THE STRUCTURAL STUDIES OF BIOACTIVE POLYSACCHARIDES FROM PLANT AND MUSHROOM

A SYNOPSIS

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SYNOPSIS

The present thesis entitled **"The structural studies of bioactive polysaccharides from plant and mushroom"** is mainly based on the determination of the structure as well as some important immunological and antioxidant activities of different polysaccharides isolated from the leaves of *Catharanthus rosea*, fruit bodies of somatic hybrid mushroom *(PfloVv1aFB)* and the mycelia of *Pleurotus ostreatus*. All the polysaccharides were purified through the Gel-permeation chromatography (GPC) technique using Sepharose-6B as column ingredient. Structural investigation of polysaccharides were carried out using chemical analysis (total hydrolysis, methylation, periodate oxidation) and NMR (^{1}H, ^{13}C, TOCSY, DQF-COSY, NOESY, ROESY, HSQC, HMQC and HMBC) experiment. The entire thesis is divided into five chapters.

Chapter-I discusses the introduction of carbohydrates, plant and mushroom polysaccharides and some of their important biological activities. Carbohydrates are essential constituents of all living organisms. These are generally classified into four classes: monosaccharides, disaccharides, oligosaccharides, and polysaccharides. The great bulk of the carbohydrates in nature are present as polysaccharides, which have relatively large molecular weights. The polysaccharides serve two principal functions: (1) these are used by both plants and animals to store glucose as a source of future food energy, and (2) they provide some of the mechanicals structure of cells.

Mushroom is a fleshy, aerial umbrella-shaped, fruiting body of macrofungi. Mushrooms are not only valued for their high nutritive value containing high quantities of protein, carbohydrate, minerals but also of their low fat content and low calorific value. Large amount of bioactive compounds like lectins, polysaccharides, polysaccharide-peptides, polysaccharide-protein complexes have been isolated from mushrooms. Among these polysaccharide is a potent mushroom derived material having various bioactive properties. Different polysaccharides from both plants and mushrooms showed their immunomodulation and antitumor properties. Mushroom polysaccharides are not only used to cure cancers of the stomach, esophagus, lungs, and colons but also act as anti-inflammatory, antiviral, hypoglycaemic and antithrombotic agents. Lentinan from *Lentinus edodes*, Schizophyllan from *Schizophyllum commune*, Agarican from *Agaricus blazei*, Lingzhi from *Ganoderma lucidum* and Maitake from *Grifola frondosa* have been used clinically as anti-tumor agents. A number of polysaccharides isolated

from the mycelia of *Antrodia camphorate*, *Agaricus blazei* and *Ganoderma tsugae* has pronounced anti-tumor effects on both in vitro and vivo. Several plant polysaccharides isolated from *Aloe barbadensis*, fruit juice of *Morinda citrifolia* (noni), *Morus alba*, *Chlamydomonas mexicana* and *Poria cocos* show immunomodulatory and antitumor activity. Different polysaccharides are used as dietary fiber. The term "dietary fiber" signifies high molecular weight indigestible materials that move through the digestive system, absorbing water. Chemically, dietary fiber consists of non-starch polysaccharides and several other plant components such as cellulose, lignin, waxes, chitins, pectins, beta-glucans, inulin and oligosaccharides. The biological activities of polysaccharides depend on the size of molecule, branching rate and form. So, it is very important to determine the exact structure of the polysaccharides, isolated either from medicinal plants or from mushrooms.

Chapter-II describes methodologies used in identification and characterization of the polysaccharides. The column chromatography technique is used for resolving a mixture of polysaccharides having different molecular weight. The neutral sugars present in the polysaccharide were identified and estimated by Gas-liquid-chromatography (GLC). The absolute configurations of sugars are identified using the method of Gerwig et al. The alditol acetates of partially methylated sugars derived from polysaccharide (using Ciucanu and Kerek Method and Purdie Method) were identified by GLC-MS to know the mode of linkages present in the polysaccharide. Periodate oxidation was carried out to confirm the mode of linkages of sugar residues. Chemical degradation (Smith degradation) was carried out to generate oligosaccharide from polysaccharide. ^{1}H, ^{13}C, DQF-COSY, TOCSY, NOESY, HMQC, 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. Splenocyte and thymocyte activation tests were carried out by the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] method. Assay of nitric oxide (NO) production was carried out in mouse monocyte cell line. The antioxidant activity of polysaccharide was evaluated through the chelating ability of ferrous ions, hydroxyl and superoxide radical scavenging activity.

Chapter-III Water-soluble polysaccharides were isolated from the hot aqueous extract of leaves of *Catharanthus rosea.* The water soluble extract was repeatedly

purified by precipitating in alcohol, dialyzing through a DEAE cellulose bag, and gel filtration. Finally, fractionation of water soluble polysaccharides through a Sephadex-6B column yielded pure polysaccharide. The molecular weight of the polysaccharide (PS-I) was determined using a calibration curve of standard dextrans and found to be ~2.0×10^5 Da. The total carbohydrate of PS-I was estimated at 98.5%. The pure PS-I had specific rotation of $[\propto]_D^{25.6}$ +98.74 (c 0.094, water). Structural studies of PS-I was performed using acid hydrolysis, methylation analysis, periodate oxidation studies along with NMR analysis (^{1}H, ^{13}C, TOCSY, DQF-COSY, NOESY, ROESY, HMQC, and HMBC). The GLC analysis of the alditol acetates of hydrolyzed PS-I showed the presence of rhamnose, glucose, and arabinose in a molar ratio of nearly 1:1:2 but the carboxyl-reduced PS-I on hydrolysis followed by GLC analysis showed the presence of glucose, arabinose, and galactose in a molar ratio of nearly 1:1:2:2. These results confirmed molar ratio of rhamnose, glucose, arabinose and galacturonic acid in PS-I was 1:1:2:2. The absolute configuration of the sugar units was determined by the method of Gerwig et al and it was found that the glucose and galaturonic acid had the D configuration but the rhamnose and arabinose were present as L. The mode of linkages of PS-I was determined by methylation analysis using Ciucanu and Kerek method followed by hydrolysis and alditol acetates preparation. The alditol acetates of methylated product were analyzed by GLC and GLC-MS which showed the presence of 1,3,4-*O*-actyl-2,5-di-*O*-methyl-arabinitol; 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methyl-arabinitol; 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-glucitol, 1,2,4,5-tetra-*O*-acetyl-3-mono-*O*-methyl-rhamnitol in a molar ratio of nearly 1:1:1:1. The above result indicated the presence of one unit of 3-acetyl arabinose and glucose as terminal residues and another unit of arabinose as (1→5)-linked arabinofuranosyl or (1→4)-linked arabinopyranosyl. It also indicated the presence of (1→2, 4)-linked rhamnopyranosyl unit. The GLC-MS analysis of the alditol acetates of methylated carboxyl reduced polysaccharide showed two new peaks of 1,2,4,5,6-penta-*O*-acetyl-3-mono-*O*-methyl galactitol and 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methyl galactitol. This result indicated that galacturonic acids were present as (1→2, 4)-linked and (1→4)-linked moieties. For further linking information, a periodate oxidation experiment was carried out with the carboxyl reduced and intact polysaccharide. The periodate oxidation experiment supported the above mode of linkages.

The 500 MHz ^{1}H NMR spectrum of this polysaccharide at 27 °C showed signals at δ 5.37, 5.22, 5.06, and 4.93 for six anomeric protons where δ 5.06 and 4.93

accommodated two protons for each signal and the rest for another two protons. The sugar residues were designated as **A**-**F** according to their decreasing anomeric proton chemical shifts. In the ^{13}C NMR spectrum (125 MHz) at 27 °C signals appeared at δ 109.6, 107.8, 100.8, and 100.0 for six anomeric carbons where signal at δ 100.8 consisted of three carbon and the rest for another three carbon signals. In the HMQC spectrum the three anomeric carbon signals at δ 109.6, 107.8, 100.0 were correlated to three anomeric protons of **B**, **D**, and **A** respectively, whereas, the signal at δ 100.8 was correlated to three anomeric protons of residues **C**, **E** and **F**. A proton signal at 5.12 ppm correlated to carbon signal at 79.4 ppm and proton signal at 2.03 correlated to carbon signal at 20.2 indicative for acetyl group. In HMBC spectrum the cross-coupling between the carbonyl carbon (171.0 ppm) of *O*-acetyl group and H-3 (5.12 ppm) of residue **D** [**OAc** (C)/**D** H-3] indicated that acetyl group attached to the 3-position of residue **D**. The proton signal at 1.23 ppm correlated to carbon signal at 17.5 and it may be for the CH_3 proton of deoxy sugar, rhamnose. The proton signals appeared at 3.78 ppm and 3.75 ppm for carbomethoxy proton ester and methoxy group respectively. The carbon signal appeared at 53.3 ppm for methyl carbon of both ester and methoxy group. Intra-residual coupling between methoxyl carbon (53.3 ppm) and H-6a and H-6b proton of residue **A** were observed. Hence from GLC, GLC-MS and NMR experiment all the sugar residues present as

Residue **A:** terminal 6-*O*-Me D-glucopyranosyl moiety

Residue **B:** (1→5)-α-L-arabinofuranosyl moiety

Residue **C:** (1→2, 4)-α-L-Rhamnopyranosyl moiety

Residue **D:** terminal 3-OAc-L-arabinofuranosyl moiety

Residue **E:** **(**1→2, 4)-methyl ester of α-D-galacturonosyl moiety

Residue **F:** **(**1→4)-methyl ester of α-D-galacturonosyl moiety

The sequence of glycosyl residues of the polysaccharide was determined from ROESY as well as NOESY experiments followed by confirmation with HMBC experiment. Residue **B** had interresidue ROESY contracts from H-1 to H-2 of residue **E**. Residue **D** had interresidual ROESY contacts from H-1 to H-5a and H-5b of residue **B**. Hence, the sequence in between the residue **B**, **D**, and **E** was established as

α-D- Gal*p*A6Me
2 **E**
↑
1
3-OAc-α-L-Ara*f* (1→5)-α-L-Ara*f*
D **B**

Again, from the interresidue ROESY contacts from **E**H-1 to **F**H-4, **F**H-1 to **C**H-2, **C**H-1 to **E**H-4, and **A**H-1 to **C**H-4 the following connectivities were established as

→4)-α-D-Gal*p*A6Me (1→4)-α-D-Gal*p*A6Me (1→2)-α-L-Rha*p* (1→
2 **E** **F** 4 **C**
↑ ↑
1
α-D-Glc*p*-6-OMe
A

From the HMBC experiment the following cross peaks were observed:
AH-1/**C**C-4; **A**C-1/**C**H-4; **B**H-1/**E**C-2; **B**C-1/**E**H-2; **C**H-1/**E**C-4; **C**C-1/**E**H-4; **D**H-1/**B**C-5; **D**C-1/**B**H-5a; **D**C-1/**B**H-5b; **E**H-1/**F**C-4; **E**C-1/**F**H-4; **F**H-1/**C**C-2; **F**C-1/**C**H-2.

So, from the ROESY and HMBC experiment the repeating unit of the polysaccharide was established as

→4)-α-D-Gal*p*A6Me (1→4)-α-D-Gal*p*A6Me (1→2)-α-L-Rha*p* (1→
2 **E** **F** 4 **C**
↑ ↑
1 1
3-OAc-α-L-Ara*f* (1→5)-α-L-Ara*f* α-D-Glc*p*-6-OMe
D **B** **A**

This polysaccharide was found to activate the macrophages. Macrophage activation was studied by NO production in culture supernatant in vitro. On treatment with

different concentrations of the polysaccharide an enhanced production of NO was observed in a dose dependent manner with optimum production of 20.5 µM NO per 5 × 10^5 macrophages at 100 µg/mL of the polysaccharide. This molecule exhibited splenocyte as well as thymocyte activation on mouse cell culture medium which was tested by the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] method. At 50 µg/mL of the polysaccharide, both the splenocyte and thymocyte proliferation index was observed maximum as compared to other concentrations. Hence, 50 µg/mL of the polysaccharide can be considered as an efficient splenocyte and thymocyte proliferators.

This work has been published in **Carbohydrate Polymers, 81, 2010, 584-591.**

Chapter-IV describes the structural characterization and immunoenhancing properties of a heteroglycan isolated from the fruit bodies of somatic hybrid mushroom (*PfloVv1aFB*). The somatic hybrid mushroom (*PfloVv1aFB*) was obtained through intergeneric protoplast fusion between *Pleurotus florida* and *Volvariella volvacea* strains. The hot water-extract of fresh fruit bodies (500 g) of *PfloVv1aFB* was cooled, filtered, and precipitated in alcohol. The residue was dialyzed, centrifuged and freeze dried to yield crude polysaccharide, which on fractionation through Sepharose-6B using water as eluant yielded pure polysaccharide (Fr-I). The pure polysaccharide showed specific rotation $[\alpha]_D^{25.8}$ +9.19 (*c* 0.849, water) and molecular mass was estimated as ~1.95 x 10^5 Da. On the basis of acid hydrolysis, methylation analysis, periodate oxidation along with ^{1}H, ^{13}C, and DEPT-135 NMR spectroscopy, including two-dimentional TOCSY, DQF-COSY, NOESY, ROESY, HMQC, and HMBC experiments, the structure of the repeating unit of this polysaccharide was determined. Acid hydrolysis of the polysaccharide followed by alditol acetates preparation and GLC analysis revealed the presence of glucose, galactose, and mannose in a molar ratio of nearly 4:1:1. The absolute configuration of all the residues were determined as D by the method of Gerwig et al. The mode of linkages of the polysaccharide was determined by methylation analysis using Ciucanu and Kerek method followed by hydrolysis and alditol acetates preparation. The GLC and GLC-MS analysis of alditol acetates of methylated polysaccharide revealed the presence of terminal D-glucopyranosyl and D-mannopyranosyl, (1→3)-linked D-glucopyranosyl, (1→6)-linked D-glucopyranosyl, (1→3, 6)-linked D-glucopyranosyl, and (1→2, 6)-linked D-galactopyranosyl moieties in

the polysaccharide. These linking modes were further confirmed by periodate oxidation experiment.

In ^{1}H NMR spectrum (500 MHz) in D_2O at 27 ºC signals appeared at δ 5.12, 4.98, 4.51, 4.50, and 4.49 for six anomeric protons where signal at δ 4.50 consisted of two protons and rest for another four protons. ^{13}C NMR spectrum (125 MHz) in D_2O at 27 ºC showed the signals at δ 103.3, 103.1, 103.0, 102.0, 100.9, and 98.5 for six anomeric carbons. In HMQC spectrum the anomeric proton at δ 5.12, 4.98, 4.51, 4.50, and 4.49 correlated to the anomric carbon at δ 98.5, 100.9, 103.3, 103.0, 103.1 and 102.0 respectively. The residues were designated as **A**, **B**, **C**, **D**, **E**, and **F** according to their decreasing anomeric proton chemical shift. All the ^{1}H and ^{13}C signals were assigned using TOCSY, DQF-COSY and HMQC experiments. The proton coupling constants were measured from DQF-COSY experiment.

On the basis of proton and carbon chemical shifts, proton-proton coupling constants and C-1, H-1 coupling constants, all the sugar residues were assigned as follows:

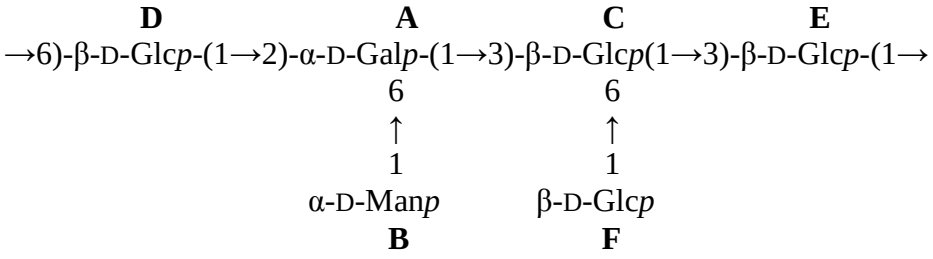
Residue **A:** (1→2, 6)-linked-α-D-galactopyranosyl moiety
Residue **B:** terminal α-D-mannopyranosyl moiety
Residue **C:** (1→3, 6)-linked β-D-glucopyranosyl moiety
Residue **D:** (1→6)-linked-β-D-glucopyranosyl moiety
Residue **E:** (1→3)-linked-β-D-glucopyranosyl moiety
Residue **F:** terminal-β-D-glucopyranosyl moiety

The cross-peaks of both the anomeric protons and carbons of each glycosyl residue were examined. In NOESY experiment, the inter residual contacts from **A**H-1 to **C**H-3, **B**H-1 to both **A**H-6a and **A**H-6b, **C**H-1 to **E**H-3, **D**H-1 to **A**H-2, **E**H-1 to both **D**H-6a and **D**H-6b, and **F**H-1 to **C**H-6a and **C**H-6b were observed. The following sequences were established as:

A (1→3) **C ; B** (1→6) **A ; C** (1→3) **E ; D** (1→2) **A ; E** (1→6) **D ; F** (1→6) **C**

The above sequences were confirmed by the HMBC experiment. In HMBC experiment, inter residual cross-peaks **A**H-1/**C**C-3; **A**C-1/**C**H-3; **B**H-1/**A**C-6; **B**C-1/**A**H-6a; **B**C-1/**A**H-6b; **C**H-1/**E**C-3; **C**C-1/**E**H-3; **D**H-1/**A**C-2; **D**C-1/**A**H-2; **E**H-1/**D**C-6; **E**C-

1/**D**H-6a; **E**C-1/**D**H-6b; **F**H-1/**C**C-6; **F**C-1/**C**H-6a; **F**C-1/**C**H-6b were observed. Thus, the HMBC and NOESY connectivities clearly supported the presence of the hexasaccharide repeating unit in polysaccharide isolated from somatic hybrid mushroom (*PfloVv1aFB*) as:



Some biological studies were carried out with this polysaccharide. Macrophage activation of the polysaccharide was observed in vitro. On treatment with different concentrations of the polysaccharide an enhanced production of NO was observed in a dose dependent manner with optimum production of 15.1 μM NO per 5 x 10^5 macrophages at100 μg/mL of the polysaccharide. Splenocyte and thymocyte activation tests were carried out in mouse cell culture medium with the polysaccharide by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method. Proliferation of splenocytes and thymocytes is an indicator of immunostimulation. At 10 μg/mL of the polysaccharide, both the splenocyte and thymocyte proliferation index was observed maximum as compared to other concentrations. Hence, 10 μg/mL of the polysaccharide can be considered as an efficient splenocyte and thymocyte proliferators.
This work has been published in **Carbohydrate research, 346, 2011, 1967-1972.**

Chapter-V consists of isolation, purification and structure determination of a heteroglycan from the mycelia of *Pleurotus ostreatus.* This heteroglycan showed the antioxidant properties. The crude polysaccharide was isolated from the mycelia of *Pleurotus ostreatus* by treatment with 2% KOH followed by ethanol precipitation, ion exchange chromatography, dialysis, and freeze-drying. The crude polysaccharide on fractionation through Sepharose-6B column yielded one fraction. The specific rotation of the pure polysaccharide (PS) was observed as $[\alpha]_D^{26.2}$ +8.5 (*c* 0.84, water). The

molecular weight was determined using calibration curve of standard dextrans and found to be ~1.8 x 10^5 Da. Monosaccharide residue was identified by GLC experiment of the complete hydrolyzed products and it was observed that the heteroglycan contains fucose, mannose, and glucose in a molar ratio of nearly 1:2:3. The absolute configuration of the monosaccharides was determined by method of Gerwig et al. and it was found that mannose and glucose had the D configuration but fucose was present as L. The mode of linkages of the sugar moieties present in the PS was determined by methylation analysis using the method of Ciucanu and Kerek through GLC and GLC-MS analysis. The results indicated that the terminal D-glucopyranosyl and D-mannopyranosyl, (1→6)-D-mannopyranosyl, (1→2)-L-fucopyranosyl, (1→4,6)-D-glucopyranosyl, and (1→3,6)-linked D-glucopyranosyl moieties were present in the PS. These linkages were further confirmed by periodate oxidation experiment.

In the anomeric region of the ^{1}H NMR spectrum (500 MHz) at 27°C five signals were observed at δ 5.37, 5.10, 4.97, 4.78, and 4.50 where the signal at δ 5.10 contained two anomeric protons. In the ^{13}C NMR spectrum (125 MHz) five signals were observed in the anomeric region at δ 103.6, 102.0, 99.6, 98.5, and 97.5 where the signal at δ 102.0 was found almost double to those of the other signals. In the HSQC spectrum the anomeric protons at δ 5.37, 4.97, and 4.50 correlated to the anomeric carbons at δ 99.6, 97.5, and 103.6 respectively. The anomeric proton at δ 5.10 correlated to both the anomeric carbons at δ 102.0 and 98.5, and the anomeric proton at δ 4.78 also correlated to the carbons at δ 102.0. So five anomeric proton and carbon signals indicated six sugar residues and designated as **A, B, C, D, E,** and **F** according to their decreasing proton chemical shifts. So from GLC, GLC-MS and NMR experiment all the sugar residues present as:

Residue **A:** (1→4, 6)-linked-α-D-glucoopyranosyl moiety

Residue **B:** (1→6)-linked-α-D-mannopyranosyl moiety

Residue **C:** (1→2)-linked-α-L-fucopyranosyl moiety

Residue **D:** terminal-α-D-glucopyranosyl moiety

Residue **E:** terminal β-D-mannopyranosyl moiety

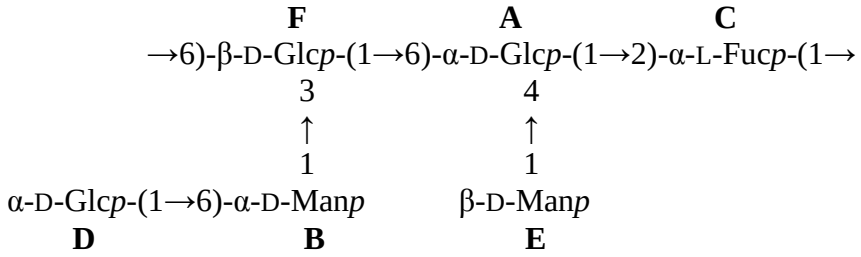
Residue **F:** (1→3, 6)-linked-β-D-glucopyranosyl moiety

The sequences of glycosyl moieties were determined from ROESY as well as NOESY experiments and it was confirmed from HMBC experiment. The following sequences were assigned:

A (1→2) **C**; **B** (1→3) **F**; **C** (1→6) **F**; **D** (1→6) **B**; **E** (1→4) **A**; **F** (1→6) **A**

From the HMBC experiment the following cross peaks were observed: **A**H-1/**C**C-2; **A**C-1/**C**H-2; **B**H-1/**F**C-3; **B**C-1/**F**H-3; **C**H-1/**F**C-6; **C**C-1/**F**H-6a, **F**H-6b; **D**H-1/**B**C-6; **D**C-1/**B**H-6a, **B**H-6b; **E**H-1/**A**C-4; **E**C-1/**A**H-4; **F**H-1/**A**C-6; **F**C1/**A**H6a, **A**H6b.

Finally, the structure of the repeating unit of the polysaccharide has been assigned as:



The antioxidant properties of the polysaccharides were carried out through scavenging capacities against hydroxyl and superoxide radicals and chelating ability of ferrous ion. The PS showed potent hydroxyl radical scavenging activity and increased with the increase of concentration. EC_{50} value of the PS was determined as 943 µg/mL. The PS was found to be a notable scavenger of superoxide radicals generated in riboflavin-nitrobluetetrazolium (NBT) light system. The EC_{50} value of the PS was determined as 553 µg/mL. Chelating effects (54.82%) of ferrous ions was observed at 1 mg/mL of the PS.

This work has been published in **Carbohydrate research, 368, 2013, 16-21.**