum as compared to other concentrations. Hence, 25 µg/mL of the LFPS-II can be considered as efficient splenocyte stimulators. Again 50 µg/mL of this same sample showed maximum effect on thymocyte proliferation. The splenocyte and thymocyte proliferation index as compared to Phosphate Buffered Saline (PBS) control if closer to 1 or below indicates low stimulatory effect on immune system [Maiti et al., 2008; Mallick et al., 2010; Shah et al., 2007]. It is noteworthy to mention that several α-L-fucose containing heteroglycan [Bhunia et al., 2010; Dey et al., 2013; Mandal et al., 2011] also showed immunoenhancing properties as reported earlier by our group.

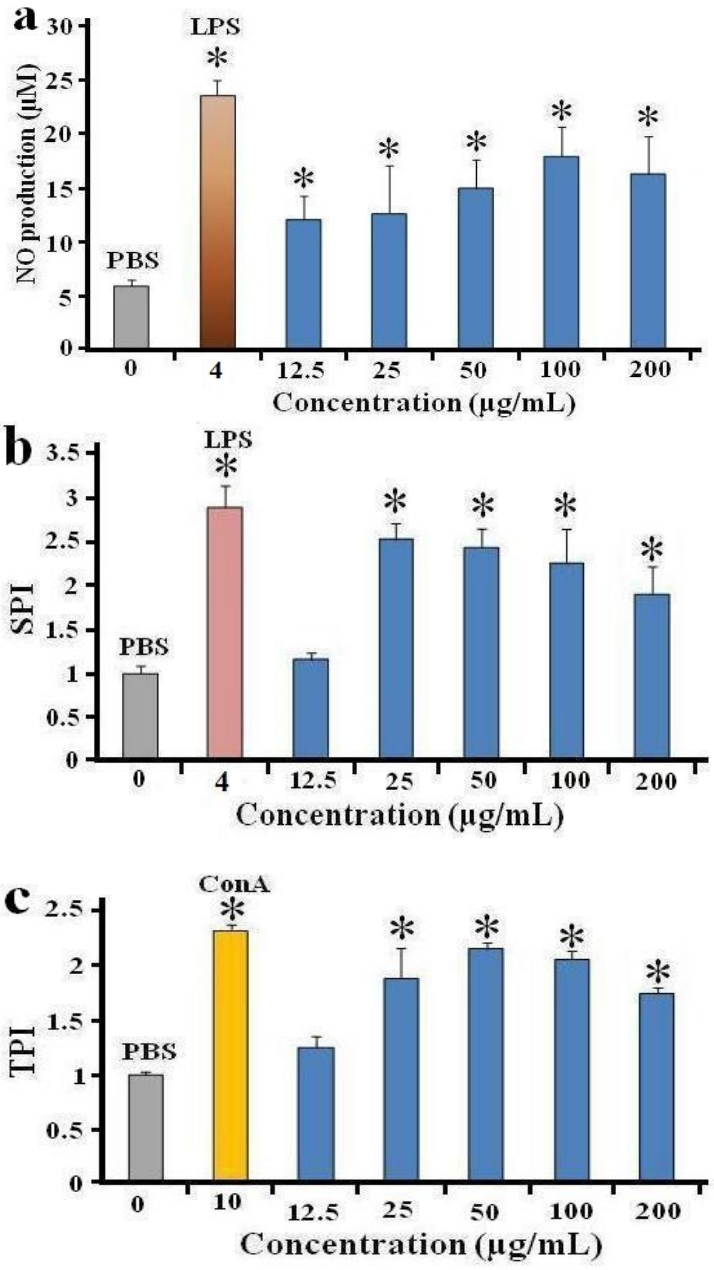


Figure 8. (a) Activation of RAW 264.7 macrophage cells with different concentrations of the LPS free PS-II (LFPS-II) in terms of NO production. Effect of different concentrations of the LPS free PS-II (LFPS-II) on proliferation of (b) splenocyte and (c) thymocyte (asterisks indicate the statistically significant compared to the PBS control).

4.3. Conclusions

A water-soluble heteroglycan (PS-II) was isolated from aqueous extract of an edible mushroom *R. albonigra* (Krombh.) Fr. Architectural details of the repeating unit of the PS-II obtained from *R. albonigra* (Krombh.) Fr. was determined from chemical and 1D/2D NMR studies. The proposed repeating unit of the PS-II had a backbone consisting of two (1→3)-β-D-glucopyranosyl, two (1→6)-α-D-galactopyranosyl, and one (1→2)-α-L-fucopyranosyl residues, out of which one (1→3)-β-D-glucopyranosyl residue was branched at *O*-4 position with terminal 2-*O*-methyl-α-L-fucopyranosyl and one (1→6)-α-D-galactopyranosyl residue was branched at *O*-2 position with terminal α-D-mannopyranosyl residue. This PS-II showed *in vitro* macrophage activation by NO production as well as splenocytes and thymocytes proliferation. So, it may be used as a source of natural immunostimulant and it also contained naturally important bioactive carbohydrate moiety α-L-fucose which have large utility in medicinal chemistry.

4.4. References

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CHAPTER-5

Antioxidant and immunostimulant β-glucan (PS) from the alkaline extract of the edible mushroom, *Russula albonigra*

5.1. Introduction and earlier work

Mushroom polysaccharides, especially, β-glucans are recognized as biological response modifier (BRM) and used for the treatment of cancer and various infectious diseases both in modern medicine and traditional chemotherapeutic drug [Chan, Chan, and Sze, 2009; Kidd, 2000; Wasser, 2002]. They have drawn the attention of chemists and immunobiologists for their immunomodulatory, anti-tumor [Wasser, 2002] as well as antioxidant activities [Kozarski et al., 2011]. Reactive oxygen species (ROS) which damage lipids, proteins, carbohydrates, and nucleic acids [Blokhina, Virolainen, and Fagerstedt, 2003] are the roots of developing diseases like cancer, Alzheimer, and Parkinson [Papas, 1999]. β-glucans from mushrooms are well-known antioxidant material [Kofuji et al., 2012] which can neutralize the harsh effect of ROS [Papas, 1999]. Modern research has been focused on utilizing the naturally occurring substances to neutralize the radical activities. Several linear (1→3) and branched (1→3)-, (1→6)-β-D-glucans [Ohno et al., 1993; Yoshioka et al., 1985] are used as immunostimulating and antitumor materials. Some immunostimulating water soluble β-D-glucans [Maity et al., 2013; Sen et al., 2013] have also been reported by our group.

Russula albonigra (Krombh.) Fr., an ectomycorrhizal, edible and non-toxic fungus [Nandi et al., 2012] contained two water-soluble polysaccharides, α,β-glucan and a heteroglycan [Nandi et al., 2012; Nandi et al., 2013] whereas alkali treated aqueous extract contained only one polysaccharide which was characterized as a β-glucan. The detailed structural investigation and study of immunostimulation as well as antioxidant activities of this β-glucan were carried out and reported in this chapter.

5.2. Present work

5.2.1. Isolation and purification of polysaccharide from *R. albonigra*

The fresh fruit bodies of edible mushroom, *Russula albonigra* (Krombh.) Fr. (500 g) were washed with distilled water, boiled with 4% NaOH solution, filtered, centrifuged, and the supernatant was precipitated in EtOH. The precipitated materials on dialysis followed by freeze drying yielded 1.6 g of crude polysaccharide. The water soluble crude polysaccharide (30 mg) was purified by gel permeation chromatography on column of Sepharose 6B yielded one homogeneous fraction [**Figure 1**]. A single homogeneous fraction was collected, and freeze-dried, yielding 20 mg of pure PS. The same procedure was repeated in several lots to yield 100 mg of pure polysaccharide.

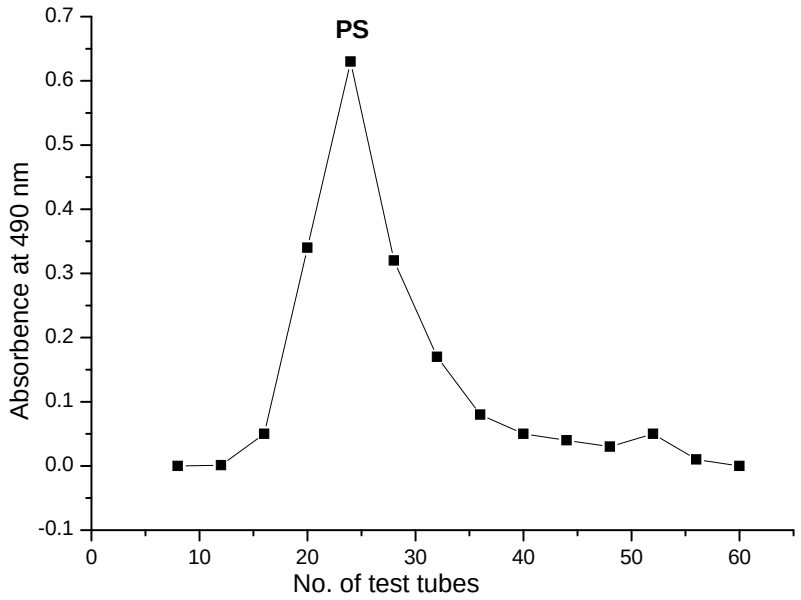


Figure 1. The gel permeation chromatogram of the polysaccharide (PS) isolated from hot alkaline aqueous extract of fruit bodies of the edible mushroom, *Russula albonigra.*

5.2.2. Optical rotation and molecular weight of β-glucan (PS)

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 [Dong et al., 2002]. The molecular weight [Hara et al., 1982] of PS was estimated as ~1.95 × 10^5 Da from a calibration curve prepared with standard dextrans [**Figure 2**].

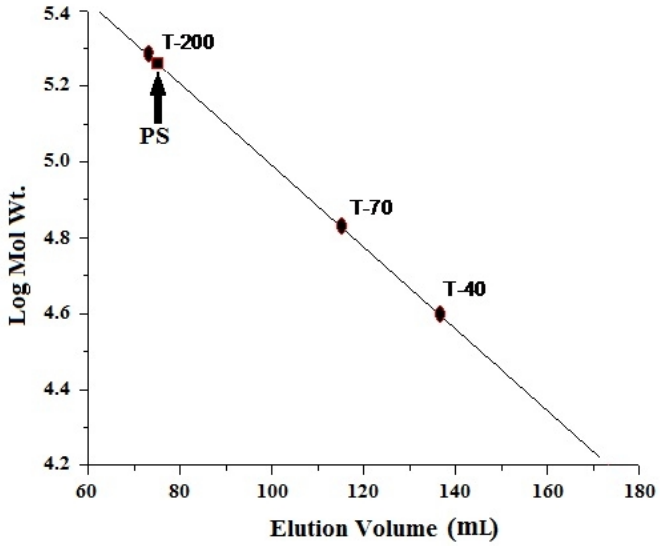


Figure 2. Determination of molecular weight of the PS isolated from alkaline extract of fruit bodies of the edible mushroom, *Russula albonigra*.

5.2.3. Structural analysis of β-glucan (PS)

5.2.3.1. Chemical analysis of β-glucan (PS)

GLC analysis of the alditol acetates of this polysaccharide revealed the presence of glucose only. The absolute configuration of the monosaccharide present in the glucan was determined by the method of Gerwig, Kamerling, and Vliegenthart [1978] and it was found that glucose had D-configuration. The polysaccharide was methylated according to the method of Ciucanu & Kerek [1984] 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 [**Figure 3, Table 1**]. 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, mild hydrolysis and periodate oxidation studies were performed for determination of the backbone present in the polysaccharide. The GLC analysis of the alditol acetates of the periodate-oxidised [Goldstein et al., 1965], 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 [**Figure 4, Table 2**]. 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 [**Figure 5**]. 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 [Dong et al., 2002] 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.

Table 1

GLC-MS data for methylated PS of the edible mushroom, *Russula albonigra*.

Methylated sugars	Linkage types	Molar ratio	Major Mass fragments (m/z)
2,3,4,6-Me_4-Glc*p*	Glc*p*-(1→	1	43, 45, 71, 87, 101, 117, 129, 145, 161, 205
2,4,6-Me_3-Glc*p*	→3)-Glc*p*-(1→	2	43, 45, 58, 71, 87, 99, 101, 117, 129, 143, 161, 201, 233
2,3,4-Me_3-Glc*p*	→6)-Glc*p*-(1→	2	43, 58, 71, 87, 99, 101, 117, 129, 161, 189, 233
2,4-Me_2-Glc*p*	→3,6)-Glc*p*-(1→	1	43, 58, 87, 99, 101, 117, 129, 139, 159, 189, 233

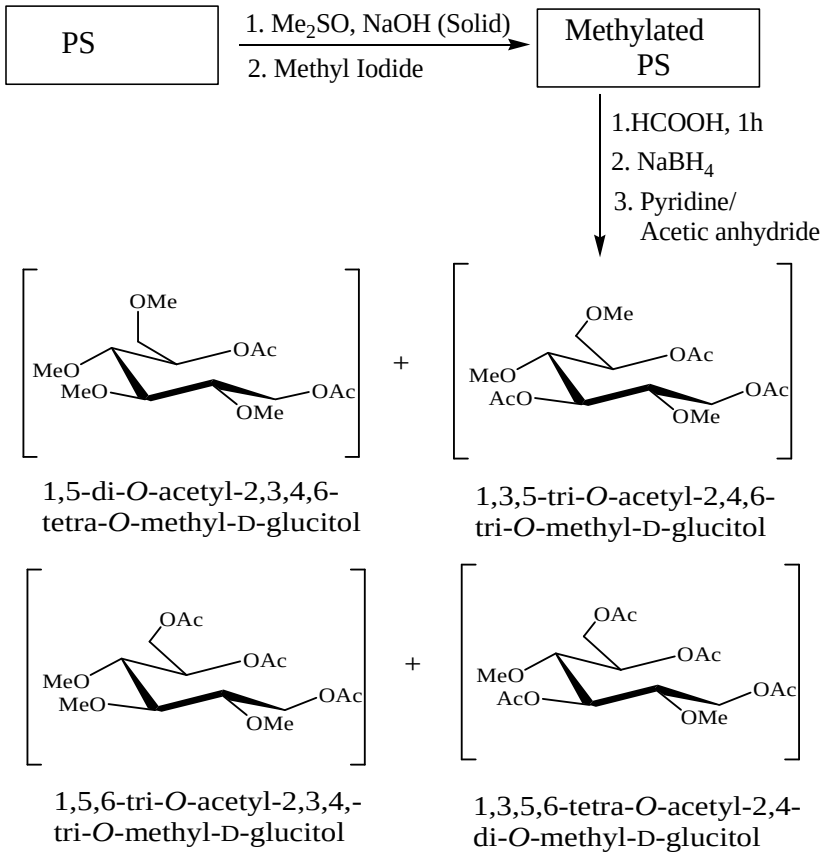


Figure 3. Schematic presentation of methylation experiment of PS isolated from the edible mushroom, *Russula albonigra.*

Table 2

GLC-MS analysis of periodate oxidized methylated PS of an edible mushroom, *Russula albonigra.*

Methylated sugars	Linkage types	Molar ratio	Major Mass fragments (m/z)
2,4,6-Me_3-Glc*p*	→3)-Glc*p*-(1→	2	43, 45, 58, 71, 87, 99, 101, 117, 129, 143, 161, 201, 233
2,4-Me_2-Glc*p*	→3,6)-Glc*p*-(1→	1	43, 58, 87, 99, 101, 117, 129, 139, 159, 189, 233

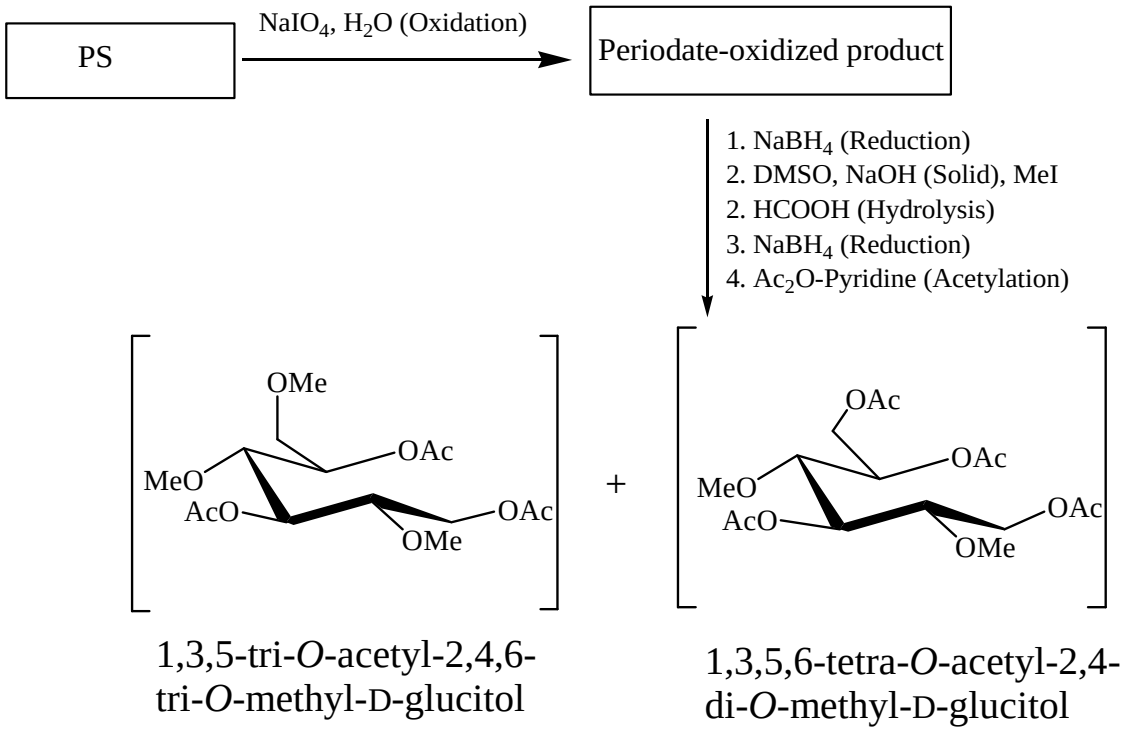


Figure 4. Schematic presentation of periodate oxidation reactions of PS isolated from the edible mushroom, *Russula albonigra.*

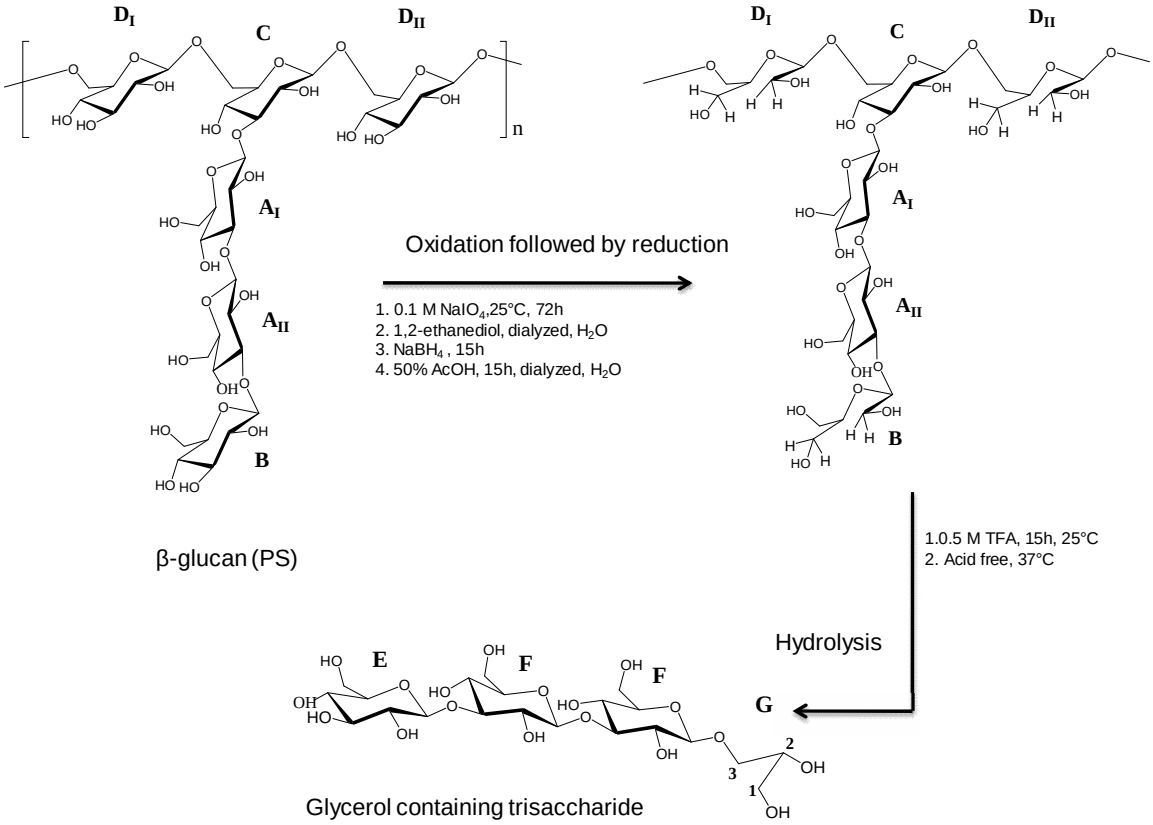


Figure 5. Schematic presentation of Smith degradation reactions of PS isolated from the edible mushroom, *Russula albonigra.*

5.2.3.2. 1D and 2D NMR analysis of β-glucan (PS)

The ^{1}H NMR (500 MHz) spectrum [**Figure 6a, Table 3**] 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 [**Figure 6b, Table 3**] 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 [**Figure 6c, Table 3**], 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 [Agrawal, 1992] 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 [Agrawal, 1992] 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 [Yoshioka et al., 1985; Maity et al., 2013] 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 [**Figure 6b**].

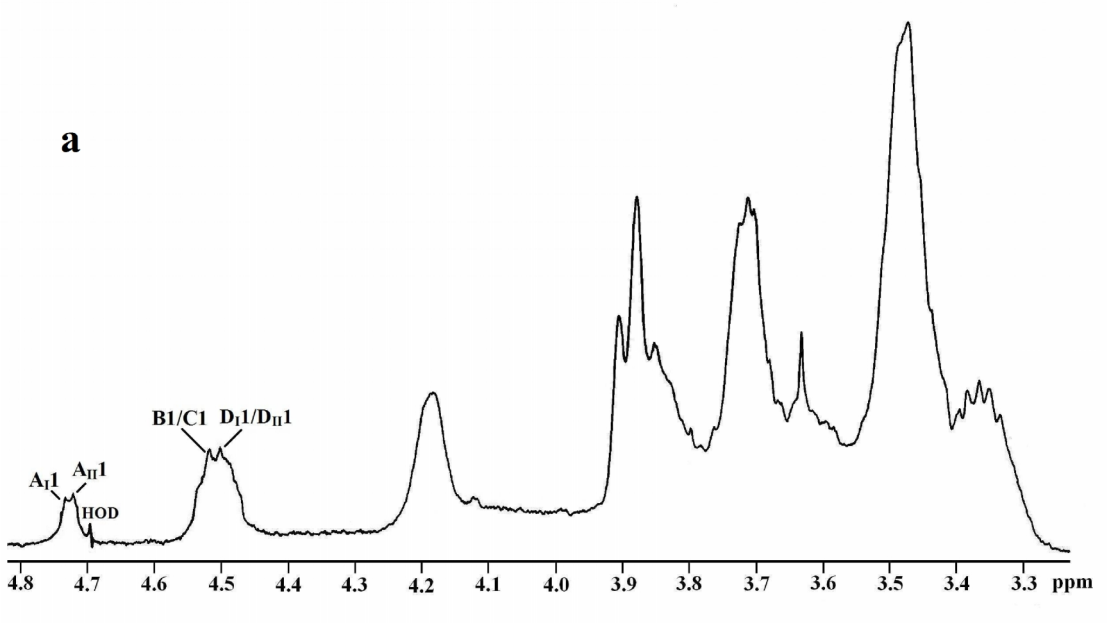


Figure 6. (a) ^{1}H NMR spectrum (500 MHz, D_2O, 30 ºC) of the β-glucan (PS) isolated from the edible mushroom, *Russula albonigra*.

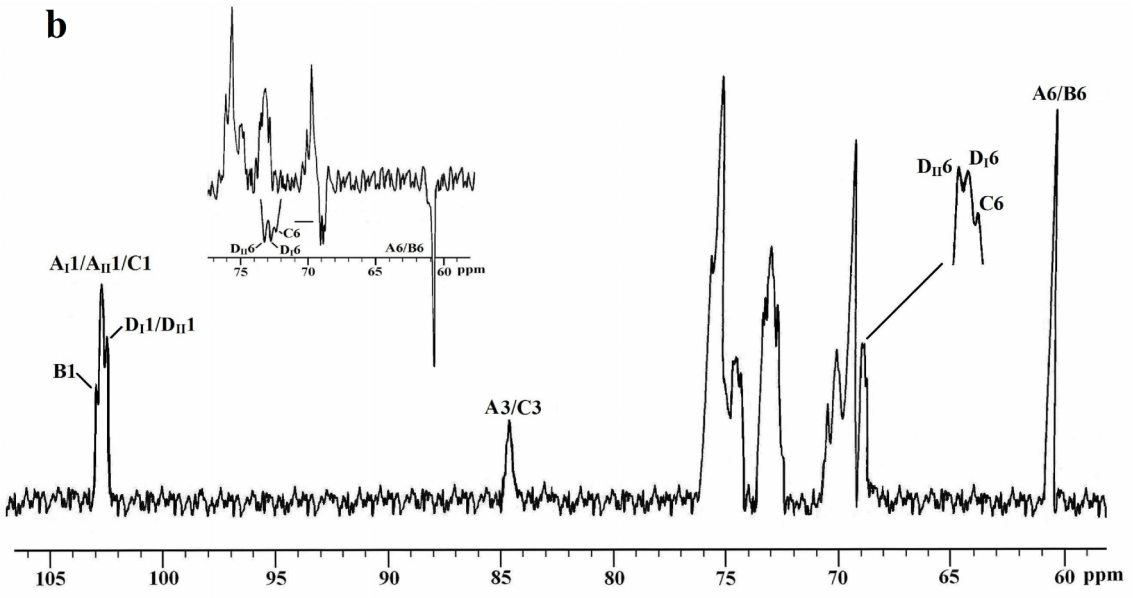


Figure 6. (b) Combination of ^{13}C NMR and DEPT-135 spectrum (125 MHz, D_2O, 30 ºC) of the β-glucan (PS) isolated from the edible mushroom *Russula albonigra.*

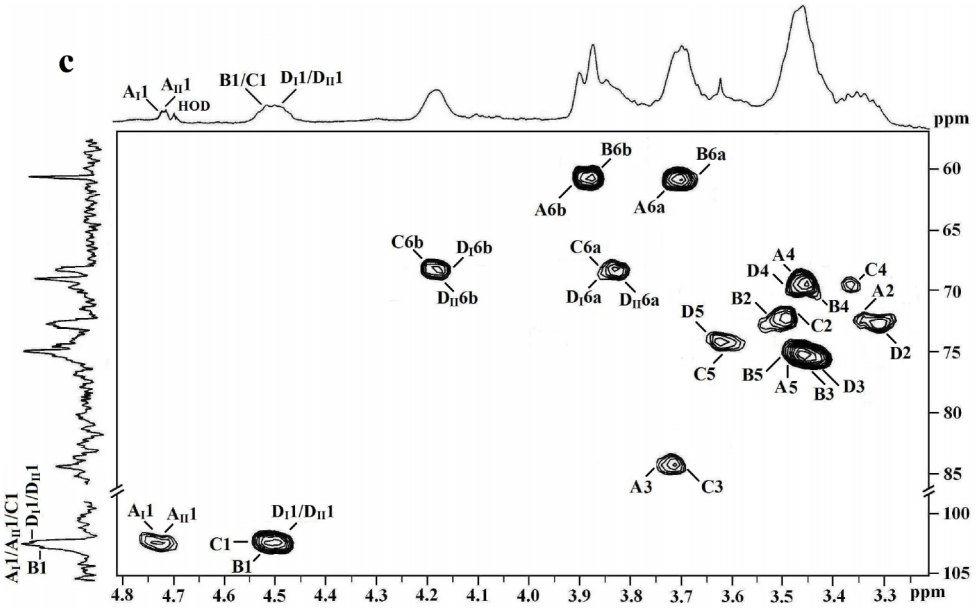


Figure 6. (c) The part of HSQC spectrum (D_2O, 30 ºC) of the β-glucan (PS) isolated from the edible mushroom, *Russula albonigra.*

Table 3

$^{1}H^{a}$ and $^{13}C^{b}$ NMR chemical shifts (δ in ppm) of the PS from *Russula albonigra* in D_2O.

Glucosyl residue	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6a,H-6b/C-6
→3)-β-D-Glc*p*-(1→	4.74^{x},4.72^{y}	3.35	3.73	3.46	3.47	3.72^{c}, 3.90^{d}
A (A_I, A_{II})	102.7	72.8	84.5	69.6	75.0	60.7
β-D-Glc*p*-(1→	4.52	3.50	3.46	3.44	3.48	3.70^{c}, 3.88^{d}
B	103.0	73.0	75.9	70.2	75.0	60.7
→3,6)-β-D-Glc*p*(1→	4.52	3.50	3.71	3.37	3.63	3.85^{c}, 4.19^{d}
C	102.7	72.8	84.5	69.6	74.5	68.7
→6)-β-D-Glc*p*-(1→	4.50	3.30	3.45	3.46	3.63	3.84_p^{c}, 4.17_p^{d}
D	102.5	73.0	75.9	70.0	74.5	68.8_p
						3.83_q^{c}, 4.18_q^{d}
						69.0_q

[a] Values of the ^{1}H chemical shifts were recorded with respect to the HOD signal fixed at δ 4.70 at 30 °C.

[b] Values of the ^{13}C chemical shifts were recorded with reference to acetone as the internal standard and fixed at δ 31.05 at 30 °C.

[c,d] Interchangeable. [x] For residue $\mathbf{A}_I$. [y] For residue $\mathbf{A}_{II}$. [p] For residue $\mathbf{D}_I$. [q] For residue $\mathbf{D}_{II}$.

The sequences of glucosyl moieties were determined from NOESY [**Figure 7a**] as well as ROESY experiments. A long range HMBC [**Figure 7b**] 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 [**Figure 7c, Table 4**]. Thus, the HMBC and NOESY connectivities confirmed the repeating unit in the PS.

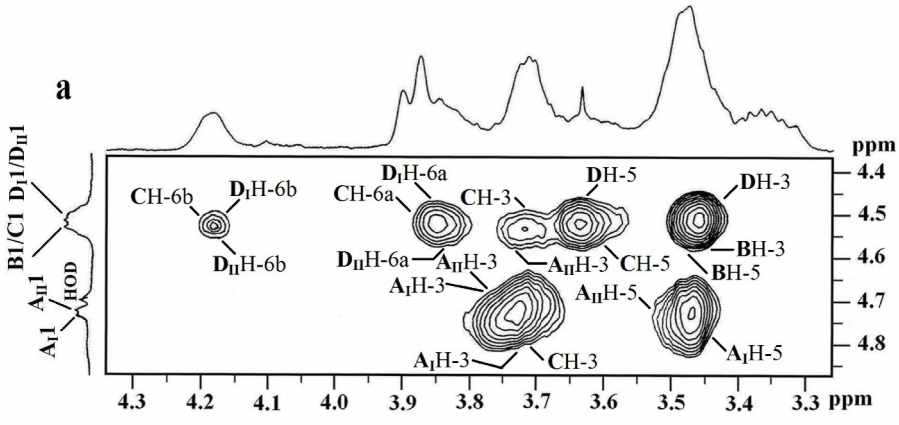


Figure 7. (a) The part of NOESY spectrum of the β-glucan (PS) isolated from the edible mushroom *Russula albonigra*. The NOESY mixing time was 300 ms.

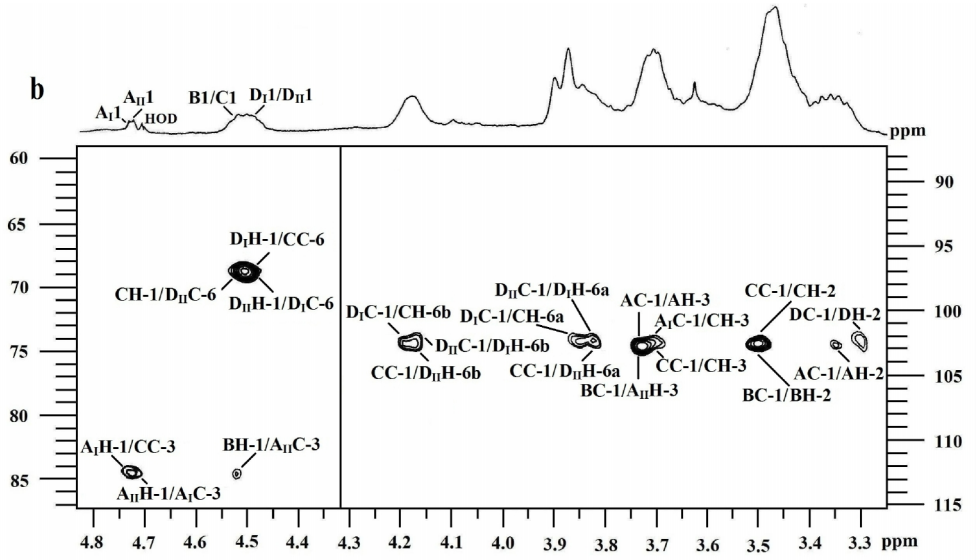


Figure 7. (b) The part of HMBC spectrum of the β-glucan (PS) isolated from the edible mushroom, *Russula albonigra*. The delay time in the HMBC experiment was 80 ms.

C

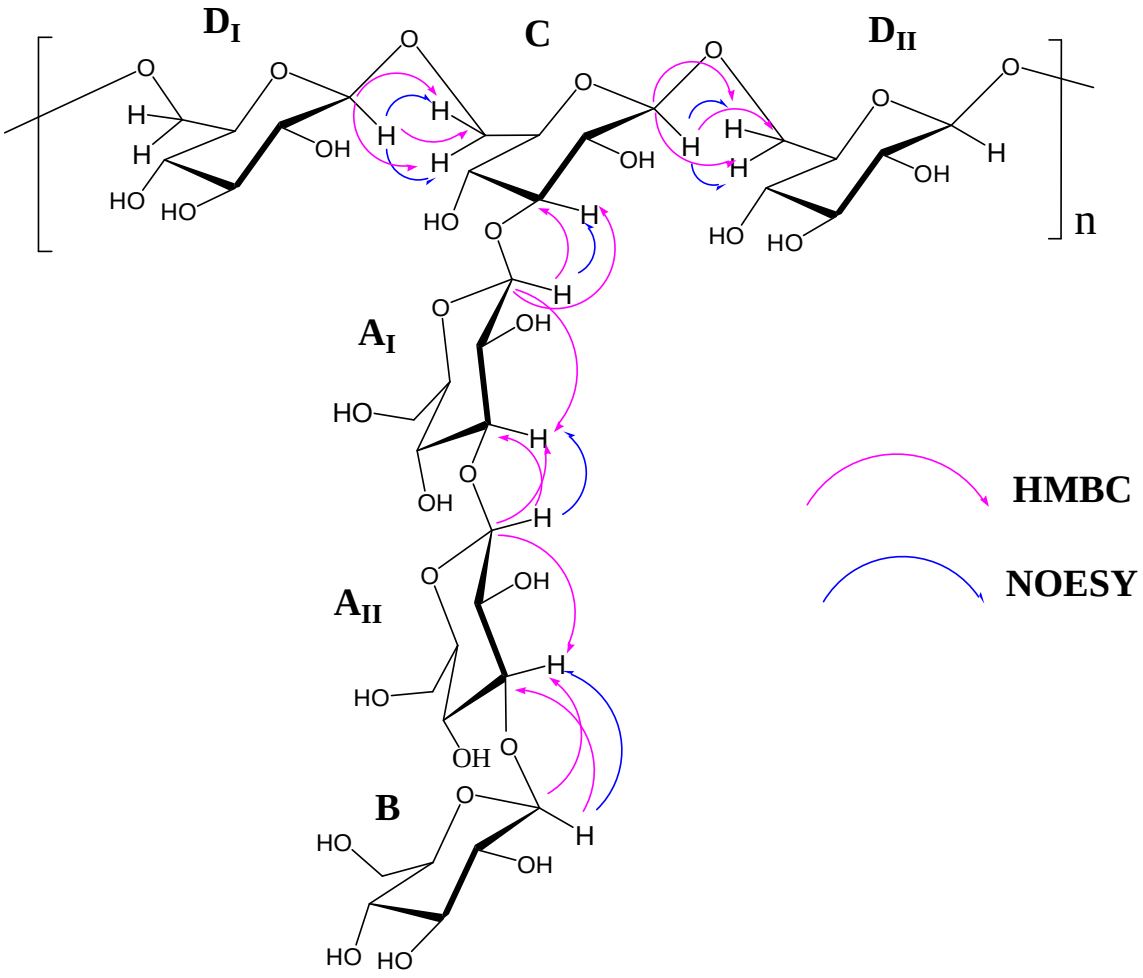


Figure 7. (c) The key 2D NMR correlations of β-glucan (PS).

Table 4

NOESY and HMBC connectivities of PS from the edible mushroom, *Russula albonigra* in D_2O at 30 ^oC.

NOESY connectivities	HMBC connectivities
A_IH-1/**C**H-3	**A_I**H-1/**C**C-3
A_IH-1/**A_I**H-3	**A_I**C-1/**C**H-3
A_IH-1/**A_I**H-5	**A_{II}**H-1/**A_I**C-3
A_{II}H-1/**A_I**H-3	**A**C-1/**A**H-3
A_{II}H-1/**A_{II}**H-3	**A**C-1/**A**H-2
A_{II}H-1/**A_{II}**H-5	
BH-1/**A_{II}**H-3	**B**H-1/**A_{II}**C-3
BH-1/**B**H-3	**B**C-1/**A_{II}**H-3
BH-1/**B**H-5	**B**C-1/**B**H-2
CH-1/**D_{II}**H-6a;**D_{II}**H-6b	**C**H-1/**D_{II}**C-6
CH-1/**C**H-3	**C**C-1/**D_{II}**H-6a;**D_{II}**H-6b
CH-1/**C**H-5	**C**C-1/**C**H-2
	CC-1/**C**H-3
D_IH-1/**C**H-6a;**C**H-6b	**D_I**H-1/**C**C-6
D_{II}H-1/**D_I**H-6a; **D_I**H-6b	**D_I**C-1/**C**H-6a;**C**H-6b
DH-1/**D**H-3	**D_{II}**H-1/**D_I**C-6
DH-1/**D**H-5	**D_{II}**C-1/**D_I**H-6a; **D_I**H-6b
	DC-1/**D**H-2

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 [**Figure 8, Table 5**] 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 [**Figure 5**]. Hence, Smith degradation resulted in the formation of an oligosaccharide unit from the parent polysaccharide and the structure of which was established as:

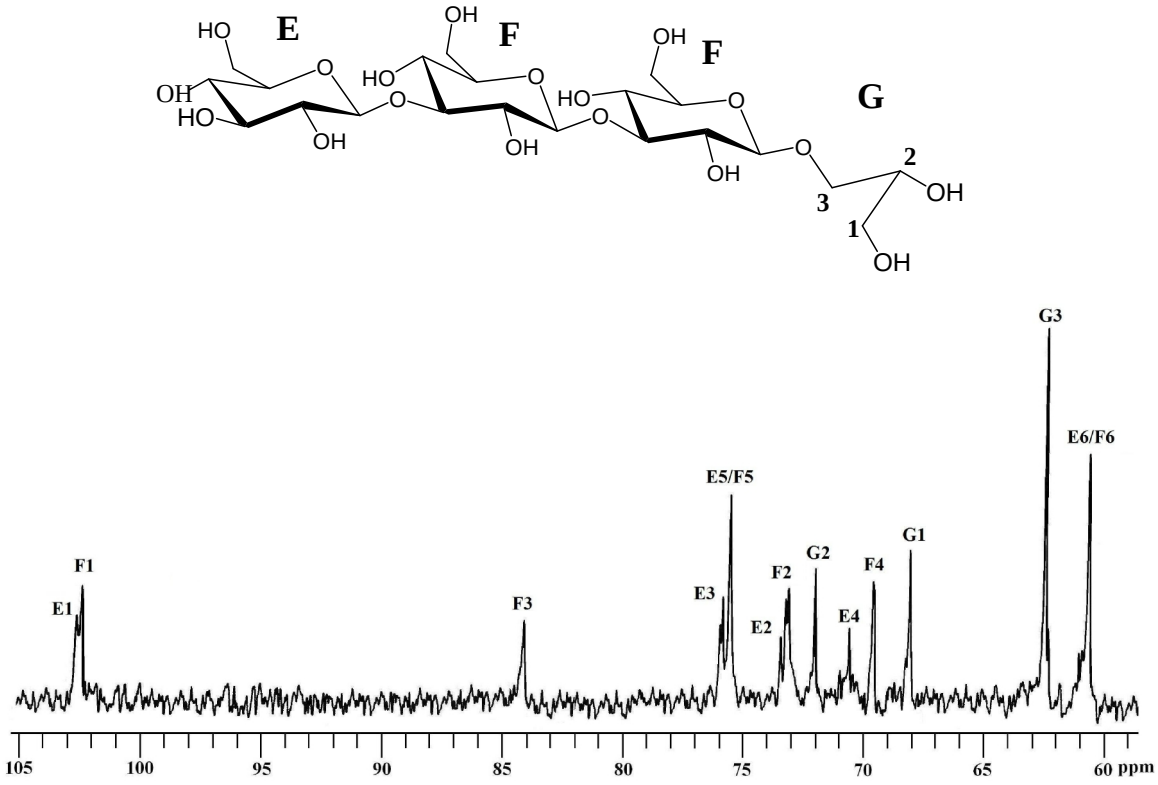


Figure 8. The ^{13}C NMR spectrum of the Smith-degraded glycerol-containing trisaccharide of the β-glucan (PS) isolated from the edible mushroom, *Russula albonigra* (Krombh.) Fr. in D_2O at 30 ^oC.

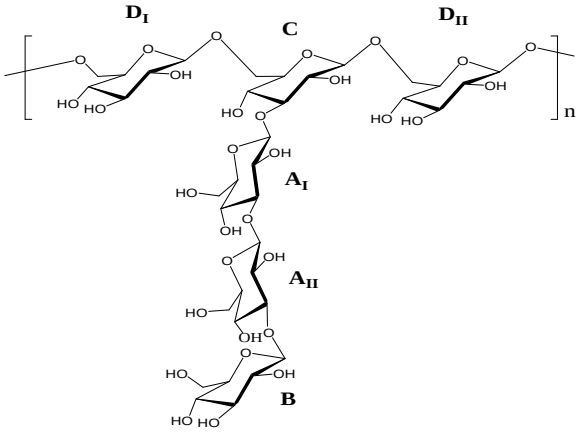
Table 5

^{13}C NMR[f] chemical shifts (δ in ppm) of Smith-degraded glycerol-containing trisaccharide of PS from the edible mushroom, *Russula albonigra* (Krombh.) Fr. in D_2O.

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6
β-D-Glc*p*-(1→ **E**	102.7	73.52	76.0	70.69	75.68	60.77
→3)-β-D-Glc*p*-(1→ **F**	102.5	73.22	84.20	69.70	75.68	60.77
Gro-(3→ **G**	68.10	72.0	62.56			

[f] The values of chemical shifts were recorded with reference to acetone as internal standard and fixed at δ 31.05 at 30 ºC.

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:



5.2.4. Immunological studies of -glucan (PS)

A negative (-) limulus amebocyte lysate (LAL) test with the PS was carried out adopting the procedure as reported in our previous publication [Nandi et al. 2013] and found that it was free from bacterial endotoxin. Immunological studies were also investigated with the PS. Macrophage activation by PS was observed *in vitro*. Upon treatment with different concentrations of the PS, enhanced production of NO was observed in a dose-dependent manner with optimum production of 22 µM NO per 5 x 10^5 macrophages at 100 µg/mL of the PS [**Figure 9a**]. The various types of β-glucan like lentinan inhibit tumor growth by stimulating the immune system [Suzuki et al., 1994] through activation of macrophages, T-helper, NK, and other cells.

Splenocytes are the cells present in the spleen that include T cells, B cells, and dendritic cells that stimulate the immune response in living organism. Thymocytes are hematopoietic cells in thymus which generate T cells. The splenocytes and thymocytes activation tests were carried out in Swiss Albino mice cell culture medium with the PS by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method [Ohno et al., 1993]. Proliferation of splenocytes and thymocytes is an indicator of immunostimulation. The PS was found to stimulate splenocytes and thymocytes as shown in **Figure 9b** and **9c**, respectively and the asterisks on the columns indicate the statistically significant differences compared to PBS control. 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. The splenocyte and thymocyte proliferation index as compared to Phosphate Buffered Saline (PBS) control if closer to 1 or below indicates low stimulatory effect on immune system.

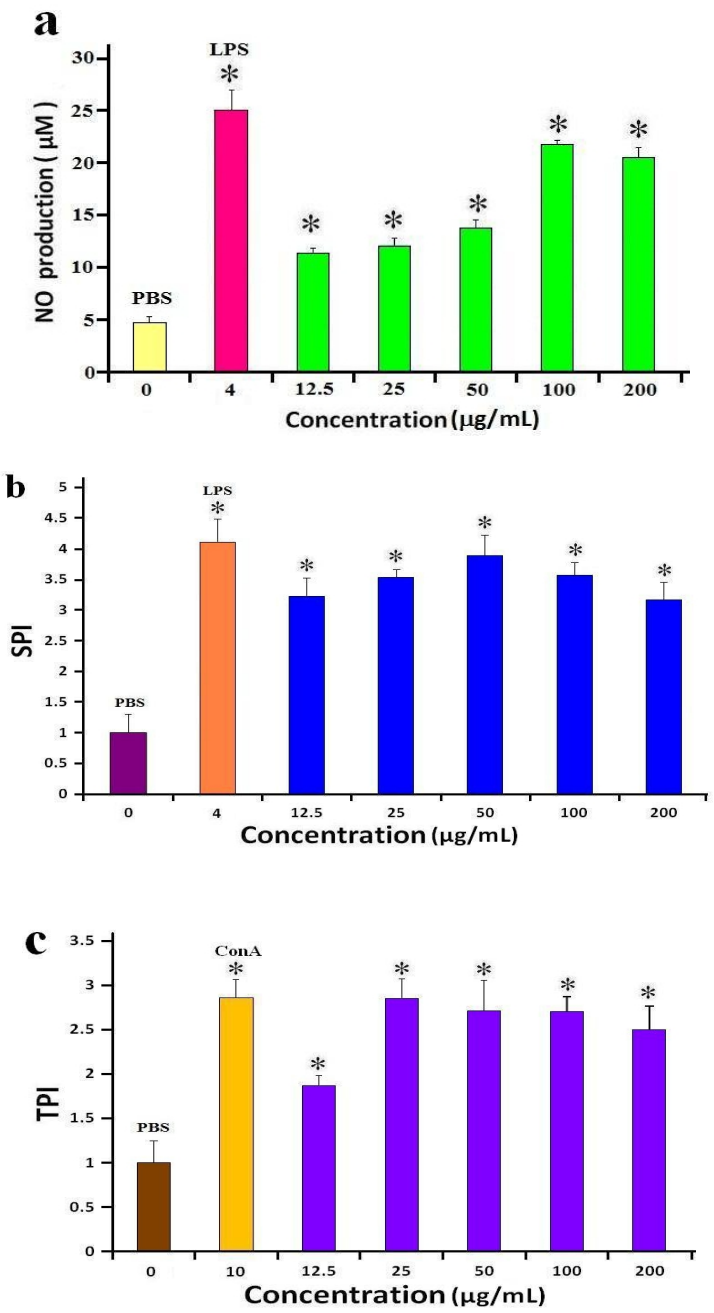


Figure 9. (a) Activation of RAW 264.7 macrophage cells with different concentrations of β-glucan (PS) in terms of NO production. Effect of different concentrations of β-glucan (PS) on proliferation of (b) splenocyte and (c) thymocyte (asterisks indicate the statistically significant compared to the PBS control with $P < 0.05$).

5.2.5. Antioxidant activities of -glucan (PS)

5.2.5.1. Assay of hydroxy radical scavenging activity

Hydroxy radical ($OH^{\cdot}$) has a very short life but it is considered to be the most toxic among all reactive oxygen species (ROS). It can damage DNA by attacking purines, pyrimidines and deoxyribose. Hydroxy radicals are formed by an electron transfer from transition metals to H_2O_2 and consequently interact with biomolecules [Ferreira, Barros, and Aberu, 2009]. In our experiment, hydroxy radicals which are generated from Fe^{2+}-ascorbate-EDTA-H_2O_2 system (Fenton's reaction) attack the deoxyribose and eventually result in the formation of malondialdehyde (MDA). The formation of MDA is measured as a pink MDA-TBA chromogen at 535 nm [Aruoma, Laughton, and Halliwell, 1989]. When test sample was added to reaction mixture, they removed hydroxy radicals and prevented sugar degradation. The PS showed potent hydroxy radical scavenging activity which rose gradually with the increase of concentration [**Figure 10a**]. The IC_{50} value of the PS was found to be 265 µg/mL [Acharya, Chatterjee, and Ghosh, 2011].

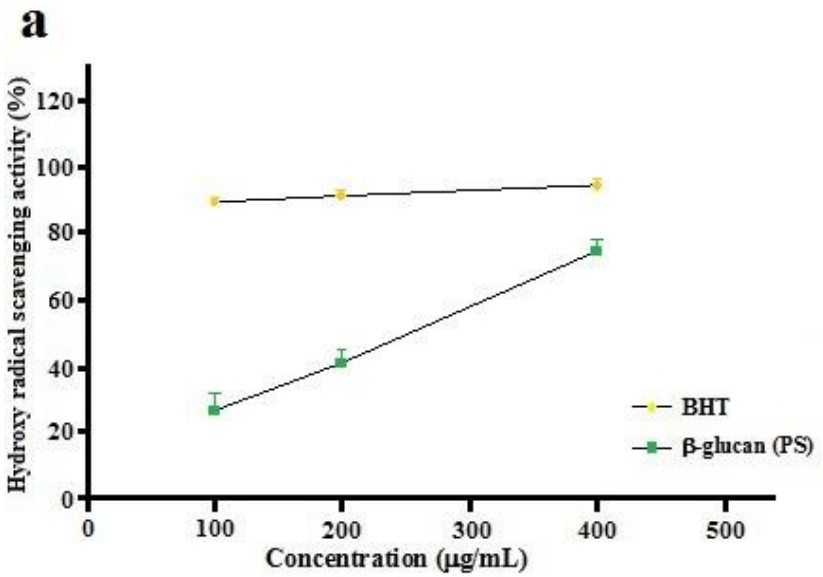


Figure 10. (a) Hydroxy radical scavenging activity of β-glucan (PS) isolated from the edible mushroom, *Russula albonigra* (All the above results are the mean ± SD of three separate experiments, each in triplicate).

5.2.5.2. Assay of superoxide radical scavenging activity

Superoxide anion ($O_2^{\cdot}$) is one of the six major reactive oxygen species causing oxidative damage in the human body [Huang, Ou, and Prior, 2005]. It is considered as primary ROS as it is a relatively weak oxidant but it can generate secondary ROS such as peroxynitrate (ONOO·), peroxy radical (LOO·), singlet oxygen, hydroxy radical and hydrogen peroxide [Chen et al., 2012; Huang, Ou, and Prior, 2005; Wootton-Beard and Ryan, 2011]. Therefore, superoxide radical scavenging activity is of great importance to exhibit potential antioxidant property. The method used by Martinez, Marcelo, Marco, & Moacyr [2001] based on generation of superoxide radical by auto-oxidation of riboflavin in presence of light which in turn reduces yellow dye NBT to produce blue formazon. Intensity of color is directly proportional to the concentration of superoxide anion. In the present study, the PS was found to act as a notable scavenger of superoxide radicals [**Figure 10b**]. The IC_{50} value of the PS was determined 130 µg/mL, whereas Patra *et al* [2013] reported that the IC_{50} value of the polysaccharide from *Pleurotus Ostreatus* was 553 µg/mL.

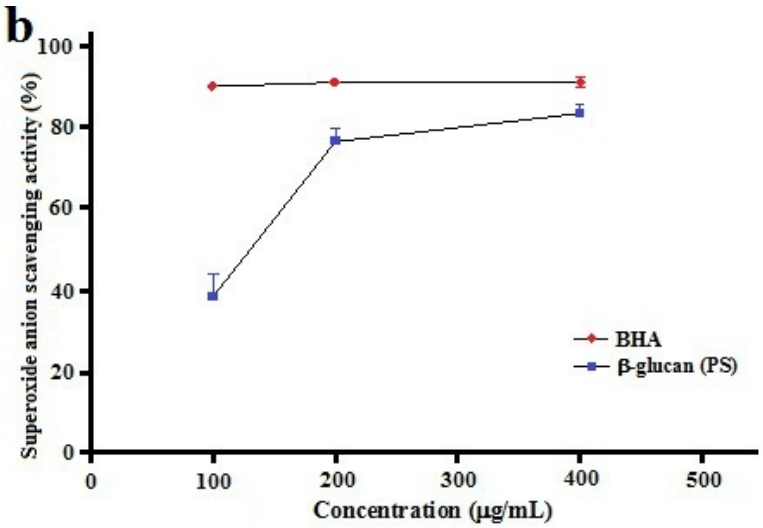


Figure 10. (b) Superoxide radical scavenging activity of β-glucan (PS) isolated from the edible mushroom, *Russula albonigra* (All the above results are the mean ± SD of three separate experiments, each in triplicate).

5.2.5.3. Chelating ability of ferrous ion

Dietary nutrients containing metal chelators may act as preventive antioxidant because some transition metals e.g. Fe^{2+}, Cu^{+}, Pb^{2+}, Co^{2+} and so on can trigger process of free radical reaction [Chen et al., 2012]. Ferrozine quantitatively forms complexes with Fe^{2+}. In the presence of chelating agent, the complex formation is disrupted, resulting in the reduction of red color. Reduction therefore allows estimation of the chelating ability of the coexisting chelator. **Figure 10c** reveals that the PS demonstrated a marked capacity for iron binding ability, where the 50% chelation was found at a concentration of 300 µg/mL.

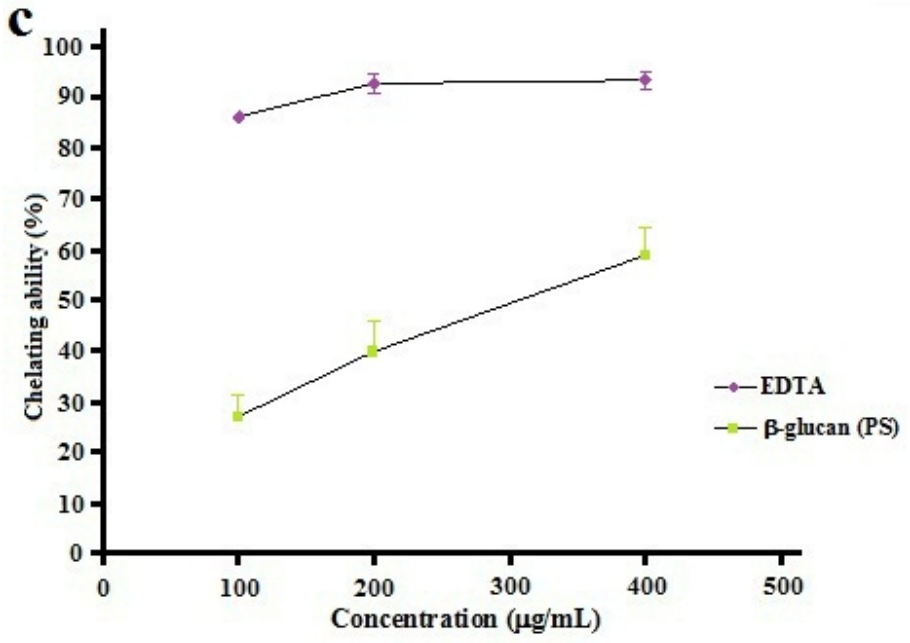


Figure 10. (c) Ferrous ion chelating ability of β-glucan (PS) isolated from the edible mushroom, *Russula albonigra* (All the above results are the mean ± SD of three separate experiments, each in triplicate).

5.2.5.4. Determination of reducing power

Reducing properties of a substance are generally associated with the presence of reductones or hydroxide groups. Such substance can exert antioxidant activity by donating hydrogen atom to break the free radical chain [Wootton-Beard and Ryan, 2011]. 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. **Figure 10d** reveals that at concentration of 500 µg/mL PS showed reducing power of 0.5.

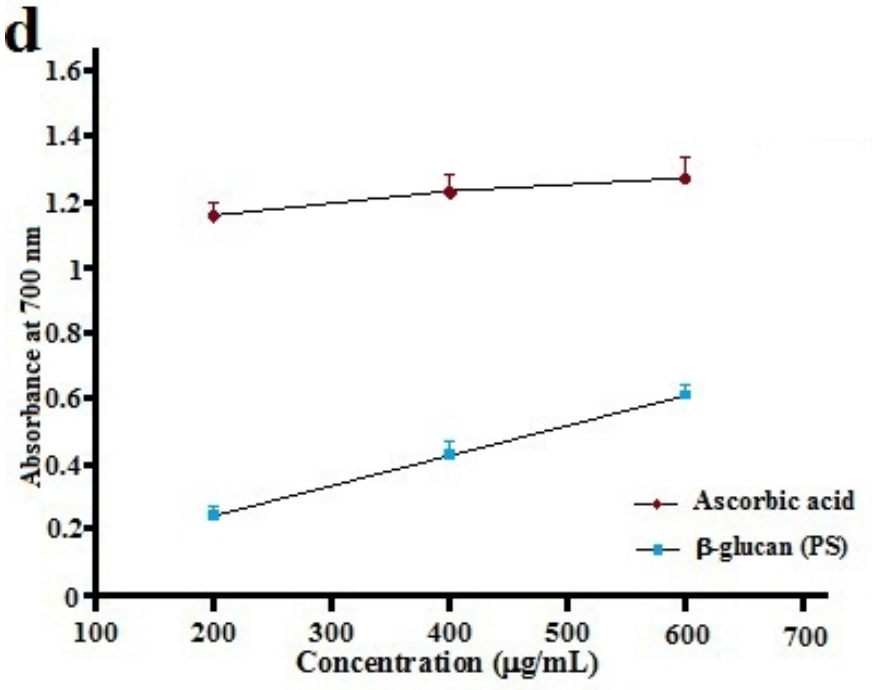


Figure 10. (d) Reducing power of β-glucan (PS) isolated from the edible mushroom, *Russula albonigra* (All the above results are the mean ± SD of three separate experiments, each in triplicate).

5.2.5.5. β carotene bleaching assay

β carotene usually undergoes rapid discoloration in absence of antioxidant. Oxidation of β carotene and linoleic acid generate free radicals. Free radicals from linoleic acid are formed by the abstraction of a hydrogen atom from one of its diallylic methylene groups which attack the highly unsaturated β carotene molecule. Hence, β carotene is oxidized, and gradually losing its orange color which is then monitored spectrophotometrically [Okoh, Sadimenko, and Afolayan, 2011]. **Figure 10e** reveals that the PS had inhibition effect on β carotene bleaching. The PS showed 50% inhibition at a concentration of 180 µg/mL.

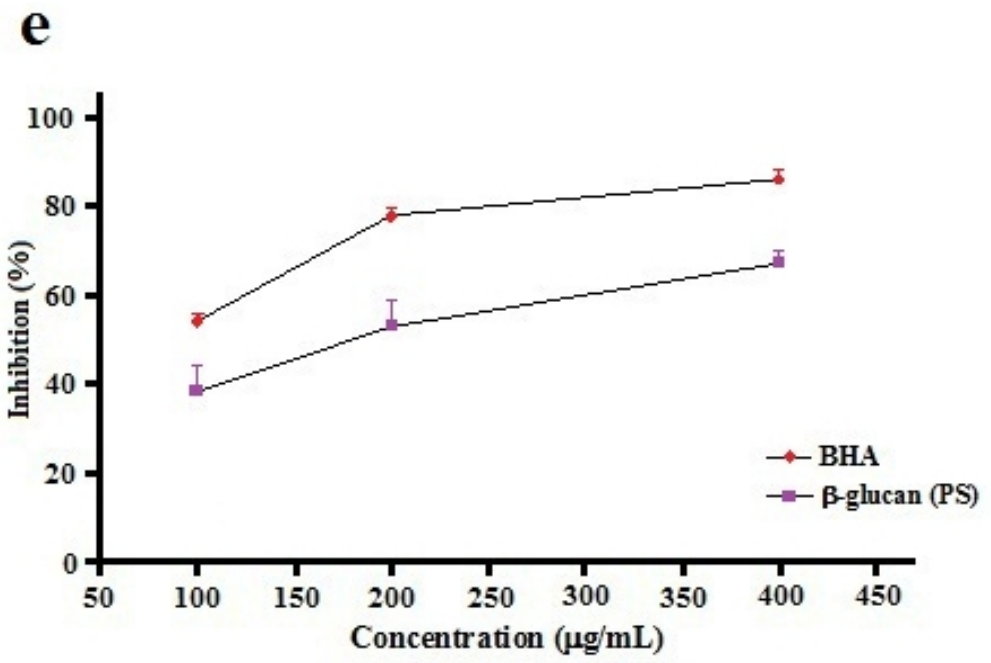


Figure 10. (e) Inhibition of β carotene bleaching of β-glucan (PS) isolated from the edible mushroom, *Russula albonigra* (All the above results are the mean ± SD of three separate experiments, each in triplicate).

5.3. Conclusions

Immunoenhancing antioxidant water soluble β-glucan (PS) was isolated from the alkaline extract of an edible mushroom, *Russula albonigra* (Krombh.) Fr. The structure of this PS was elucidated on the basis of total hydrolysis, methylation analysis, Smith degradation, partial hydrolysis and 1D/2D NMR studies. These results indicated that the repeating unit of the PS contained a backbone 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 PS activated the macrophages, splenocytes, and thymocytes and also showed several potent antioxidant activities. Hence, on the basis of these activities it could be used as a natural immunostimulant and antioxidant material. Further, this mushroom can also be recommended as an excellent food for consumption.

5.4. References

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APPENDIX

Paper-1: Glucan from hot aqueous extract of an ectomycorrhizal edible mushroom, *Russula albonigra* (Krombh.) Fr.: structural characterization and study of immunoenhancing properties. **Ashis K. Nandi**, Ipsita K. Sen, Surajit Samanta, Kousik Maity, K. Sanjana P. Devi , Shreemoyee Mukherjee, Tapas K. Maiti, Krishnendu Acharya, Syed S. Islam, ***Carbohydrate Research***, **2012**, *363*, 43-50.

Paper-2: Structural elucidation of an immunoenhancing heteroglycan isolated from *Russula albonigra* (Krombh.) Fr. **Ashis K. Nandi**, Surajit Samanta, Ipsita K. Sen, K. Sanjana P. Devi, Tapas K. Maiti, Krishnendu Acharya, Syed S. Islam, ***Carbohydrate Polymers***, **2013**, *94*, 918-926.

Paper-3: Antioxidant and immunostimulant β-glucan from edible mushroom *Russula albonigra* (Krombh.) Fr. **Ashis K. Nandi**, Surajit Samanta, Saikat Maity, Ipsita K. Sen, Somanjana Khatua, K. Sanjana P. Devi, Krishnendu Acharya, Tapas K. Maiti, Syed S. Islam, ***Carbohydrate Polymers***, **2014**, *99*, 774–782.

Carbohydrate Research 363 (2012) 43–50

Contents lists available at SciVerse ScienceDirect

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Glucan from hot aqueous extract of an ectomycorrhizal edible mushroom, *Russula albonigra* (Krombh.) Fr.: structural characterization and study of immunoenhancing properties

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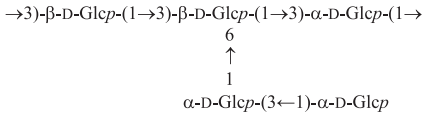
ARTICLE INFO

Article history:
Received 8 August 2012
Received in revised form 27 September 2012
Accepted 3 October 2012
Available online 12 October 2012

Keywords:
Russula albonigra (Krombh.) Fr.
Glucan
NMR spectroscopy
Immunoenhancing properties

ABSTRACT

A water soluble glucan (PS-I) was isolated from the hot aqueous extract of the fruit bodies of an ectomycorrhizal edible mushroom, *Russula albonigra* (Krombh.) Fr. The total hydrolysis, methylation analysis, periodate oxidation, and NMR (^{1}H, ^{13}C, DEPT-135, TOCSY, DQF-COSY, NOESY, ROESY, HSQC, and HMBC) studies revealed the presence of the following repeating unit in the polysaccharide:



This glucan showed excellent activations of macrophages as well as splenocytes and thymocytes in vitro.

1. Introduction

The *Russula albonigra* (Krombh.) Fr., an ectomycorrhizal[1–3] basidiomycetes[4] fungus, family Russulaceae[5] grows in symbiotic relationship with the roots of Sal (Shorea robusta) and other coniferous trees in the forest during rainy and autumn seasons throughout the world. Ectomycorrhizal fungi assist the plants in extracting nutrients like phosphorus and other minerals and in return received photosynthetically fixed sugars from the host plant for their growth and development. *Russula albonigra* (Krombh.) Fr. is edible and non-toxic[6] one and turns into black when handled or aged.[7] Several species of the genus *Russula* like *R. aeruginea* Lindblad. Fr., *R. alutacea* (Pers.:Fr.) Fr., *R. cyanoxantha* (Schaeff. ex Secr.) Fr., *R. decolorans* (Fr.) Fr., and *R. fragilis* (Pers.:Fr.) Fr are also edible[8] and their comparable taxonomic studies along with this mushroom have been reported.[7,9]

Mushrooms have not only been used as food or food flavoring materials, but also recognized as an important source of biologically active compounds on account of their immunomodulatory and antitumor properties,[10,11] especially, polysaccharides and polysaccharide–protein complexes exhibit anticancer activities.[12] Several (1→3)-β-D-glucan,[13] (1→3)-, (1→6)-β-D-glucan,[14] (1→6)-β-D-glucan,[15,16] (1→4)-, (1→6)-α-D-glucan[17], and (1→3)-α-, (1→6)-β-D-glucan[13] are widely used as immunoenhancing and antitumor materials. New (1→3)-, (1→6)-branched α,β-D-glucan[18–20] and water insoluble (1→3)-, (1→6)-β-D-glucan[21] were also reported by our group.

Two water soluble polysaccharides (PS-I and PS-II) have been isolated from the fruit bodies of the edible fungus, *Russula albonigra* (Krombh.) Fr. and the detailed structural investigation and study of immunoenhancing properties of PS-I were only carried out and reported herein.

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0008-6215/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carres.2012.10.002

2. Results and discussion

2.1. Isolation, purification, and chemical analysis

Five hundred grams of fresh mushroom fruit bodies were washed thoroughly with distilled water and boiled with distilled water for 12 h followed by centrifugation, precipitation in EtOH, and freeze drying to yield 750 mg of crude polysaccharide. Fractionation of water soluble crude polysaccharide (30 mg) through Sepharose 6B column yielded two polysaccharides. Fraction I (test tubes, 20–32) and fraction II (test tubes, 36–45) were collected and freeze dried, yielding purified polysaccharide PS-I (10 mg) and PS-II (7 mg), respectively. The PS-I showed specific rotation $[\alpha]_D^{31}$ −13.9 (*c* 0.6, water). The molecular weight[22] of PS-I was estimated as ~1.94×10^5 Da from a calibration curve prepared with standard dextrans.

PS-I was hydrolyzed with 2 M trifluoroacetic acid and then alditol acetate[23] was 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.[24] The mode of linkages of the PS-I was determined by the methylation analysis using the Ciucanu and Kerek method[25] 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, and 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[26,27], $NaBH_4$-reduced, methylated[28] 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.

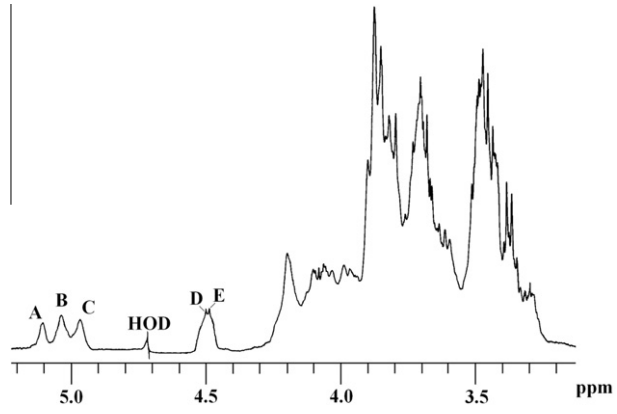


Figure 1. ^{1}H NMR spectrum (500 MHz, D_2O, 30 °C) of the PS-I isolated from an edible mushroom *Russula albonigra* (Krombh.) Fr.

2.2. NMR and structural analysis of glucan

The ^{1}H NMR (500 MHz) spectrum (Fig. 1, Table 1) at 30 °C 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 (Fig. 2a, Table 1) and DEPT-135 (Fig. 2b) NMR (125 MHz) spectrum at 30 °C 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 (Fig. 3), 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, and 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 constants, $J_{H\text{-}1,H\text{-}2}$ ~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.[29,30] 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 of **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 the 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 neighboring effect[21,31] 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.

Table 1
The ^{1}H[a] and ^{13}C[b] NMR chemical shifts for the polysaccharide isolated from *Russula albonigra* (Krombh.) Fr. (PS-I) in D_2O at 30 °C

Glycosyl residue	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6a,H-6b/C-6
α-D-Glc*p* (1→ **A**	5.10 100.2	3.52 71.49	3.80 74.18	3.62 70.3	3.85 72.7	3.72[c], 3.91[d] 60.8
→3)-α-D-Glc*p*-(1→ **B**	5.04 98.0	3.83 71.0	3.88 81.0	3.49 68.3	3.68 72.8	3.70[c], 3.86[d] 60.8
→3)-α-D-Glc*p*-(1→ **C**	4.97 97.8	3.83 71.0	3.87 81.2	3.49 68.3	3.68 72.8	3.70[c], 3.86[d] 60.8
→3,6)-β-D-Glc*p*-(1→ **D**	4.51 102.4	3.50 73.1	3.74 84.3	3.45 69.6	3.48 76.0	3.87[c], 4.20[d] 68.8
→3)-β-D-Glc*p*-(1→ **E**	4.49 102.2	3.30 73.2	3.73 85.0	3.39 69.6	3.47 75.6	3.68[c], 3.80[d] 60.8

[a] Values of the ^{1}H chemical shifts were recorded with respect to the HOD signal fixed at δ 4.70 ppm at 30 °C.
[b] Values of the ^{13}C chemical shifts were recorded with reference to acetone as the internal standard and fixed at δ 31.05 ppm at 30 °C.
[c,d] Interchangeable.

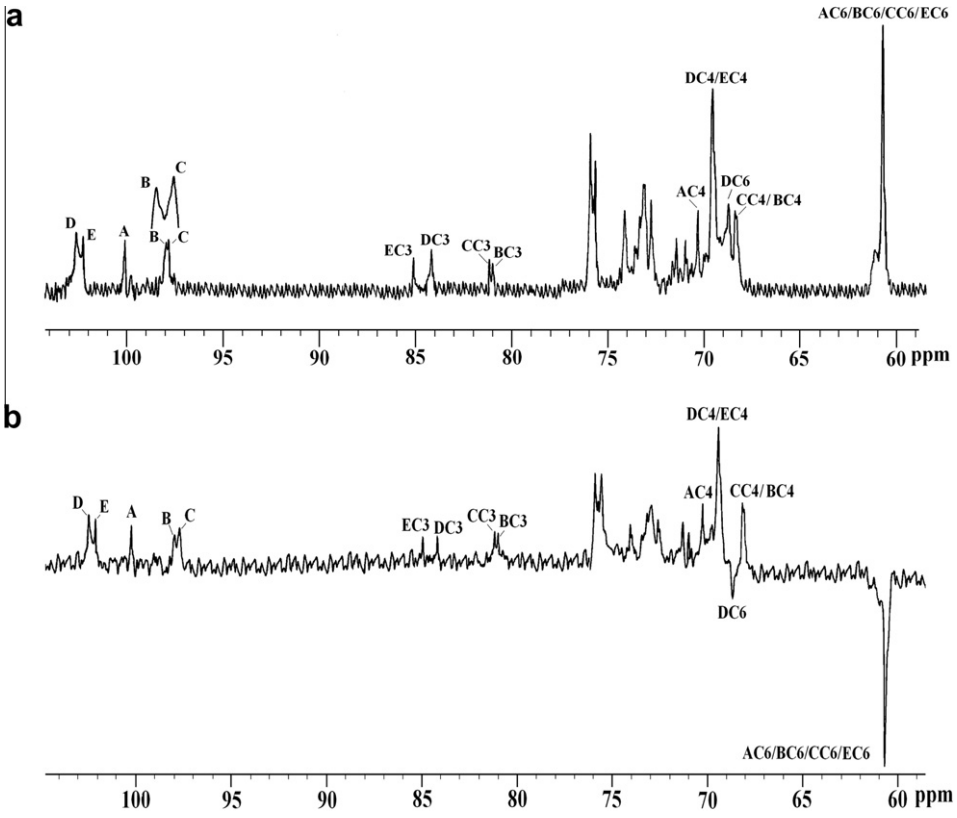


Figure 2. (a) ^{13}C NMR spectrum (125 MHz, D_2O, 30 °C) of the PS-I isolated from an edible mushroom *Russula albonigra* (Krombh.) Fr. (b) DEPT-135 spectrum (D_2O, 30 °C) of the PS-I isolated from an edible mushroom *Russula albonigra* (Krombh.) Fr.

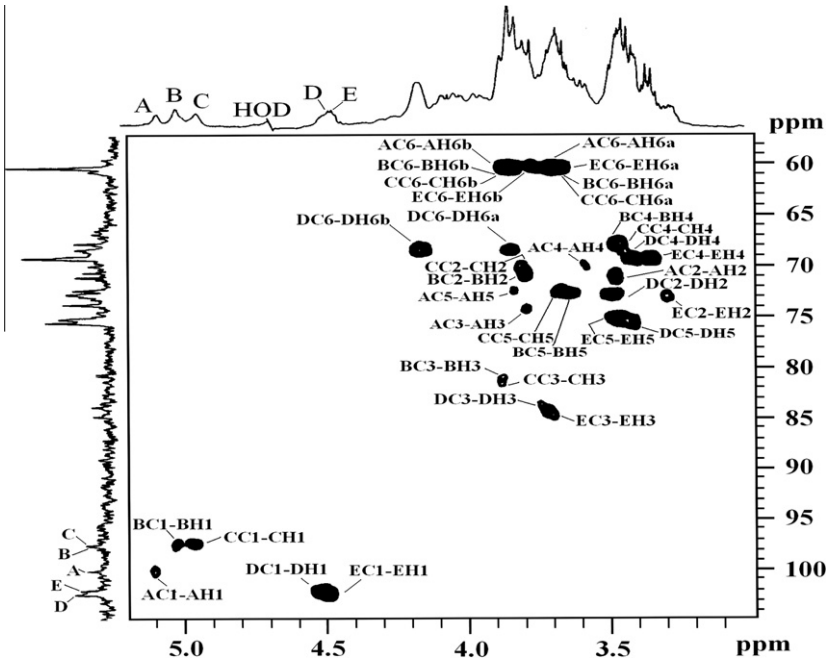


Figure 3. HSQC spectrum (D_2O, 30 °C) of the PS-I isolated from an edible mushroom *Russula albonigra* (Krombh.) Fr.

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.

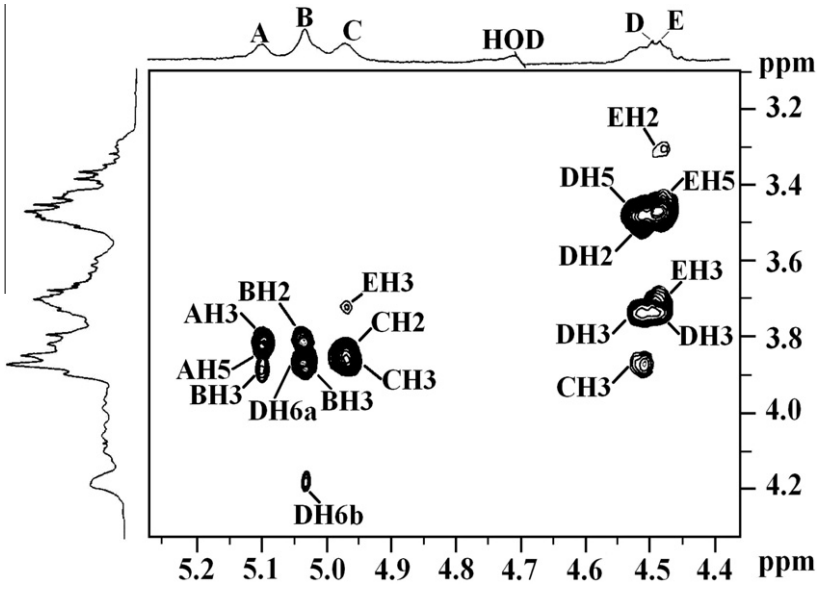


Figure 4. The part of ROESY spectrum of the PS-I isolated from an edible mushroom *Russula albonigra* (Krombh.) Fr. The ROESY mixing time was 300 ms.

The linking of residue **D** at C-6 was further confirmed from DEPT-135 spectrum (Fig. 2b). 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, its 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:

Table 2
ROE data for the polysaccharide isolated from *Russula albonigra* (Krombh.) Fr. (PS-I) in D_2O at 30 °C

Glycosyl residue	Anomeric proton	ROE contact protons	
	δ	δ	Residue, atom
α-D-Glc*p*-(1→ **A**	5.10	3.80	**A**H-3
		3.85	**A**H-5
		3.88	**B**H-3
→3)-α-D-Glc*p*-(1→ **B**	5.04	3.83	**B**H-2
		3.88	**B**H-3
		3.87	**D**H-6a
		4.20	**D**H-6b
→3)-α-D-Glc*p*-(1→ **C**	4.97	3.83	**C**H-2
		3.87	**C**H-3
		3.73	**E**H-3
→3,6)-β-D-Glc*p*-(1→ **D**	4.51	3.50	**D**H-2
		3.74	**D**H-3
		3.48	**D**H-5
		3.87	**C**H-3
→3)-β-D-Glc*p*-(1→ **E**	4.49	3.30	**E**H-2
		3.73	**E**H-3
		3.47	**E**H-5
		3.74	**D**H-3

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 (Fig. 5, Table 3), 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 was 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;

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

2.3. Biological characterization

Some immunological studies were also investigated with the PS-I. Macrophage activation of the PS-I was observed in vitro. On treatment with different concentrations of the PS-I an enhanced production of NO was observed in a dose-dependent manner with optimum production of 24.5 μM NO per 5×10^5 macrophages at 100 μg/mL of the PS-I (Fig. 6a, red bar). In order to establish that iNOS expression was responsible for NO production, the amount

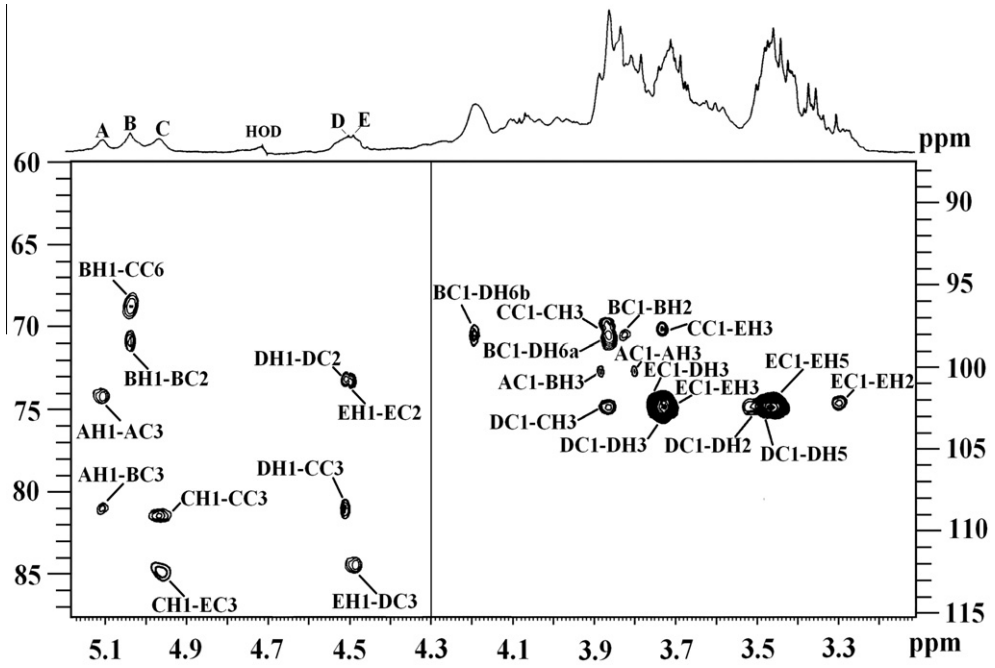


Fig. 5. The part of HMBC spectrum of the PS-I isolated from an edible mushroom *Russula albonigra* (Krombh.) Fr. The delay time in the HMBC experiment was 80 ms.

of NO produced was normalized to the cell numbers at the end of the treatment. After 48 h of treatment, the number of cells in the control increased from 1×10^5 to 2×10^5 cells/mL, whereas the number of cells increased from 1×10^5 to $2 \times 1.4 \times 10^5$ cells/mL, $2 \times 1.7 \times 10^5$ cells/mL, $2 \times 1.8 \times 10^5$ cells/mL, and $2 \times 1.8 \times 10^5$ cells/mL on treatment with 12.5, 25, 50, 100, and 200 μg/mL of PS-I, respectively. The NO production was normalized with respect to the increase of cell number as shown in Figure 6a, blue bar. But, actually the nitric oxide induction was measured to be 14, 15, 24, 24.5, and 23 μM on treatment of 12.5, 25, 50, 100, and 200 μg/mL of PS-I, respectively. Thus, fold increase of nitric oxide induction in treatments of 12.5, 25, 50, 100, and 200 μg/mL of PS-I will be 14/6 (2.3), 15/7(2.14), 24/10(2.4), 24.5/11(2.22), and 23/11(2.09), respectively. Proliferation index of macrophages on treatment with different concentration of PS-I was shown in Figure 6b.

Table 3
The significant $^3J_{H,C}$ connectivities observed in an HMBC spectrum for the anomeric protons/carbons of the sugar residues of the polysaccharide isolated from *Russula albonigra* (Krombh.) Fr. (PS-I) in D_2O at 30 °C

Residues	Sugar linkage	H-1/C-1	Observed connectivities		
		δ_H/δ_C	δ_H/δ_C	Residue	Atom
A	α-D-Glc*p*-(1→	5.10	81.0	**B**	C-3
		100.2	74.18	**A**	C-3
			3.88	**B**	H-3
			3.80	**A**	H-3
B	→3)-α-D-Glc*p*-(1→	5.04	68.8	**D**	C-6
		98.0	71.0	**B**	C-2
			3.87	**D**	H-6a
			4.20	**D**	H-6b
			3.83	**B**	H-2
C	→3)-α-D-Glc*p*-(1→	4.97	85.0	**E**	C-3
		97.8	81.2	**C**	C-3
			3.73	**E**	H-3
			3.87	**C**	H-3
D	→3,6)-β-D-Glc*p*-(1→	4.51	81.2	**C**	C-3
		102.4	73.1	**D**	C-2
			3.87	**C**	H-3
			3.50	**D**	H-2
			3.74	**D**	H-3
			3.48	**D**	H-5
E	→3)-β-D-Glc*p*-(1→	4.49	84.3	**D**	C-3
		102.2	73.2	**E**	C-2
			3.74	**D**	H-3
			3.30	**E**	H-2
			3.73	**E**	H-3
			3.47	**E**	H-5

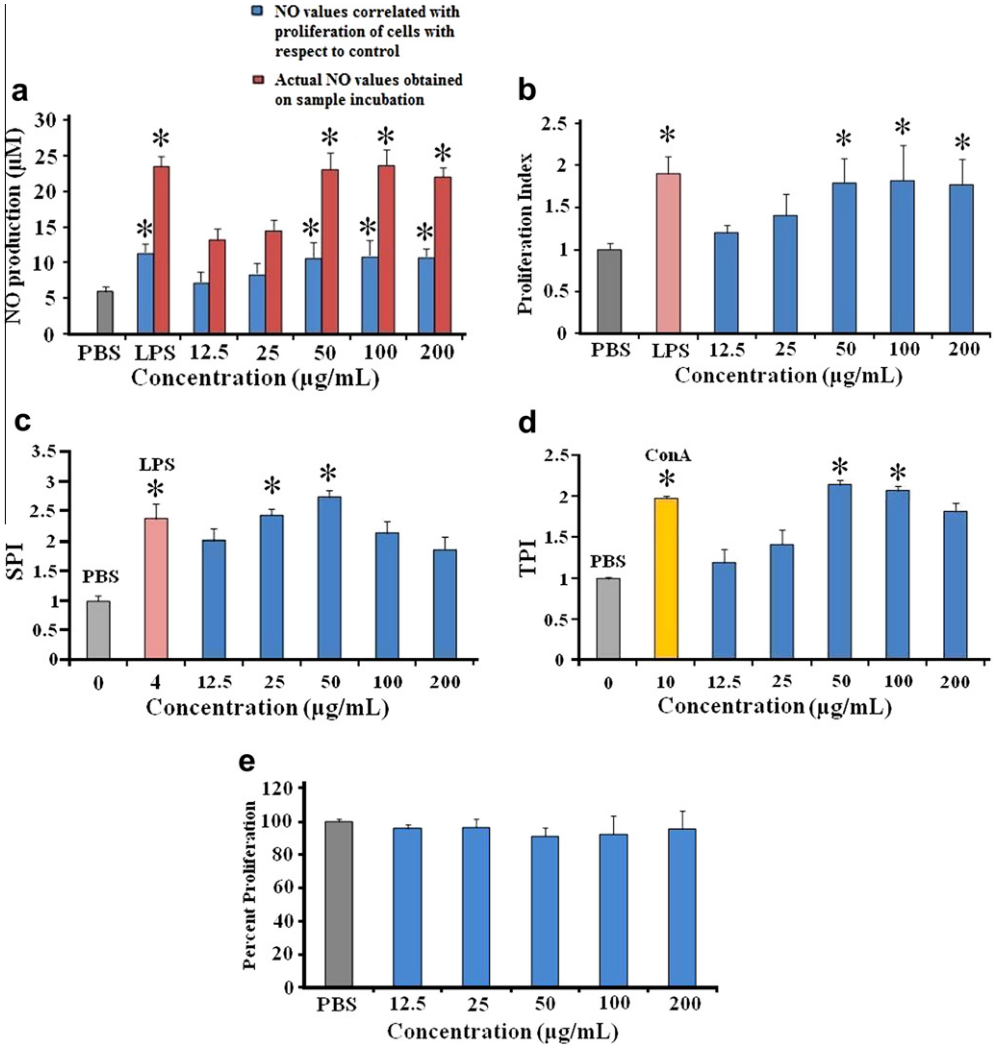


Figure 6. In vitro activation of raw macrophage stimulated with (a) different concentrations of PS-I in terms of nitric oxide (NO) obtained after correlating with proliferation of cells and actual NO production obtained on sample incubation and (b) proliferation index of macrophages on treatment with (12.5–200 μg/mL) of PS-I isolated from an edible mushroom *Russula albonigra* (Krombh.) Fr. Effect of different concentrations of PS-I on (c) splenocyte, (d) thymocyte proliferation and (e) percent proliferation of non-immune (HeLa cells) cells after the treatment. (Significant compared to the PBS control).

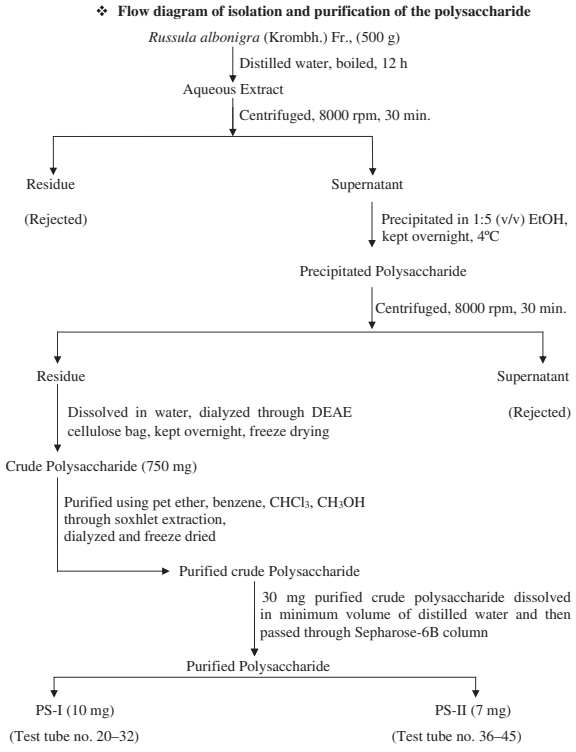
Splenocytes are the cells present in the spleen that include T cells, B cells, dendritic cells, and macrophages that stimulate the immune response in living organism. Thymocytes are hematopoietic cells in thymus which generate T cells. The splenocyte and thymocyte activation tests were carried out in mouse cell culture medium with the glucan by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method.[32] Proliferation of splenocytes and thymocytes is an indicator of immunostimulation. The splenocyte and thymocyte proliferation index as compared to Phosphate Buffered Saline (PBS) control if closer to 1 or below indicates low stimulatory effect on immune system. The PS-I was found to stimulate splenocytes and thymocytes as shown in Fig. 6c and d, respectively and the asterisks on the columns indicate the statistically significant differences compared to PBS control. At 50 μg/mL of the PS-I, both splenocyte and thymocyte proliferation indices were found maximum as compared to other concentrations. Hence, 50 μg/mL of the PS-I can be considered as efficient splenocyte and thymocyte stimulators. To establish the immunoenhancing activity of PS-I, HeLa cells which are not part of the immune system were used as control cell line and treated with different concentrations (12.5–200 μg/mL) of PS-I. It was observed that the treatment of PS-I did not show any proliferation (Fig. 6e) after 48 h of treatment and it was ruled out that PS-I can stimulate cell proliferation regardless of cell types.

3. Experimental

3.1. Isolation and purification of the polysaccharide

Fresh fruit bodies of the mushroom *R. albonigra* (Krombh.) Fr. (500 g) were washed, crushed, and boiled in 500 mL of distilled water for 12 h. The aqueous extract was kept overnight at 4 °C and filtered through linen cloth. Then crude polysaccharide was isolated and purified by the following flow diagram. Finally, the crude polysaccharide (30 mg) was purified by gel permeation chromatography on column (90 × 2.1 cm) of Sepharose 6B using water as eluant (0.5 mL/min) by Redifrac fraction collector. Ninety five test tubes were collected and monitored by the phenol–H_2SO_4 method[33] at 490 nm using a Shimadzu UV–vis spectrophotometer, model-1601. Two homogeneous fractions were obtained i.e. PS-I (test tube no. 20–32) and PS-II (test tube no. 36–45), collected, and freeze-dried, yielding 10 mg and 7 mg of pure polysaccharide respectively. The present work deals with the PS-I only. The same

procedure was repeated six times to yield 60 mg of pure polysaccharide



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3.2. General methods

The molecular weight was measured as reported earlier.[19–21] The optical rotation was measured on a Jasco Polarimeter model P-1020 at 31 °C. For sugar analysis, the PS-I (3.0 mg) was hydrolyzed with 2 M CF_3COOH (2 mL) in a round-bottom flask at 100 °C for 18 h in a boiling water bath and the analysis was carried out as described in previous papers.[19] The absolute configuration of the monosaccharide constituents was determined by the method of Gerwig et al.[24] The PS-I was methylated according to the method of Ciucanu and Kerek[25] where distilled DMSO and finely ground NaOH were used and then converted into alditol acetates as reported earlier.[19] Periodate oxidation experiment was carried out with this PS-I as described in the earlier report.[19] A Gas–Liquid Chromatographic analysis (GLC) was done using Hewlett-Packard model 5730 A, having a flame ionization detector and glass columns (1.8 m × 6 mm) packed with 3% ECNSS-M (A) on Gas Chrom Q (100–120 mesh) and 1% OV-225 (B) on Gas Chrom Q (100–120 mesh). All GLC analyses were performed at 170 °C. The Gas–liquid chromatography-mass spectrometric (GLC–MS) analysis was performed using a Shimadzu GLC–MS Model QP-2010 Plus automatic system, using ZB-5MS capillary column (30 m × 0.25 mm). The program was isothermal at 150 °C; hold time 5 min, with a temperature gradient of 2 °C min^{-1} up to a final temperature of 200 °C. The NMR experiments were carried out as reported earlier.[19–21]

3.3. Test for macrophage by nitric oxide (NO) assay

RAW 264.7 growing in Dulbecco's modified Eagle's medium (DMEM) was seeded in 96 well flat bottom tissue culture plate at 5×10^5 cells/mL concentration (180 μL). Cells were left overnight for attachment and treatment with different concentrations (12.5, 25, 50, 100, or 200 μg/mL) of glucan was given. After 48 h of treatment culture supernatant of each well was collected and NO content was estimated using Griess Reagent[34] at 540 nm. (1:1 of 0.1% in 1-napthylethylenediamine in 5% phosphoric acid and 1% sulfanilamide in 5% phosphoric acid). The amount of NO produced was normalized to the cell numbers at the end of the treatment in order to confirm that iNOS expression was responsible for PS-I induced NO production. Lipopolysaccharide (LPS), L6511 of *Salmonella enterica* serotype typhimurium (sigma, St. Louis, USA) was used as positive control.

3.4. Splenocyte and thymocyte proliferation assay

A single cell suspension of spleen and thymus was prepared from normal mice under aseptic conditions by homogenization in Hank's balanced salt solution (HBSS). The suspension was centrifuged to obtain cell pellet. The contaminating RBC was removed by hemolytic Gey's solution. After two washes in HBSS the cells were resuspended in complete RPMI. Cell concentration was adjusted to 1×10^6 cells/mL and viability of splenocytes and thymocytes (as tested by trypan blue dye exclusion) was always over 90%. The cells (180 μL) were plated in 96 well flat bottom tissue culture plates and incubated with 20 μL of various concentrations of polysaccharide (12.5, 25, 50, 100, or 200 μg/mL). PBS (10 mM, Phosphate Buffer Saline, pH-7.4) was taken as negative control whereas LPS (4 μg/mL, Sigma) and Concavalin A (Con A, 10 μg/mL) served as positive controls. All cultures were set up in triplicate for 72 h at 37 °C in a humidified atmosphere of 5% CO_2. Proliferation of splenocytes indicated as Splenocyte Proliferation Index (SPI) and Thymocytes written as Thymocyte Proliferation Index (TPI) were checked by standard MTT assay method. To establish the immunoenhancing activity of PS-I, HeLa cancer cell line which was not a part of the immune system was chosen as control and treated with different concentrations (12.5–200 μg/mL) of PS-I and proliferation was measured.

Acknowledgements

The authors are grateful to Dr. S. Roy, Director, IICB, Kolkata, for providing instrumental facilities. Mr. Barun Majumdar of Bose Institute, Kolkata, is acknowledged for preparing NMR spectra. A.K.N (one of the authors) thanks the CSIR for offering junior research fellowship (CSIR-09/599(0049)/2012-EMR-I).

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Carbohydrate Polymers 94 (2013) 918–926

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

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

Structural elucidation of an immunoenhancing heteroglycan isolated from *Russula albonigra* (Krombh.) Fr.

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ARTICLE INFO

Article history:
Received 22 November 2012
Received in revised form 12 February 2013
Accepted 13 February 2013
Available online 21 February 2013

Keywords:
Russula albonigra (Krombh.) Fr.
Heteroglycan
NMR spectroscopy
Immunostimulation

ABSTRACT

A water soluble heteroglycan (PS-II) of average molecular weight ~1.45×10^5 Da was isolated from the aqueous extract of an ectomycorrhizal edible mushroom, *Russula albonigra* (Krombh.) Fr. Structural characterization of PS-II was carried out using acid hydrolysis, methylation analysis, periodate oxidation, and 1D/2D NMR studies. Structural analysis revealed that PS-II was composed of terminal 2-*O*-methyl-Fuc*p*, terminal Man*p*, (1→2)-Fuc*p*, (1→3)-Glc*p*, (1→3,4)-Glc*p*, (1→6)-Gal*p*, and (1→2,6)-Gal*p* residues in a relative proportion of approximately 1:1:1:1:1:1:1. The proposed repeating unit of the PS-II had a backbone consisting of two (1→3)-β-D-glucopyranosyl, two (1→6)-α-D-galactopyranosyl, and one (1→2)-α-L-fucopyranosyl residues, out of which one (1→3)-β-D-glucopyranosyl residue was branched at *O*-4 position with terminal 2-*O*-methyl-α-L-fucopyranosyl and one (1→6)-α-D-galactopyranosyl residue was branched at *O*-2 position with terminal α-D-mannopyranosyl residue. This PS-II showed *in vitro* macrophage activation by NO production as well as splenocytes and thymocytes proliferation.

1. Introduction

Edible mushrooms have been used as a delicious food or food flavoring materials from ancient times. The important biologically active compounds like polysaccharides and polysaccharide–protein complexes from mushroom are being investigated widely for their immunomodulatory (Borchers, Stern, Hackman, Keen, & Gershwin, 1999; Wasser & Weis, 1999) as well as anticancer activities (Ooi & Liu, 2000; Sun & Liu, 2009). Mushroom polysaccharides do not attack cancer cells directly but exhibit antitumor activity through the activation of T cells, B cells, NK cells and macrophage-dependent immune systems in living organism (Wasser, 2002). *Russula albonigra* (Krombh.) Fr., an ectomycorrhizal (Chakraborty, Mondal, Pramanik, Rout, & Islam, 2004; Lilleskov, Bruns, Dawson, & Camacho, 2009) edible and non-toxic (Pradhan, Banerjee, Roy, & Acharya, 2010) fungus, grows in symbiotic relationship with the roots of Sal (*Shorea robusta*) and other coniferous trees in the forest during rainy and autumn season throughout the world. Two water-soluble fractions (PS-I and PS-II) were isolated from the fruit bodies of the edible mushroom, *R. albonigra* (Krombh.) Fr. The first fraction, PS-I was identified as glucan which showed excellent immunoenhancing properties and reported recently (Nandi et al., 2012). The second fraction, PS-II was characterized as heteroglycan which contained α-L-fucose as an important bioactive natural compound. The important bioactive carbohydrate moiety α-L-fucose is essential for novel treatment approaches in human breast cancer (Jay, Gene, & Catherine, 2011) and also involved during *in vitro* fertilization (Jennifer, Jennifer, & Barry, 2010) for improvement of infertility treatments. Several heteropolysaccharides with α-L-fucose from different mushrooms like *Lentinus edodes* (Carbonero et al., 2008), *Hericium erinaceus* (Zhang, Fu, Xu, Sun, & Zhang, 2012), *Agaricus blazei* Murill (Liu & Sun, 2011), *Fomitella fraxinea* (Imaz.) (Cho, Koshino, Yu, & Yoo, 1998), *Agaricus bisporus* (Ruthes et al., 2013), *Phellinus baumii* Pilát (Ge, Zhang, & Sun, 2009), *Lentinus squarrosulus* (Mont.) Singer (Bhunia et al., 2010), *Calocybe indica* var. APK2 (Mandal et al., 2011) have been reported. Moreover, PS-II showed *in vitro* macrophage activation by NO production in RAW 264.7 cell line as well as splenocyte and thymocyte proliferations for which it can be used as immunostimulating material in future. The detailed structural investigation and study of immunostimulating properties of PS-II were carried out in the present investigation and reported herein.

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http://dx.doi.org/10.1016/j.carbpol.2013.02.019

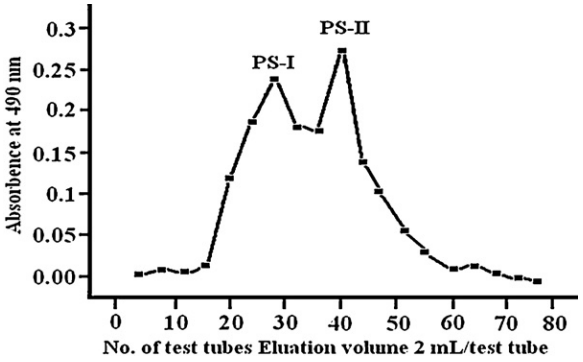


Fig. 1. Gel permeation chromatogram of crude polysaccharide isolated from an edible mushroom *Russula albonigra* (Krombh.) Fr. using Sepharose 6B column.

2. Materials and methods

2.1. Isolation and purification of the crude polysaccharide

Fresh fruit bodies of the mushroom *R. albonigra* (Krombh.) Fr. (500 g) were washed, crushed and boiled with distilled water for 12 h. The aqueous extract was kept overnight at 4 °C and filtered through linen cloth. The crude polysaccharide was isolated and purified by the method described previously (Nandi et al., 2012). Finally, the crude polysaccharide (30 mg) was purified by gel permeation chromatography on a Sepharose 6B column and two homogeneous fractions (Fig. 1) were obtained *i.e.* PS-I (test tubes 20–32) and PS-II (test tubes 36–45), collected, and freeze-dried, yielding 10 mg and 7 mg of pure polysaccharide respectively. The PS-II was further purified by passing through Sepharose 6B column in several lots to yield 70 mg of pure polysaccharide.

2.2. General analyses

Optical rotation was measured on a Jasco Polarimeter model P-1020 at 28 °C. Paper chromatographic studies were performed on Whatmann Nos. 1 and 3 mm sheets. Solvent systems used were (X) BuOH–HOAc–H_2O (v/v/v, 4:1:5, upper phase) and (Y) EtOAc–pyridine–H_2O (v/v/v, 8:2:1). Silver nitrate in acetone (1.2%), methanol in sodium hydroxide solution, and 5% sodium thiosulphate solution were used as spray reagents (Hoffman, Lindberg, & Svensson, 1972). Alditol acetates of monosaccharides and the methyl sugar were analyzed by GC and GC–MS (Jansson, Kenne, Liedgren, Lindberg, & Lönngren, 1976). A gas–liquid chromatography Hewlett-Packard 5730 A was used, having a flame ionization detector and glass columns (1.8 m × 6 mm) packed with 3% ECNSS-M (A) on Gas Chrom Q (100–120 mesh) and 1% OV-225 (B) on Gas Chrom Q (100–120 mesh). All GC analyzes were performed at 170 °C. Gas–liquid chromatography–mass spectrometric (GC–MS) analysis was performed on Shimadzu GC–MS Model QP-2010 Plus automatic system, using ZB-5MS capillary column (30 m × 0.25 mm). The program was isothermal at 150 °C; hold time 5 min, with a temperature gradient of 2 °C/min up to a final temperature of 200 °C.

2.3. Determination of molecular weight

The molecular weight of the polysaccharide was determined by gel-permeation chromatography. Standard dextrans (Hara, Kiho, Tanaka, & Ukai, 1982) T-200, T-70, and T-40 were passed through a Sepharose 6B column, and then the elution volumes were plotted against the logarithms of their respective molecular weights. The elution volume of the polysaccharide was then plotted in the same graph, and molecular weight of polysaccharide was determined.

2.4. Absolute configuration of monosaccharides

The absolute sugar configuration was determined by the method of Gerwig, Kamerling, and Vliegenthart (1978). The polysaccharide (1.0 mg) was hydrolyzed with CF_3COOH, and then the acid was removed. A solution of 250 μL of 0.625 (M) HCl in R-(+)-2-butanol was added and heated at 80 °C for 16 h. Then the reactants were evaporated and TMS-derivatives were prepared with *N,O*-bis(trimethylsilyl)trifluroacetamide (BSTFA). The products were analyzed by GC using a capillary column SPB-1 (30 m × 0.26 mm), a temperature program (3 °C/min) from 150 to 210 °C. The 2,3,4,6-tetra-*O*-TMS-(+)-2-butylglycosides obtained were identified by comparison with those prepared from the D and L enantiomers of different monosaccharides.

2.5. Constituent sugar analysis

The polysaccharide (PS-II, 3.0 mg) was hydrolyzed with 2 M CF_3COOH (2 mL) in a round-bottomed flask at 100 °C for 18 h in a boiling water bath. The excess of acid was completely removed by co-distillation with water. The hydrolyzed product was divided into two parts. One part was examined by paper chromatography in solvent systems X and Y. Another part was reduced with $NaBH_4$ (9 mg), followed by acidification with dilute CH_3COOH, and then co-distilled with pure CH_3OH to remove excess boric acid. The reduced sugars (alditols) were acetylated with 1:1 pyridine–acetic anhydride in a boiling water bath for 1 h to give the alditol acetates, which were analyzed by GC and GC–MS.

2.6. Methylation analysis

The polysaccharide (PS-II) was methylated using the method described by Ciucanu and Kerek (1984). The polysaccharide (4.0 mg) was kept on P_2O_5 in a vacuum decicator for several days and then dissolved in 0.5 mL of distilled DMSO. Finely powdered anhydrous NaOH was added and stirring for 30 min. Then 1.0 mL CH_3I was added, stirring for 1.5 h. The methylated products were isolated by partitioning between $CHCl_3$ and H_2O (5:2, v/v). The organic layer containing products was washed with 3 mL water for three times and dried. The methylated products were then formolyzed with 90% formic acid (1 mL) at 100 °C for 1 h, and excess formic acid was evaporated by co-distillation with distilled water, and then reduced with $NaBH_4$, acetylated with (1:1) acetic anhydride–pyridine, and analyzed by GC and GC–MS.

2.7. Periodate oxidation

The polysaccharide (PS-II, 5 mg) was oxidized with 0.1 M sodium metaperiodate (2 mL) at 27 °C in the dark during 48 h. The excess periodate was destroyed by adding 1,2-ethanediol, and the solution was dialyzed against distilled water. The dialyzed material was reduced with $NaBH_4$ for 15 h and neutralized with acetic acid. The resulting material was obtained by co-distillation with methanol. The periodate-oxidized-reduced (Goldstein, Hay, Lewis, & Smith, 1965; Hay, Lewis, & Smith, 1965) material was divided into two portions. One portion was hydrolyzed with 2 M CF_3COOH and used for alditol acetate preparation. Another portion was methylated by Ciucanu and Kerek method (1984), and alditol acetate of the methylated product was prepared and analyzed by GC and GC–MS.

2.8. NMR studies

The pure polysaccharide (PS-II) was kept over P_2O_5 under vacuum for several days, and then exchanged with deuterium (Dueñas-Chaso et al., 1997) by lyophilizing with D_2O (99.96% atom ^2H, Aldrich) for four times. Samples were dissolved in D_2O and

NMR spectra were recorded on a Bruker Avance DPX-500 spectrometer at 30 °C. The ^{1}H and ^{13}C (both ^{1}H coupled and decoupled) NMR spectra were recorded at 30 °C. The ^{1}H NMR spectrum was recorded by suppressing the HOD signal (fixed at δ 4.70) using the WEFT pulse sequence (Hård, Zadelhoff, Moonen, Kamerling, & Vliegenthart, 1992). The 2D-DQF-COSY experiment was carried out using standard BRUKER software. The TOCSY experiment was recorded at mixing time of 150 ms, and complete assignment required several TOCSY experiments having mixing times ranging from 60 to 300 ms. The NOESY and ROESY mixing delay was 300 ms. The ^{13}C chemical shifts were measured using acetone as internal standard, fixing the methyl carbon signal at δ 31.05. The delay time in the HMBC experiment was 80 ms.

2.9. Preparation of LPS free polysaccharide for immunological studies

Prior to immunoactivation studies, LPS which may contaminate during isolation and purification process was removed from PS-II in order to discard the contribution of LPS in immunostimulation. The PS-II was passed through polymyxin-B agarose matrix (Sigma 160 and Aldrich, USA) packed in 2 mL column (1 cm × 2 cm), with 0.5 mL/min flow rate. It was equilibrated with 10 mM phosphate buffer, pH 7.4. The bacterial lipopolysaccharides (LPSs) were bound to the matrix and the unbound LPS free PS-II (LFPS-II) were eluted and collected for immunoenhancing studies.

2.10. Limulus amebocyte lysate (LAL) test

Limulus amebocyte lysate (LAL) test was carried out *in vitro* for detection of bacterial endotoxin. The test was performed using gel clot technique (Liu et al., 2009). Limulus amebocyte lysate (LAL) (G2125, sensitivity: 0.125 EU/mL) was purchased from Quantum Biotech, Mumbai, India. The control standard endotoxin (CSE) (code E0125) and water (code W1004) for the bacterial endotoxin test (BET) were provided by Quantum Biotech, Mumbai, India. Four tubes were taken, each containing 0.1 mL of LAL reagent. In two tubes, 0.1 mL LFPS-II aqueous solution were added, meanwhile 0.1 mL BET water and 0.1 mL CSE were added to the rest two tubes as negative control and positive control, respectively. All tubes were incubated for 1 h in a water bath at 37 °C. After the test tube was inverted 180° slowly, it is positive (+) if the gel in tube is not deformed and does not slip from the wall and a negative (−) test is characterized in the absence of a gel or by the formation of a viscous mass which does not hold when the tube is inverted. Test is invalid when positive control is (−) or negative control is (+).

2.11. Test for macrophage by nitric oxide (NO) assay

RAW 264.7, a murine macrophage cell line obtained from National Centre for Cell Sciences (NCCS), Pune, India, was growing in Dulbecco's modified Eagle's medium (DMEM) and seeded in 96-well flat bottom tissue culture plate (Ohno, Hasimato, Adachi, & Yadomae, 1996; Sarangi, Ghosh, Bhutia, Mallick, & Maiti, 2006) at 5×10^5 cells/mL concentration (180 μL). Cells were left overnight for the attachment and then LFPS-II were treated with different concentrations (12.5, 25, 50, 100 or 200 μg/mL) to the wells. After 48 h of treatment culture supernatant of each well was collected and NO production was estimated using Griess Reagent (Green et al., 1982) at 540 nm (1:1 of 0.1% in 1-napthylethylenediamine in 5% phosphoric acid and 1% sulfanilamide in 5% phosphoric acid). Lipopolysaccharide (LPS), L6511 of *Salmonella enterica* serotype typhimurium (Sigma, St. Louis, USA) was used as positive control.

2.12. Splenocyte and thymocyte proliferation assay

A single cell suspension of spleen and thymus was prepared from Swiss Albino mice under aseptic conditions by homogenization in Hank's balanced salt solution (HBSS). This study was approved by the ethics committee. The suspension was centrifuged to obtain cell pellet. The contaminating RBC was removed by hemolytic Gey's solution. After washing two times in HBSS the cells were resuspended in complete RPMI (Roswell Park Memorial Institute) with serum and antibiotics added. RPMI and fetal bovine serum (FBS) has been obtained from Gibco whereas antibiotics were obtained from Himedia. Cell concentration was adjusted to 1×10^6 cells/mL and viability of splenocytes and thymocytes (as tested by trypan blue dye exclusion) was always over 90%. The cells (180 μL) were plated in 96-well flat bottom tissue culture plates and incubated with 20 μL of various concentrations of the LFPS-II (12.5, 25, 50, 100 or 200 μg/mL). PBS (10 mM, phosphate buffer saline, pH 7.4) was taken as negative control whereas LPS (4 μg/mL, Sigma) and Concavalin A (Con A, 10 μg/mL, Himedia) served as positive controls. All cultures were set up in triplicate for 72 h at 37 °C in a humidified atmosphere of 5% CO_2. Proliferation of splenocytes indicated as Splenocyte Proliferation Index (SPI) and Thymocytes written as Thymocyte Proliferation Index (TPI) were checked by standard MTT assay method (Ohno et al., 1993). The data are reported as the mean ± standard deviation of different observations and compared against PBS control (Maiti et al., 2008; Mallick, Maiti, Bhutia, & Maiti, 2010; Shah et al., 2007).

3. Results and discussion

3.1. Isolation, purification, and chemical analysis of the polysaccharide

Five hundred grams of fresh mushroom fruit bodies were washed thoroughly with distilled water, boiled with water for 12 h followed by centrifugation, precipitation in EtOH, and finally freeze dried to yield 750 mg of crude polysaccharide. Fractionation of water soluble crude polysaccharide (30 mg) through Sepharose 6B column yielded two polysaccharides (Fig. 1). Fraction I (test tubes, 20–32) and fraction II (test tubes, 36–45) were collected and freeze dried, yielding purified polysaccharide PS-I (10 mg) and PS-II (7 mg), respectively. The pure PS-II showed a specific rotation $[\alpha]_D^{28}$ + 34.5 (*c* 0.05, water). The molecular weight (Hara et al., 1982) of PS-II was estimated as ~1.45×10^5 Da from a calibration curve prepared with standard dextrans.

The sugar analysis of PS-II by paper chromatography and GC 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. (1978) 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 and Kerek (1984) followed by hydrolysis and alditol acetate conversion. The GC–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 (Table 1a). These linkages were further confirmed by periodate

Table 1a
GC–MS analysis of methylated polysaccharide (PS-II) isolated from *Russula albonigra* (Krombh.) Fr.

	Methylated sugars	Linkage type	Major mass fragments (*m*/*z*)
Rasidue **A**	3,4-Me_2-Gal	→2,6)-α-D-Gal*p*-(1→	43,71,87,99,129,159,173,189,233
Rasidue **B**	2,3,4-Me_3-Gal	→6)-α-D-Gal*p*-(1→	43,71,87,101,117,129,161,173,189,233
Rasidue **C**	2,3,4,6-Me_4-Man	α-D-Man*p*-(1→	43,59,71,87,101,117,129,145,161,205
Rasidue **D**	2,3,4-Me_3-Fuc	α-L-Fuc*p*-(1→	43,72,89,101,115,117,131,161,175
Rasidue **E**	3,4-Me_2-Fuc	→2)-α-L-Fuc*p*-(1→	43,59,71,89,99,115,129,131,173,189
Rasidue **F**	2,6-Me_2-Glc	→3,4)-β-D-Glc*p*-(1→	43,58,74,87,101,117,129,143,159,173,189,233
Rasidue **G**	2,4,6-Me_3-Glc	→3)-β-D-Glc*p*-(1→	43,74,87,101,117,129,143,161,173,203,217,233

Table 1b
GC–MS analysis of methylated polysaccharide (PS-II) after oxidation of sodium periodate isolated from *Russula albonigra* (Krombh.) Fr.

	Methylated sugars	Linkage type	Major mass fragments (*m*/*z*)
Rasidue **F**	2,6-Me_2-Glc	→3,4)-β-D-Glc*p*-(1→	43,58,74,87,101,117,129,143,159,173,189,201,233
Rasidue **G**	2,4,6-Me_3-Glc	→3)-β-D-Glc*p*-(1→	43,58,74,87,101,117,129,143,161,173,203,217,233

oxidation experiment. GC analysis of alditol acetates of periodate-oxidized (Goldstein et al., 1965; Hay et al., 1965), 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 GC and GC–MS analysis of periodate-oxidized and methylated (Abdel-Akher & Smith, 1950) 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 (Table 1b). 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.

3.2. NMR and structural analysis of the polysaccharide (PS-II)

The ^{1}H NMR (500 MHz) spectrum (Fig. 2a, Table 1c) at 30 °C 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 (Fig. 2b, Table 1c) and DEPT-135 (Fig. 2c) NMR (125 MHz) spectrum at 30 °C 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 (Fig. 2d), 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 (Fig. 2d and e) experiments. Coupling constants were measured from DQF-COSY spectrum.

Based on the coupling constant, $J_{H\text{-}1,H\text{-}2} \sim 3.1$ Hz and $J_{C\text{-}1,H\text{-}1} \sim 171$ 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 (Agrawal, 1992; Rinaudo & Vincendon, 1982) 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 (Fig. 2c). 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

Table 1c
The ^{1}H[a] and ^{13}C[b] NMR chemical shifts for the polysaccharide (PS-II) isolated from *Russula albonigra* (Krombh.) Fr. in D_2O at 30 °C.

Glycosyl residue	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6a,H-6b/C-6	-*O*Me
→2,6)-α-D-Gal*p*-(1→	5.10	3.87	4.00	4.12	4.20	3.67[c], 3.67[d]	
A	100.8	75.6	69.8	69.5	69.0	66.5	
→6)-α-D-Gal*p*-(1→	5.04	3.82	3.97	4.12	4.20	3.64[c], 3.64[d]	
B	98.0	70.5	70.0	69.5	69.0	66.7	
α-D-Man*p*-(1→	5.04	4.05	3.86	3.54	3.82	3.71[c], 3.88[d]	
C	102.2	70.4	72.0	67.7	73.0	61.2	
α-L-Fuc*p*-2*O*Me (1→	4.97	3.80	3.88	4.06	4.21	1.24	3.43
D	97.8	78.2	69.5	70.2	67.1	15.6	56.0
→2)-α-L-Fuc*p*-(1→	4.97	3.80	3.88	4.06	4.21	1.24	
E	97.8	78.0	69.5	70.2	67.1	15.6	
→3,4)-β-D-Glc*p*-(1→	4.52	3.50	3.73	3.65	3.48	3.70[c], 3.90[d]	
F	102.2	73.2	84.5	75.2	75.6	60.8	
→3)-β-D-Glc*p*-(1→	4.50	3.35	3.74	3.39	3.46	3.70[c], 3.90[d]	
G	102.5	73.2	85.0	69.6	76.0	60.8	

[a] Values of the ^{1}H chemical shifts were recorded with respect to the HOD signal fixed at δ 4.70 ppm at 30 °C.
[b] Values of the ^{13}C chemical shifts were recorded with reference to acetone as the internal standard and fixed at δ 31.05 ppm at 30 °C.
[c] Interchangeable.
[d] Interchangeable.

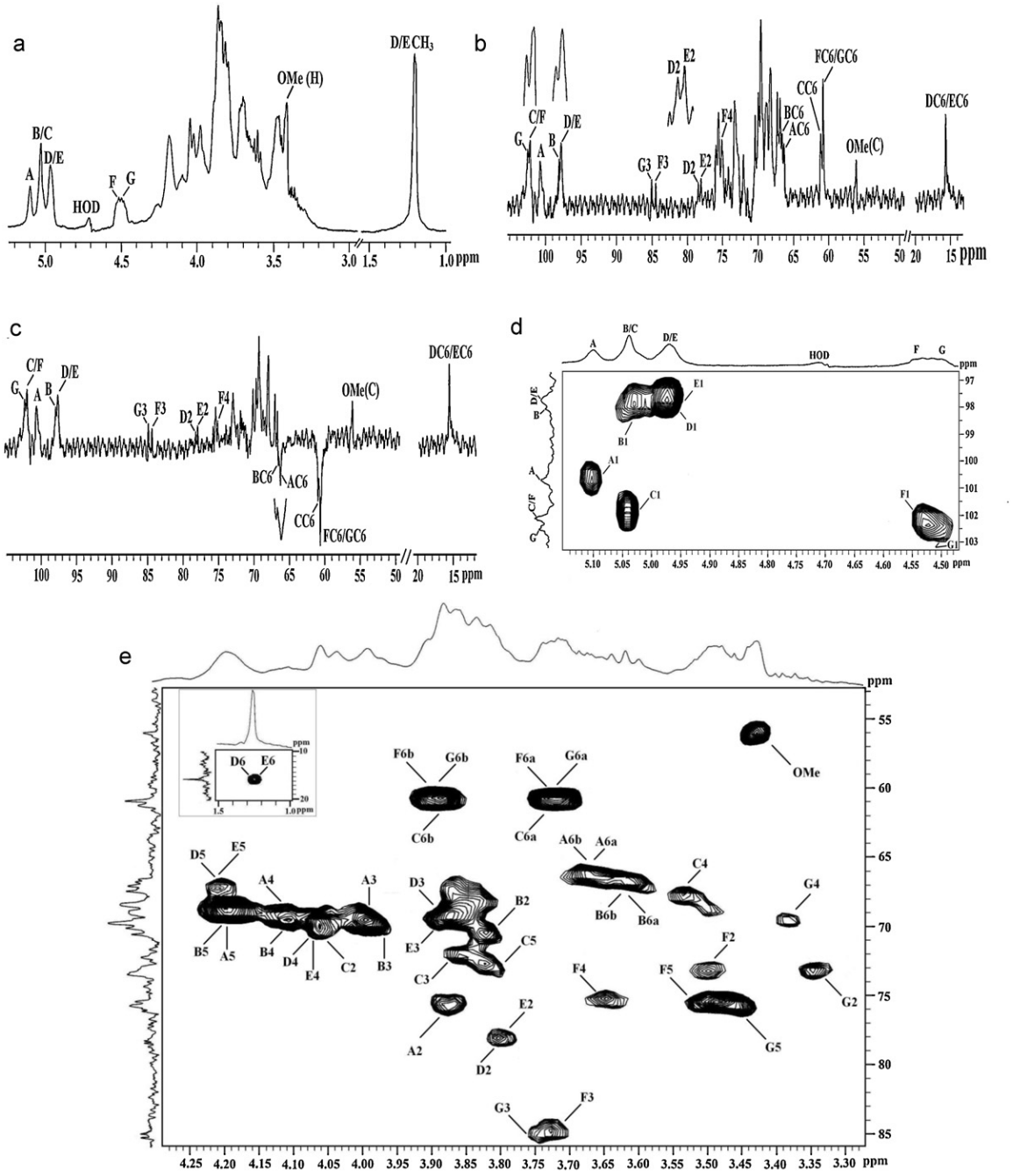


Fig. 2. (a) ^{1}H NMR spectrum (500 MHz, D_2O, 30 °C) of the PS-II isolated from an edible mushroom *Russula albonigra* (Krombh.) Fr. (b) ^{13}C NMR spectrum (125 MHz, D_2O, 30 °C) of the PS-II isolated from an edible mushroom *Russula albonigra* (Krombh.) Fr. (c) DEPT-135 spectrum (D_2O, 30 °C) of the PS-II isolated from an edible mushroom *Russula albonigra* (Krombh.) Fr. The HSQC spectrum (D_2O, 30 °C) of (d) anomeric part and (e) other than anomeric part of the PS-II isolated from an edible mushroom *Russula albonigra* (Krombh.) Fr.

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–^{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 (Fig. 4, Table 3). On the other hand, the downfield shift of C-2 (δ 78.0) with respect to standard values of methyl glycosides (Agrawal, 1992; Rinaudo & Vincendon, 1982) indicated that the residue **E** was also linked at C-2 position with residue **A** which further confirmed by the ROESY (Fig. 3, Table 2) as well as HMBC experiment (Fig. 4, Table 3). So the moiety **E** was (1→2)-linked unit. The C-2 chemical shift values of the residues

Table 2
ROE data for the polysaccharide (PS-II) isolated from *Russula albonigra* (Krombh.) Fr. in D_2O at 30 °C.

Glycosyl residue	Anomeric proton	ROE contact protons	
	δ	δ	Residue, atom
→2,6)-α-D-Gal*p*-(1→	5.10	3.87	**A**H-2
A		3.80	**E**H-2
→6)-α-D-Gal*p*-(1→	5.04	3.82	**B**H-2
B		3.74	**G**H-3
α-D-Man*p*-(1→	5.04	4.05	**C**H-2
C		3.87	**A**H-2
α-L-Fuc*p*-2*O*Me (1→	4.97	3.80	**D**H-2
D		3.65	**F**H-4
→2)-α-L-Fuc*p*-(1→	4.97	3.80	**E**H-2
E		3.64	**B**H-6a/6b
→3,4)-β-D-Glc*p*-(1→	4.52	3.73	**F**H-3
F		3.48	**F**H-5
		3.67	**A**H-6a/6b
→3)-β-D-Glc*p*-(1→	4.50	3.74	**G**H-3
G		3.46	**G**H-5
		3.73	**F**H-3

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 $J_{H-1,H-2}$ (~8 Hz), and $J_{C-1,H-1}$ (~160 Hz) and the large coupling constant values $J_{H-2,H-3}$ and $J_{H-3,H-4}$ (~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 (Agrawal, 1992; Rinaudo & Vincendon, 1982) 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 (Fig. 3, Table 2) as well as NOESY (not shown) 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 (Fig. 4, Table 3), 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:

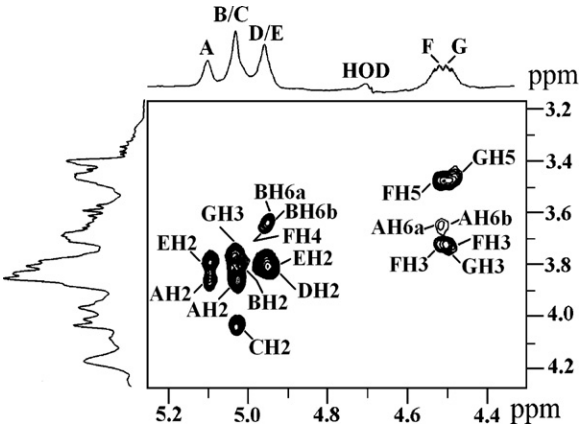
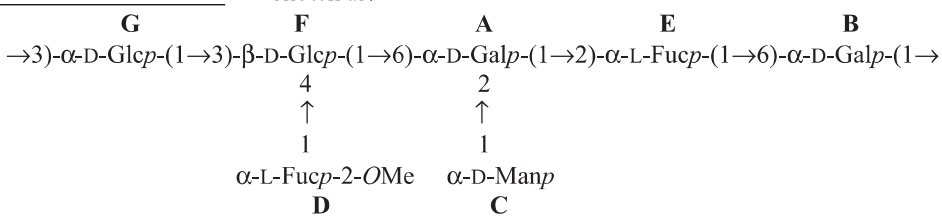


Fig. 3. The part of ROESY spectrum of the PS-II isolated an edible mushroom *Russula albonigra* (Krombh.) Fr. The ROESY mixing time was 300 ms.



3.3. 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. Immunological studies were also investigated with the LFPS-II. Macrophage activation by LFPS-II was observed *in vitro*. Upon treatment with different concentrations of the LFPS-II, enhanced production of NO was observed in a dose-dependent manner with optimum production of 18 μM NO per 5×10^5 macrophages at 100 μg/mL of the LFPS-II

Table 3
The significant $^{3}J_{H,C}$ connectivities observed in an HMBC spectrum for the anomeric protons/carbons of the sugar residues of the polysaccharide (PS-II) isolated from *Russula albonigra* (Krombh.) Fr. in D_2O at 30 °C.

Residues	Sugar linkage	H-1/C-1	Observed connectivities		
		δ_H/δ_C	δ_H/δ_C	Residue	Atom
A	→2,6)-α-D-Gal*p*-(1→	5.10	78.0	**E**	C-2
			75.6	**A**	C-2
		100.8	3.80	**E**	H-2
			3.87	**A**	H-2
B	→6)-α-D-Gal*p*-(1→	5.04	85.0	**G**	C-3
			70.5	**B**	C-2
		98.0	3.74	**G**	H-3
			3.82	**B**	H-2
C	α-D-Man*p*-(1→	5.04	75.6	**A**	C-2
		102.2	3.87	**A**	H-2
D	α-L-Fuc*p*-2OMe (1→	4.97	75.2	**F**	C-4
		97.8	3.65	**F**	H-4
			3.80	**D**	H-2
E	→2)-α-L-Fuc*p*-(1→	4.97	66.7	**B**	C-6
			78.0	**E**	C-2
		97.8	3.64	**B**	H-6a/6b
			3.80	**E**	H-2
F	→3,4)-β-D-Glc*p*-(1→	4.52	66.5	**A**	C-6
			73.2	**F**	C-2
		102.2	3.67	**A**	H-6a/6b
			3.50	**F**	H-2
			3.73	**F**	H-3
			3.48	**F**	H-5
G	→3)-β-D-Glc*p*-(1→	4.50	84.5	**F**	C-3
		102.5	3.73	**F**	H-3
			3.74	**G**	H-3
			3.46	**G**	H-5
		-OCH_3 δ_H/δ_C			
D	α-L-Fuc*p*-2OMe(1→	3.43	78.2	**D**	C-2
		56.0	3.80	**D**	H-2

(Fig. 5a). Lentinan obtained from *L. edodes* (Berk.) Sing., inhibit the tumor growth by stimulating the immune system (Suzuki, Takatsuki, Maeda, Hamuro, & Chihara, 1994) through activation of macrophages, T-helper, NK, and other cells.

Splenocytes are the cells present in the spleen that include T cells, B cells, and dendritic cells that stimulate the immune response in living organism. Thymocytes are hematopoietic cells in thymus which generate T cells. The splenocytes and thymocytes activation tests were carried out in Swiss Albino mice cell culture medium with the LFPS-II by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method (Ohno et al., 1993). Proliferation of splenocytes and thymocytes is an indicator of

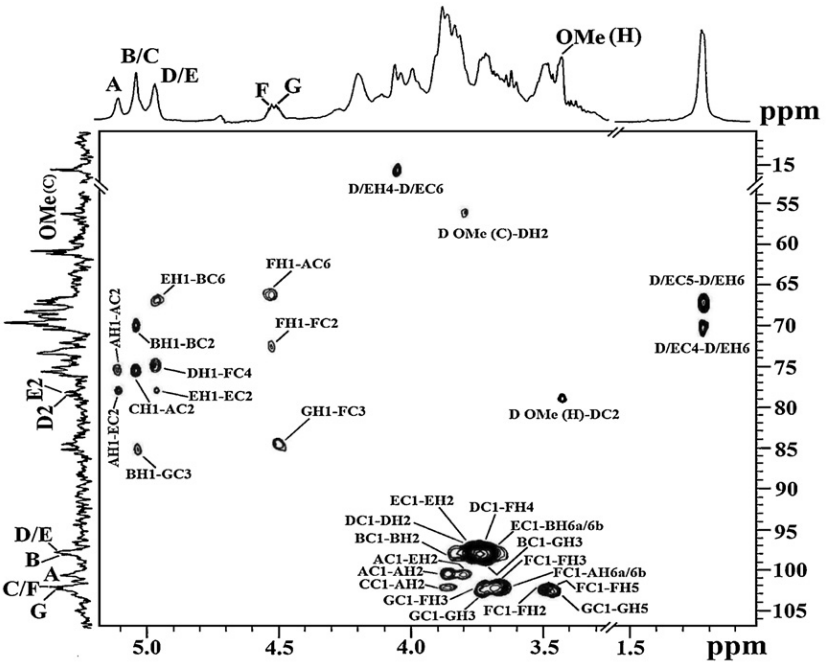
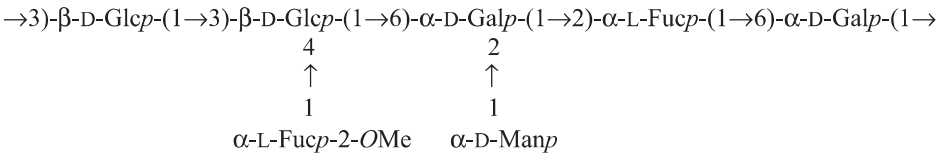


Fig. 4. The part of HMBC spectrum of the PS-II isolated from an edible mushroom *Russula albonigra* (Krombh.) Fr. The delay time in the HMBC experiment was 80 ms.

immunostimulation. The LFPS-II was found to stimulate splenocytes and thymocytes as shown in Fig. 5b and c, respectively and the asterisks on the columns indicate the statistically significant differences compared to PBS control. At 25 μg/mL of the LFPS-II, splenocyte proliferation index was found to be maximum as compared to other concentrations. Hence, 25 μg/mL of the LFPS-II can be considered as efficient splenocyte stimulators. Again 50 μg/mL of this same sample showed maximum effect on thymocyte proliferation. The splenocyte and thymocyte proliferation index as compared to PBS control if closer to 1 or below indicates low stimulatory effect on immune system. It is noteworthy to mention that several α-L-fucose containing heteroglycan (Bhunia et al., 2010; Dey et al., 2013; Mandal et al., 2011) also showed immunoenhancing properties as reported earlier by our group.

4. Conclusions

A water-soluble heteroglycan (PS-II) was isolated from aqueous extract of an edible mushroom *R. albonigra* (Krombh.) Fr. This PS-II showed splenocyte, thymocyte, and macrophage activation. So, it may be used as a source of natural immunostimulant and it also contained naturally important bioactive carbohydrate moiety α-L-fucose which have large utility in medicinal chemistry. Architectural details of the repeating unit of the PS-II obtained from *R. albonigra* (Krombh.) Fr. was determined from chemical and NMR studies (^{1}H, ^{13}C, DEPT-135, TOCSY, DQF-COSY, NOESY, ROESY, HSQC, and HMBC) and the structure of the repeating unit of the PS-II was established as:



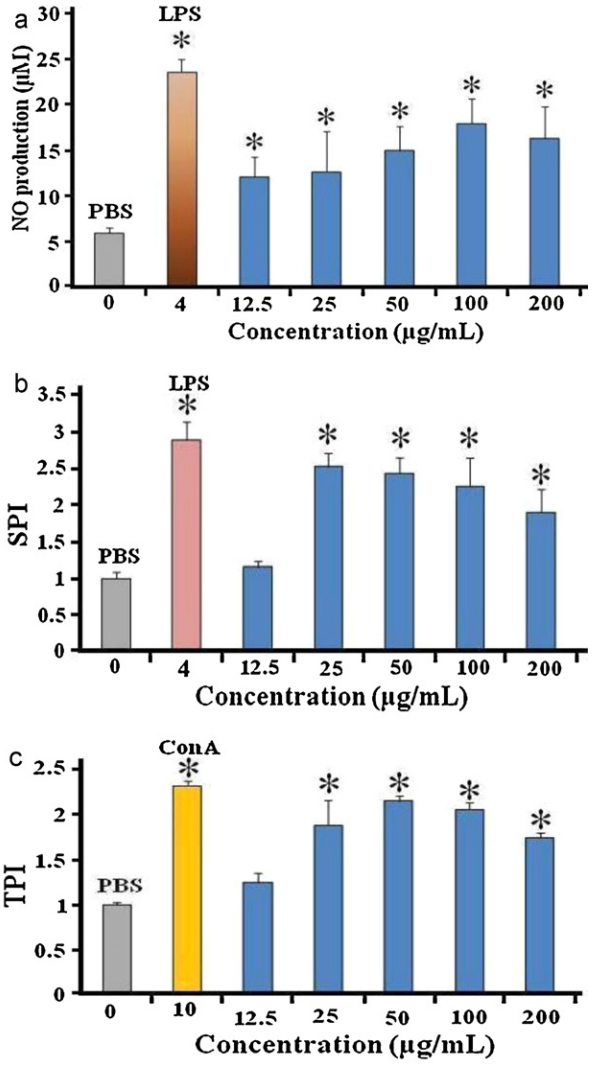


Fig. 5. (a) Activation of RAW 264.7 macrophage cells with different concentrations of the LPS free PS-II (LFPS-II) in terms of NO production. Effect of different concentrations of the LPS free PS-II (LFPS-II) on proliferation of (b) splenocyte and (c) thymocyte (asterisks indicate the statistically significant compared to the PBS control).

Acknowledgements

Dr. S. Roy, Director, IICB, Kolkata, is gratefully acknowledged for providing instrumental facilities. The authors acknowledge Mr. Barun Majumder of Bose Institute, Kolkata, for preparing NMR spectra. A.K.N. (one of the authors) thanks the CSIR for offering junior research fellowship (CSIR-09/599(0049)/2012-EMR-I).

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Carbohydrate Polymers 99 (2014) 774–782

Contents lists available at ScienceDirect

Carbohydrate Polymers

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Antioxidant and immunostimulant β-glucan from edible mushroom *Russula albonigra* (Krombh.) Fr.

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ARTICLE INFO

Article history:
Received 6 August 2013
Received in revised form 3 September 2013
Accepted 6 September 2013
Available online 11 September 2013

Keywords:
Russula albonigra (Krombh.) Fr.
β-Glucan
NMR studies
Immunostimulation
Antioxidant activities

ABSTRACT

A water soluble β-glucan (PS) with an average molecular weight ~1.95×10^5 Da was isolated from the alkaline extract of ectomycorrhizal edible mushroom, *Russula albonigra* (Krombh.) Fr. and found to consist of terminal, (1 → 3)-, (1 → 6)-, and (1 → 3,6)-linked β-D-glucopyranosyl moieties in a ratio of nearly 1:2:2:1. The structure of this PS was elucidated on the basis of total hydrolysis, methylation analysis, Smith degradation, partial hydrolysis, and 1D/2D NMR experiments. On the basis of these experiments, the repeating unit of the PS was found to contain a backbone 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. This PS showed *in vitro* macrophage activation by NO production as well as splenocytes and thymocytes proliferation. Moreover, it also exhibited potent antioxidant activities.

1. Introduction

Mushroom polysaccharides, especially, β-glucans are recognized as biological response modifier (BRM) and used for the treatment of cancer and various infectious diseases both in modern medicine and traditional chemotherapeutic drug (Chan, Chan, & Sze, 2009; Kidd, 2000; Wasser, 2002). They have drawn the attention of chemists and immunobiologists for their immunomodulatory, anti-tumor (Wasser, 2002) as well as antioxidant activities (Kozarski et al., 2011). Reactive oxygen species (ROS) which damage lipids, proteins, carbohydrates, and nucleic acids (Blokhina, Virolainen & Fagerstedt, 2003) are the roots of developing diseases like cancer, Alzheimer, and Parkinson (Papas, 1999). β-Glucans from mushrooms are well-known antioxidant material (Kofuji et al., 2012) which can neutralize the harsh effect of ROS (Papas, 1999). Modern research has been focused on utilizing the naturally occurring substances to neutralize the radical activities. Several linear (1 → 3) and branched (1 → 3)-, (1 → 6)-β-D-glucans (Ohno et al., 1993; Yoshioka, Tabeta, Saito, Uehara, & Fukuoka, 1985) are used as immunostimulating and antitumor materials. Some immunostimulating water soluble β-D-glucans (Maity et al., 2013; Sen et al., 2013) have been also reported by our group.

Russula albonigra (Krombh.) Fr., an ectomycorrhizal, edible and non-toxic fungus (Nandi et al., 2012) contained two water-soluble polysaccharides, α,β-glucan and a heteroglycan (Nandi et al., 2012, 2013) whereas alkali treated aqueous extract contained only one polysaccharide which was characterized as a β-glucan. The detailed structural investigation and study of immunostimulation as well as antioxidant activities of this β-glucan were carried out and reported herein.

2. Materials and methods

2.1. Isolation and purification of the polysaccharide

Fresh fruit bodies of mushroom *R. albonigra* (Krombh.) Fr. (500 g) were gently washed with distilled water and then boiled with 4% NaOH solution for 1 h. The alkaline extract was kept overnight at 4 °C and filtered through linen cloth. The crude polysaccharide was isolated and purified by the method described earlier (Maity et al., 2013; Sen et al., 2013) and it (30 mg) was purified by gel permeation chromatography on column of Sepharose 6B using water as eluant by Redifrac fraction collector. A single homogeneous fraction (test tube 20–32, Fig. 1a) was collected, and freeze-dried, yielding 20 mg of pure PS. The same procedure was repeated in several lots to yield

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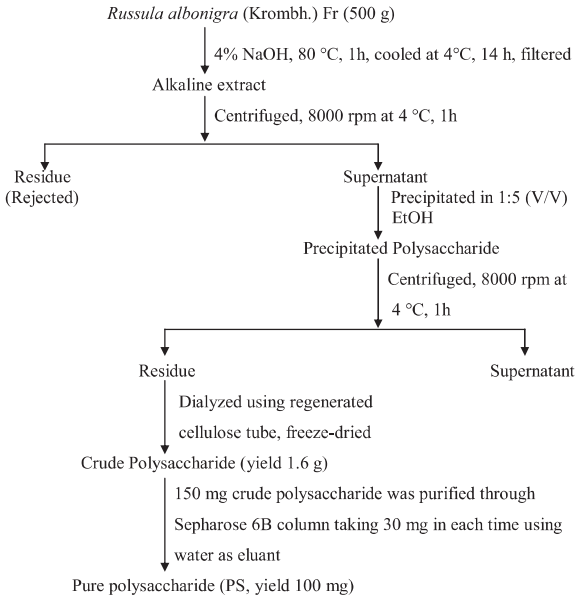
http://dx.doi.org/10.1016/j.carbpol.2013.09.016

100 mg of pure polysaccharide. Isolation and purification steps are shown in the following diagram.

❖ **Flow diagram of isolation and purification of the polysaccharide**



2.2. Determination of molecular weight

The molecular weight of the polysaccharide was determined by gel-permeation chromatography. Standard dextrans (Hara, Kiho, Tanaka, & Ukai, 1982) T-200, T-70, and T-40 were passed through a Sepharose 6B column, and then the elution volumes were plotted against the logarithms of their respective molecular weights. The elution volume of the polysaccharide was then plotted in the same graph, and molecular weight of polysaccharide was determined.

2.3. Absolute configuration of monosaccharides

The absolute sugar configuration was determined by the method of Gerwig, Kamerling, and Vliegenthart (1978). The polysaccharide (1.0 mg) was hydrolyzed with CF_3COOH, and then the acid was removed. A solution of 250 μL of 0.625 (M) HCl in R-(−)-2-butanol was added and heated at 80 °C for 16 h. Then the reactants were evaporated and TMS-derivatives were prepared with *N*,*O*-bis(trimethylsilyl)trifluroacetamide (BSTFA). The products were analyzed by GC using a capillary column SPB-1 (30 m × 0.26 mm), a temperature program (3 °C/min) from 150 to 210 °C. The 2,3,4,6-tetra-*O*-TMS-(+)-2-butylglycosides obtained were identified by comparison with those prepared from the D and L enantiomers of different monosaccharides.

2.4. Optical rotation

Optical rotation was measured on a Jasco Polarimeter model P-1020 at 31 °C.

2.5. Constituent sugar, methylation, and periodate oxidation analysis

For sugar analysis, the PS (3.0 mg) was hydrolyzed with 2 M CF_3COOH (2 mL) in a round-bottom flask at 100 °C for 18 h in a boiling water bath and the analysis was carried out as described in previous paper (Nandi et al., 2013). The PS (4 mg) was methylated according to the method of Ciucanu and Kerek method (1984) where distilled DMSO and finely grounded NaOH were used and then converted to alditol acetates as reported earlier (Nandi et al., 2013). Periodate oxidation experiment was carried out with this PS (5 mg) as described in the earlier report (Nandi et al., 2013). Alditol acetates of monosaccharides and the methyl sugar were analyzed by GC and GC–MS. A gas–liquid chromatography Hewlett-Packard 5730 A was used, having a flame ionization detector and glass columns (1.8 m × 6 mm) packed with 3% ECNSS-M (A) on Gas Chrom Q (100–120 mesh) and 1% OV-225 (B) on Gas Chrom Q (100–120 mesh). All GC analyzes were performed at 170 °C. Gas-liquid chromatography–mass spectrometric (GC–MS) analysis was performed on Shimadzu GC-MS Model QP-2010 Plus automatic system, using ZB-5MS capillary column (30 m × 0.25 mm). The program was isothermal at 150 °C; hold time 5 min, with a temperature gradient of 2 °C/min up to a final temperature of 200 °C.

2.6. Smith degradation

The polysaccharide (25 mg) was oxidized with 0.1 M sodium metaperiodate (20 mL) at 25 °C in the dark during 72 h. The oxidation was stopped by the addition of 1,2-ethanediol, and the solution was dialyzed against distilled H_2O. Thereafter, $NaBH_4$ was added and kept at room temperature for 15 h, with intermittent stirring. The mixture was neutralized with 50% AcOH and again dialyzed against distilled water, and freeze dried and was subjected to mild hydrolysis with 0.5 M TFA for 15 h at 25 °C. TFA was removed by repeated evaporation of water at 37 °C. Finally, it was purified by passing through a Sephadex G-25 column. A part of this material was subjected to methylation analysis and the remainder was used for ^{13}C NMR studies.

2.7. Partial acid hydrolysis

The polysaccharide (30 mg) dissolved in 0.1 M TFA (6 mL) was hydrolyzed at 100 °C for 1 h. Acid was removed by repeated evaporation of water at 37 °C. The residue was dissolved in water (4 mL), to which three volumes of ethanol were added. The precipitate was washed with ethanol and then freeze-dried (F1), was used for methylation and ^{13}C NMR analysis. The supernatant was dried by evaporation, and residue was dissolved in water, and reduced with $NaBH_4$ at 25 °C for 2 h. After neutralization with 1 M AcOH, it was desalted by passing through a Sephadex G-25 column. The carbohydrate containing eluate (F2) was collected, freeze-dried and subjected to methylation analysis.

2.8. NMR studies

The pure polysaccharide (PS) was kept over P_2O_5 under vacuum for several days, and then exchanged with deuterium by lyophilizing with D_2O (99.96% atom ^2H, Aldrich) for four times. Samples were dissolved in D_2O and NMR spectra were recorded on a Bruker Avance DPX-500 spectrometer at 30 °C. The ^1H and ^{13}C (both ^1H coupled and decoupled) NMR spectra were recorded at 30 °C. The ^1H NMR spectrum was recorded by suppressing the HOD signal (fixed at δ 4.70) using the WEFT pulse sequence. The 2D-DQF-COSY experiment was carried out using standard BRUKER software. The TOCSY experiment was recorded at mixing time of 150 ms, and complete assignment required several TOCSY experiments having mixing times ranging from 60 to 300 ms. The NOESY and ROESY mixing delay was 300 ms whereas the delay time in the HMBC experiment was 80 ms. The ^{13}C chemical shifts were measured using acetone as internal standard, fixing the methyl carbon signal at δ 31.05.

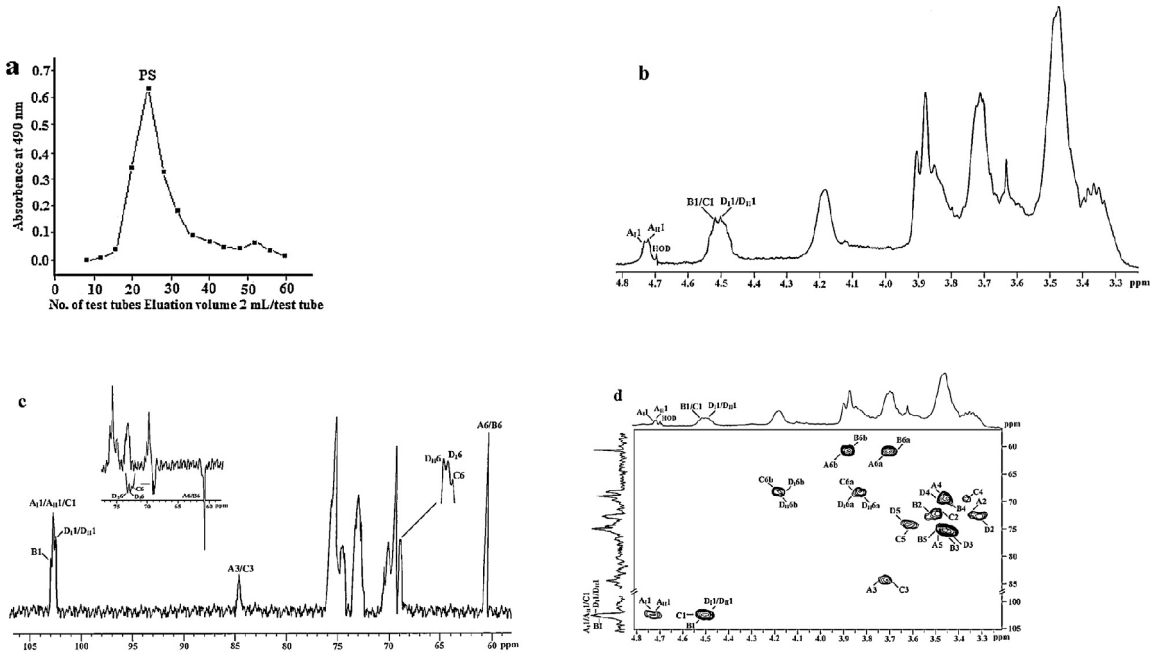


Fig. 1. (a) Gel permeation chromatogram, (b) ^{1}H NMR spectrum (500 MHz, D_2O, 30 °C), (c) combination of ^{13}C NMR and DEPT-135 spectrum (125 MHz, D_2O, 30 °C) and (d) part of HSQC spectrum (D_2O, 30 °C) of the β-glucan (PS) isolated from an edible mushroom *Russula albonigra* (Krombh.) Fr.

2.9. Immunostimulating properties

2.9.1. Test for macrophage by nitric oxide (NO) assay

RAW 264.7, a murine macrophage cell line obtained from National Centre for Cell Sciences (NCCS), Pune, India, was grown in Dulbecco's modified Eagle's medium (DMEM) and seeded in 96 well flat bottom tissue culture plate at 5×10^5 cells/mL concentration (180 μL). Cells were left overnight for the attachment and then PS were treated with different concentrations (12.5, 25, 50, 100, and 200 μg/mL) to the wells. After 48 h of treatment culture supernatant of each well were collected and NO production was estimated using Griess Reagent (Green et al., 1982) at 540 nm (1:1 of 0.1% in 1-napthylethylenediamine in 5% phosphoric acid and 1% sulfanilamide in 5% phosphoric acid). Lipopolysaccharide (LPS), L6511 of *Salmonella enterica* serovar Typhimurium (Sigma, St. Louis, USA) was used as positive control (Nandi et al., 2012, 2013).

2.9.2. Splenocyte and thymocyte proliferation assay

A single cell suspension of spleen and thymus was prepared from Swiss Albino mice under aseptic conditions by homogenization in Hank's balanced salt solution (HBSS). The suspension was centrifuged to obtain cell pellet. The contaminating RBC was removed by hemolytic Gey's solution. After washing two times in HBSS the cells were resuspended in complete RPMI (Roswell Park Memorial Institute) with serum and antibiotics added. RPMI and fetal bovine serum (FBS) has been obtained from Gibco whereas antibiotics were obtained from Himedia. Cell concentration was adjusted to 1×10^6 cells/mL and viability of splenocytes and thymocytes (as tested by trypan blue dye exclusion) was always over 90%. The cells (180 μL) were plated in 96 well flat bottom tissue culture plates and incubated with 20 μL of various concentrations of the PS (12.5, 25, 50, 100, and 200 μg/mL). PBS (10 mM, Phosphate Buffer Saline, pH-7.4) was taken as negative control whereas LPS (4 μg/mL, Sigma) and Concavalin A (Con A, 10 μg/mL, Himedia) served as positive controls. All cultures were set up in triplicate for 72 h at 37 °C in a humidified atmosphere of 5% CO_2. Proliferation of splenocytes indicated as Splenocyte Proliferation Index (SPI) and Thymocytes written as Thymocyte Proliferation Index (TPI) were checked by standard MTT assay method (Ohno et al., 1993). The data are reported as the mean ± standard deviation of different observations and compared against PBS control.

2.10. Antioxidant properties

2.10.1. Hydroxy radical scavenging activity

The reaction mixture (1 mL) consisted of KH_2PO_4–KOH buffer (20 mM, pH 7.4), 2-deoxy-D-ribose (2.8 mM), variable concentration (100–400 μg/mL) of PS, $FeCl_3$ (100 mM), EDTA (104 μM), ascorbate (100 μM) and H_2O_2 (1 mM). It was incubated at 37 °C for 1 h. 2 mL thiobarbituric acid (TBA) and trichloroacetic acid (TCA) solution (100 mL contained 375 mg TBA, 15 mg TCA; 2 mL concentrated HCL added to 98 ml of TBA–TCA solution) was added and incubated at boiling water bath for 15 min. After cooling, absorbance was measured at 535 nm (Acharya, Chatterjee, & Ghosh, 2011). Butylated hydroxytoluene (BHT) was used as positive control.

2.10.2. Superoxide radical scavenging activity

The method by Martinez, Marcelo, Marco, and Moacyr (2001) for determination of the superoxide anion was followed with modification in the riboflavin-light-nitrobluetetrazolium (NBT) system. Each 3 mL reaction mixture contained 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, various concentrations (100–400 μg/mL) of PS, 100 μM EDTA, 75 μM NBT and 2 μM riboflavin. Reaction started by illuminating sample with light and the increased absorbance was measured at 560 nm after 10 min of illumination. Identical tubes with the reaction mixture were kept in the dark and served as blank. Butylated hydroxyanisole (BHA) was used as a positive control.

2.10.3. Chelating ability of ferrous ions

Chelating ability was determined according to the method of Dinis, Madeira, and Almeida (1994). Reaction mixture (4 mL) contained different concentrations of PS (100–400 μg/mL) mixed with 3.7 mL of water and 0.1 mL of 2 mM ferrous chloride. The reaction was initiated by the addition of 0.2 mL of 5 mM ferrozine. After 10 min incubation at room temperature, the absorbance was determined at 562 nm against a blank. EDTA was used as positive control. The percentage of inhibition of ferrozine–Fe^{2+} complex formation is given by this formula:

$$\%\,\text{inhibition} = \left\{\frac{A_0 - A_1}{A_0}\right\} \times 100.$$

where A_0 is the absorbance of the control and A_1 is the absorbance in the presence of mushroom fractions.

2.10.4. Determination of reducing power

Reducing power was determined according to the method of Oyaizu (1986). Various concentrations of PS (200–600 μg/mL) were mixed with 2.5 mL sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide (1%). The mixture was incubated for 20 min and then 2.5 mL of trichloroacetic acid (10%) was added. 2.5 mL of this solution was mixed with 2.5 mL distilled water and 0.5 mL $FeCl_3$ (0.1%) and incubated for 15 min. The absorbance was measured at 700 nm against buffer. Ascorbic acid was used as standard. IC_{50} value is the effective concentration at which the absorbance was 0.5 for reducing power.

2.10.5. β carotene bleaching assay

Inhibition of β carotene bleaching was determined according to the method of Dapkevicius, Venskutonis, Van Beek, and Linssen (1998). Reaction mixture consisted of 0.5 mg β carotene in 1 ml HPLC grade chloroform, 25 μL linoleic acid and 200 mg Tween 40. Chloroform was completely evaporated. Then 50 mL distilled water saturated with O_2 was added with vigorous shaking. Aliquots (4 mL) of this emulsion were transferred into different tubes containing different concentrations of PS (100–400 μg/mL) and absorbance was read at 490 nm against water. The tubes were placed at 50 °C for 2 h and again absorbance was taken. Butylated hydroxyanisole (BHA) was used as positive control.

3. Results and discussion

3.1. Isolation, purification and chemical analysis

The fresh fruit bodies of edible mushroom, *Russula albonigra* (Krombh.) Fr. (500 g) were washed with distilled water, boiled with 4% NaOH solution, filtered, centrifuged, and the supernatant was precipitated in EtOH. The precipitated materials on dialysis followed by freeze drying yielded 1.6 g of crude polysaccharide. The water soluble crude polysaccharide on fractionation through Sepharose 6B column yielded one homogeneous fraction (PS). The molecular weight (Hara et al., 1982) of PS was estimated as $\sim 1.95 \times 10^5$ Da from a calibration curve prepared with standard dextrans. GC 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 (Dong, Yao, Yang, & Fang, 2002). The absolute configuration of the monosaccharide present in the glucan was determined by the method of Gerwig et al. (1978) and it was found that glucose had D-configuration. The polysaccharide was methylated according to the method of Ciucanu and Kerek (1984) followed by hydrolysis and then converted to alditol acetate. The GC–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 GC analysis of the alditol acetates of the periodate-oxidised (Goldstein, Hay, Lewis, & Smith, 1965), 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 GC analysis of the alditol acetates of Smith degraded hydrolyzed product showed the presence of D-glucose and D-glycerol. The GC–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 (Dong et al., 2002) 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.

3.2. NMR and structural analysis of β-glucan (PS)

The ^{1}H NMR (500 MHz) spectrum (Fig. 1b and Table 1) at 30 °C 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 $\mathbf{A_I}$, $\mathbf{A_{II}}$, **B**, **C**, $\mathbf{D_I}$, and $\mathbf{D_{II}}$ according to their decreasing proton chemical shifts. In the ^{13}C (125 MHz) spectrum (Fig. 1c and Table 1) at 30 °C 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 (Fig. 1d and Table 1), the anomeric carbon signal at δ 103.0 corresponded to **B**, whereas the signal at δ 102.7 corresponded to $\mathbf{A_I}$, $\mathbf{A_{II}}$, and **C** and the peak at δ 102.5 was correlated to $\mathbf{D_I}$ and $\mathbf{D_{II}}$ 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 $J_{H\text{-}1,H\text{-}2} \sim 8$ Hz, and $J_{C\text{-}1,H\text{-}1} \sim 160$ Hz. In residues **A** ($\mathbf{A_I}$ and $\mathbf{A_{II}}$), the downfield shift of C-3 (δ 84.5) with respect to standard value of methyl glycosides (Agrawal, 1992) 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 (Agrawal, 1992) 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,

Table 1
^{1}H[a] and ^{13}C[b] NMR chemical shifts (δ in ppm) of PS from *Russula albonigra* (Krombh.) Fr. in D_2O.

Glucosyl residue	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6a,H-6b/C-6
→ 3)-β-D-Glc*p*-(1 →	4.74[d], 4.72[e]	3.35	3.73	3.46	3.47	3.72[c], 3.90[c]
A (**A_I**, **A_{II}**)	102.7	72.8	84.5	69.6	75.0	60.7
β-D-Glc*p*-(1 →	4.52	3.50	3.46	3.44	3.48	3.70[c], 3.88[c]
B	103.0	73.0	75.9	70.2	75.0	60.7
→ 3,6)-β-D-Glc*p*-(1 →	4.52	3.50	3.71	3.37	3.63	3.85[c], 4.19[c]
C	102.7	72.8	84.5	69.6	74.5	68.7
→ 6)-β-D-Glc*p*-(1 →	4.50	3.30	3.45	3.46	3.63	3.84[c,f], 4.17[c,f]
D	102.5	73.0	75.9	70.0	74.5	68.8[f]
						3.83[c,g], 4.18[c,g]
						69.0[g]

[a] Values of the ^{1}H chemical shifts were recorded with respect to the HOD signal fixed at δ 4.70 at 30 °C.
[b] Values of the ^{13}C chemical shifts were recorded with reference to acetone as the internal standard and fixed at δ 31.05 at 30 °C.
[c] Interchangeable.
[d] For residue **A_I**.
[e] For residue **A_{II}**.
[f] For residue **D_I**.
[g] For residue **D_{II}**.

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 neighboring effect (Maity et al., 2013; Yoshioka, Tabeta, Saito, Uehara, & Fukuoka, 1985) 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 (Fig. 1c).

The sequences of glucosyl moieties were determined from NOESY (Fig. 2a) as well as ROESY experiments. A long range HMBC (Fig. 2b) 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 (Fig. 2c and Table 2). 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 (Fig. 3 and Table 3) at 30 °C of SDPS showed two anomeric carbon signals

Table 2
NOESY and HMBC connectivities of PS from *Russula albonigra* (Krombh.) Fr. in D_2O at 30 °C.

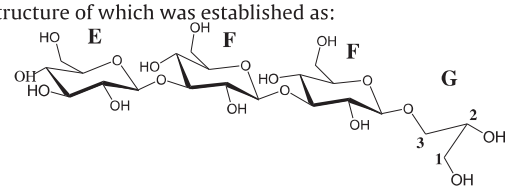
NOESY connectivities	HMBC connectivities
A_IH-1/**C**H-3	**A_I**H-1/**C**C-3
A_IH-1/**A_I**H-3	**A_I**C-1/**C**H-3
A_IH-1/**A_I**H-5	**A_{II}**H-1/**A_I**C-3
A_{II}H-1/**A_I**H-3	**A**C-1/**A**H-3
A_{II}H-1/**A_{II}**H-3	**A**C-1/**A**H-2
A_{II}H-1/**A_{II}**H-5	
BH-1/**A_{II}**H-3	**B**H-1/**A_{II}**C-3
BH-1/**B**H-3	**B**C-1/**A_{II}**H-3
BH-1/**B**H-5	**B**C-1/**B**H-2
CH-1/**D_{II}**H-6a; **D_{II}**H-6b	**C**H-1/**D_{II}**C-6
CH-1/**C**H-3	**C**C-1/**D_{II}**H-6a; **D_{II}**H-6b
CH-1/**C**H-5	**C**C-1/**C**H-2
	CC-1/**C**H-3
D_IH-1/**C**H-6a; **C**H-6b	**D_I**H-1/**C**C-6
D_{II}H-1/**D_I**H-6a; **D_I**H-6b	**D_I**C-1/**C**H-6a; **C**H-6b
DH-1/**D**H-3	**D_{II}**H-1/**D_I**C-6
DH-1/**D**H-5	**D_{II}**C-1/**D_I**H-6a; **D_I**H-6b
	DC-1/**D**H-2

Table 3
^{13}C NMR[a] chemical shifts (δ in ppm) of Smith-degraded glycerol-containing trisaccharide of PS from *Russula albonigra* (Krombh.) Fr. in D_2O.

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6
β-D-Glc*p*-(1 → **E**	102.7	73.52	76.0	70.69	75.68	60.77
→ 3)-β-D-Glc*p*-(1 → **F**	102.5	73.22	84.20	69.70	75.68	60.77
Gro-(3 → **G**	68.10	72.0	62.56			

[a] The values of chemical shifts were recorded with reference to acetone as internal standard and fixed at δ 31.05 at 30 °C.

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

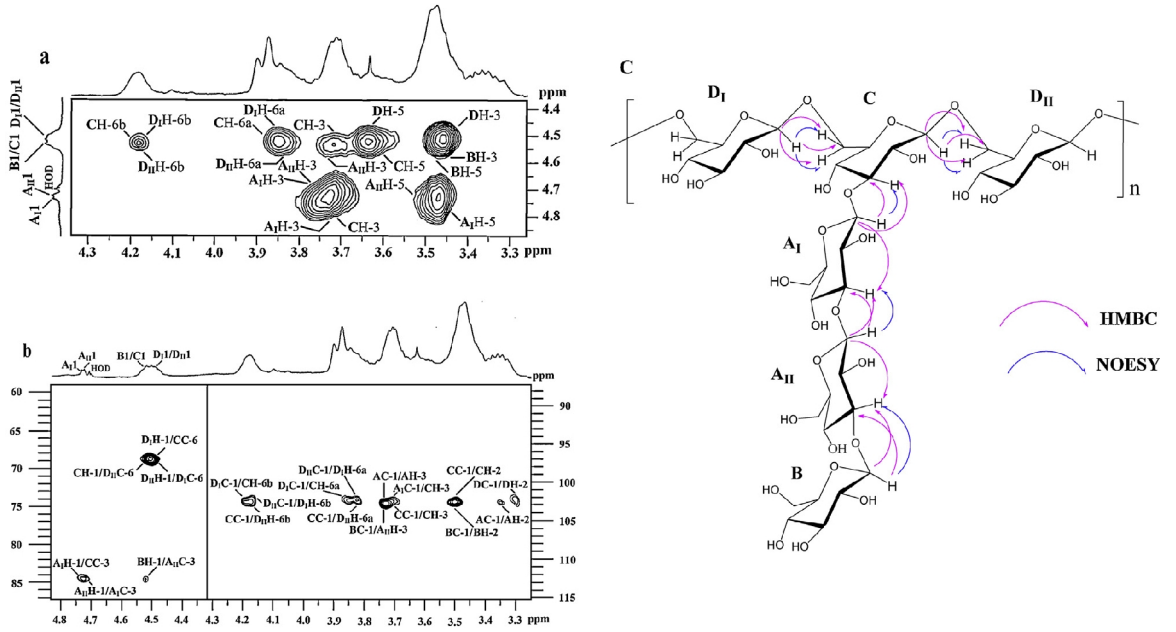
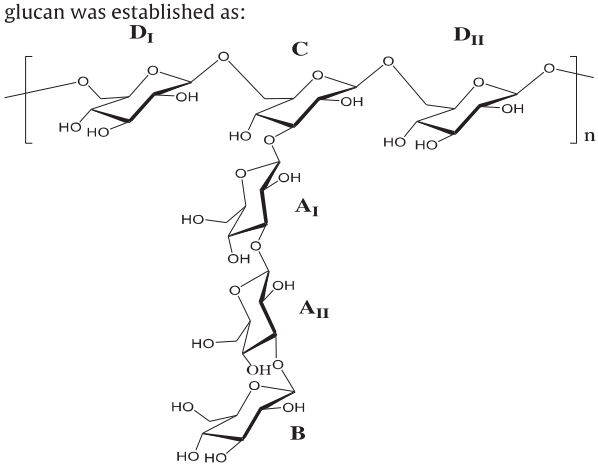


Fig. 2. (a) The part of NOESY spectrum of the β-glucan (PS) isolated an edible mushroom *Russula albonigra* (Krombh.) Fr. The NOESY mixing time was 300 ms. (b) The part of HMBC spectrum of the β-glucan (PS) isolated from an edible mushroom *Russula albonigra* (Krombh.) Fr. The delay time in the HMBC experiment was 80 ms. (c) The key 2D NMR correlations of β-glucan (PS).

spectroscopic evidences, the structure of repeating unit of the β-glucan was established as:



3.3. Immunological studies of β-glucan (PS)

A negative (−) limulus amebocyte lysate (LAL) test with the PS was carried out adopting the procedure as reported in our previous publication (Nandi et al., 2013) and found that it was free from bacterial endotoxin. Immunological studies were also investigated with the PS. Macrophage activation by PS was observed *in vitro*. Upon treatment with different concentrations of the PS, enhanced production of NO was observed in a dose-dependent manner with optimum production of 22 μM NO per 5×10^5 macrophages at 100 μg/mL of the PS (Fig. 4a). The various types of β-glucan like lentinan inhibit tumor growth by stimulating the immune system (Suzuki, Takatsuki, Maeda, Hamuro, & Chihara, 1994) through activation of macrophages, T-helper, NK, and other cells.

Splenocytes are the cells present in the spleen that include T cells, B cells, and dendritic cells that stimulate the immune response in living organism. Thymocytes are hematopoietic cells in thymus which generate T cells. The splenocytes and thymocytes activation tests were carried out in Swiss Albino mice cell culture medium with the PS by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method (Ohno et al., 1993). Proliferation of splenocytes and thymocytes is an indicator of immunostimulation. The PS was found to stimulate splenocytes and thymocytes as shown in Fig. 4b and c, respectively and the asterisks on the columns indicate the statistically significant differences compared to PBS control. 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. The splenocyte and thymocyte proliferation index as compared to Phosphate Buffered Saline (PBS) control if closer to 1 or below indicates low stimulatory effect on immune system.

3.4. Antioxidant activities of β-glucan (PS)

3.4.1. Assay of hydroxy radical scavenging activity

Hydroxy radical (OH•) has a very short life but it is considered to be the most toxic among all reactive oxygen species (ROS). It can damage DNA by attacking purines, pyrimidines and deoxyribose. Hydroxy radicals are formed by an electron transfer from transition metals to H_2O_2 and consequently interact with biomolecules (Ferreira, Barros, & Aberu, 2009). In our experiment, hydroxy radicals which are generated from Fe^{2+}–ascorbate–EDTA–H_2O_2 system (Fenton's reaction) attack the deoxyribose and eventually result in the formation of malondialdehyde (MDA). The formation of MDA is measured as a pink MDA–TBA chromogen at 535 nm (Aruoma, Laughton, & Halliwell, 1989). When test sample was added to reaction mixture, they removed hydroxy radicals and prevented sugar degradation. The PS showed potent hydroxy radical scavenging

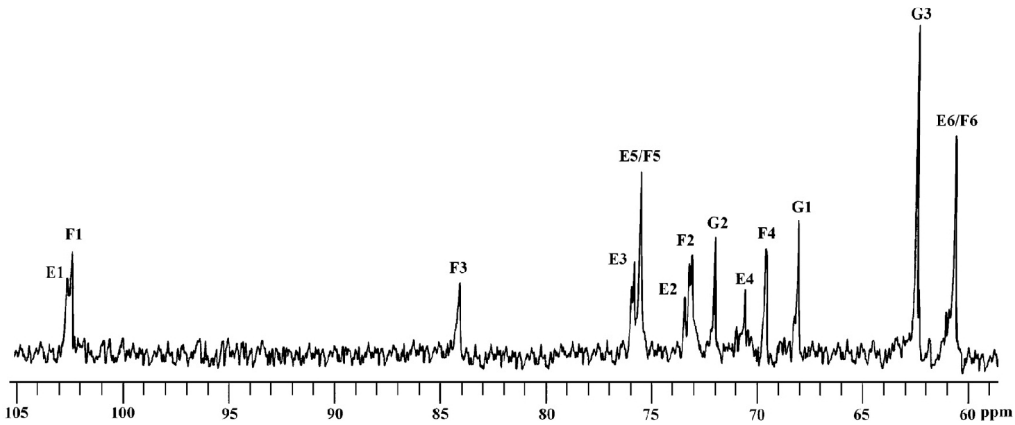


Fig. 3. The ^{13}C NMR spectrum of the Smith-degraded glycerol-containing trisaccharide of the β-glucan (PS) isolated from *Russula albonigra* (Krombh.) Fr. in D_2O at 30 °C.

activity which rose gradually with the increase of concentration (Fig. 5a). The IC_{50} value of the PS was found to be 265 μg/mL.

3.4.2. Assay of superoxide radical scavenging activity

Superoxide anion ($O_2^{\bullet}$) is one of the six major reactive oxygen species causing oxidative damage in the human body (Huang, Ou, & Prior, 2005). It is considered as primary ROS as it is a relatively weak oxidant but it can generate secondary ROS such as peroxynitrate ($ONOO^{\bullet}$), peroxy radical ($LOO^{\bullet}$), singlet oxygen, hydroxy radical and hydrogen peroxide (Chen, Ju, Li, & Yu, 2012; Huang, Ou, & Prior, 2005; Wootton-Beard & Ryan, 2011). Therefore, superoxide radical scavenging activity is of great importance to exhibit potential antioxidant property. The method used by Martinez, Marcelo, Marco, and Moacyr (2001) based on generation of superoxide radical by auto-oxidation of riboflavin in presence of light which in turn reduces yellow dye NBT to produce blue formazon. Intensity of color is directly proportional to the concentration of superoxide anion. In the present study, the PS was found to act as a notable scavenger of superoxide radicals (Fig. 5b). The IC_{50} value of the PS was determined 130 μg/mL, whereas Patra et al., 2013 reported that the IC_{50} value of the polysaccharide from *Pleurotus ostreatus* was 553 μg/mL.

3.4.3. Chelating ability of ferrous ion

Dietary nutrients containing metal chelators may act as preventive antioxidant because some transition metals, *e.g.* Fe^{2+}, Cu^{+}, Pb^{2+}, Co^{2+} and so on can trigger process of free radical reaction (Chen et al., 2012). Ferrozine quantitatively forms complexes with Fe^{2+}. In the presence of chelating agent, the complex formation is disrupted, resulting in the reduction of red color. Reduction therefore allows estimation of the chelating ability of the coexisting chelator. Fig. 5c reveals that the PS demonstrated a marked capacity for iron binding ability, where the 50% chelation was found at a concentration of 300 μg/mL.

3.4.4. Determination of reducing power

Reducing properties of a substance are generally associated with the presence of reductones or hydroxide groups. Such substance can exert antioxidant activity by donating hydrogen atom to break the free radical chain (Wootton-Beard & Ryan, 2011). In the reducing power assay, the more antioxidant compounds convert the

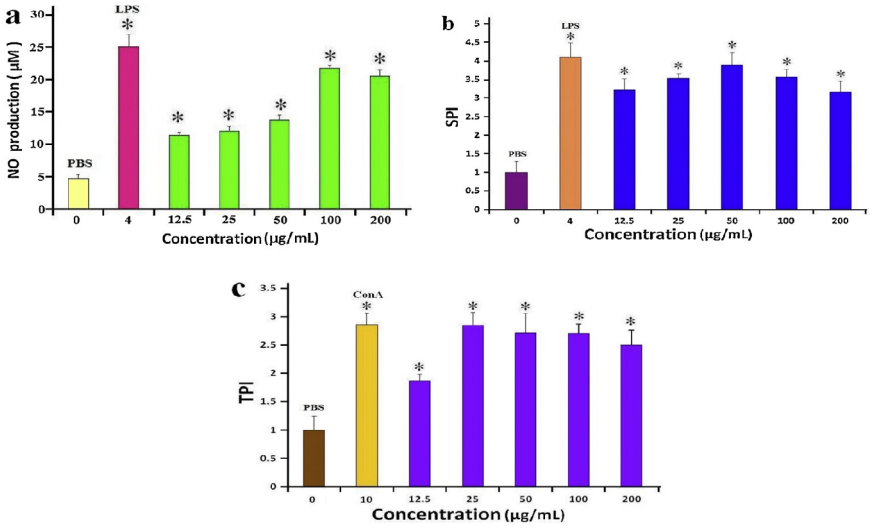


Fig. 4. (a) Activation of RAW 264.7 macrophage cells with different concentrations of β-glucan (PS) in terms of NO production. Effect of different concentrations of β-glucan (PS) on proliferation of (b) splenocyte and (c) thymocyte (asterisks indicate the statistically significant compared to the PBS control with $P < 0.05$).

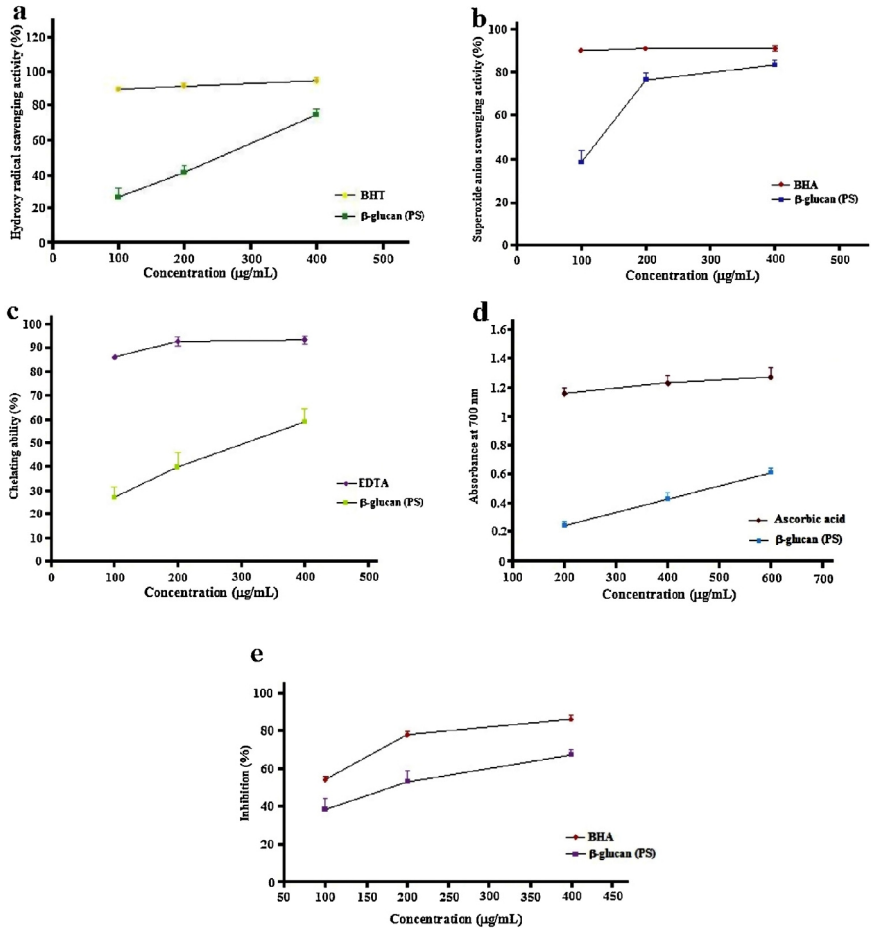


Fig. 5. (a) Hydroxy radical scavenging activity, (b) superoxide radical scavenging activity, (c) ferrous ion chelating ability, (d) reducing power and (e) inhibition of β carotene bleaching of β-glucan (PS) isolated from *Russula albonigra* (Krombh.) Fr. (All the above results are the mean ± SD of three separate experiments, each in triplicate.).

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. Fig. 5d reveals that at concentration of 500 μg/mL PS showed reducing power of 0.5.

3.4.5. *β carotene bleaching assay*

β carotene usually undergoes rapid discoloration in absence of antioxidant. Oxidation of β carotene and linoleic acid generate free radicals. Free radicals from linoleic acid are formed by the abstraction of a hydrogen atom from one of its diallylic methylene groups which attack the highly unsaturated β carotene molecule. Hence, β carotene is oxidized, and gradually losing its orange color which is then monitored spectrophotometrically (Okoh, Sadimenko, & Afolayan, 2011). Fig. 5e reveals that the PS had inhibition effect on β carotene bleaching. The PS showed 50% inhibition at a concentration of 180 μg/mL.

4. Conclusions

An immunoenhancing antioxidant water soluble β-glucan (PS) was isolated from the alkaline extract of an edible mushroom, *Russula albonigra* (Krombh.) Fr. The structure of this PS was elucidated on the basis of total hydrolysis, methylation analysis, Smith degradation, partial hydrolysis and 1D/2D NMR studies. These results indicated that the repeating unit of the PS contained a backbone 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 PS activated the macrophages, splenocytes, and thymocytes and also showed several potent antioxidant activities. Hence, on the basis of these activities it could be used as a natural immunostimulant and antioxidant material, further, this mushroom can also be recommended as an excellent food for consumption.

Acknowledgements

The authors are grateful to Dr. S. Roy, Director, IICB, Kolkata, for providing instrumental facilities. Mr. Barun Majumdar of Bose Institute, Kolkata, is acknowledged for preparing NMR spectra. A.K. N. (one of the authors) thanks the CSIR for offering junior research fellowship (CSIR-09/599(0049)/2012-EMR-I).

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