# STUDIES ON STRUCTURAL INVESTIGATION OF PLANT AND MUSHROOM POLYSACCHARIDES

**A SYNOPSIS**

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**BY**

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The present thesis entitled "**STUDIES ON STRUCTURAL INVESTIGATION OF PLANT AND MUSHROOM POLYSACCHARIDES**" deals with determination of the structural characterization of some biologically important plant and mushroom polysaccharides. The entire thesis is divided into five chapters.

**Chapter-I**: It represents the general introduction to carbohydrates, polysaccharides, plant, and mushroom polysaccharides and their biological activities.

**Chapter-II**: It represents the general methodologies adopted during the entire thesis work.

**Chapter-III**: It represents the isolation, purification and structural characterization of the polysaccharide isolated from the rhizomes of *Curcuma zedoaria* and published in *Carbohydrate polymers*, **2011**, *86*, 1252-1259.

**Chapter-IV**: It describes the isolation and structural characterization of the polysaccharide isolated from the edible mushroom *Agaricus bitorquis*. This work has been published in *Carbohydrate Research*, **2008**, *343*, 3120-3122.

**Chapter-V**: This is one of the major parts of the thesis which describes the isolation and chemical analysis of the polysaccharide isolated from a hybrid mushroom. This work has been published in *Carbohydrate Research*, **2011**, *346*, 2451-2456.

## *Chapter-I*

A **carbohydrate** is an organic compound with the empirical formula $C_m(H_2O)_n$ (where *m* could be different from *n*) consisting of carbon, hydrogen, and oxygen. Carbohydrates perform a numerous roles in living things. There are three different types saccharide (a synonym of carbohydrate) namely monosaccharide, oligosaccharide, and polysaccharide. 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 (2) They provide some of the mechanicals structure of cells.

Like all fungi, mushrooms are not plants and do not undergo photosynthesis. By the term 'mushroom' we mean "*a macrofungus with a distinctive fruiting body which can be either hypogeous or epigeous, large enough to be seen with the naked eye and to be picked by hand*". Mushrooms are nutritionally beneficial and are used as folk medicine in different countries throughout the years. They are also important for their immunomodulatory and antitumor activities. Polysaccharides isolated from both the mushroom and plant origin showed their great role as an antitumor and immunomodulatory effects. Mushroom polysaccharides are used against cancers of stomach, esophagus, lungs, and colons and also act as anti-inflammatory, antiviral (against AIDS), hypoglycaemic, and antithrombotic agents. Some mushroom polysaccharides like **Lentinan** from *Lentinus edodes* (**Japan**), **Schizophyllan** from *Schizophyllum commune*, **Agarican** from *Agaricus blazei* (**USA**), **Maitake** from *Grifola frondosa* (**Japan**) itself have been used clinically as anti-tumor agents.

## *Chapter-II*

This chapter deals with the methodologies adopted during the structural elucidation of the polysaccharide. Before the structural characterization, polysaccharide first isolated from the mushroom or the plant origin. The polysaccharides are purified using different chromatographic techniques. The exact structure of the polysaccharides is determined using two types of methods: (1) Chemical method that includes total acid hydrolysis, Paper chromatography, methylation study, periodate oxidation etc. (2) Spectroscopic method comprising of 1D ($^{1}H$, $^{13}C$) and 2D NMR (DQF-COSY, TOCSY, NOESY, ROESY, HMQC, HMBC etc), and mass spectroscopic experiments (GLC and GLC-MS).

## *Chapter-III*

*Curcuma zedoaria* is an important medicinal tuber plant belonging to the family Zingiberaceae. Plants of the ginger family (Zingiberaceae) have been widely used as spices and as traditional medicines in many Asian countries, and their medicinal

functions have been broadly discussed and accepted in many traditional recipes. Different parts of *Curcuma zedoaria* have been used in Ayurveda and other traditional medicines and also the rhizomes of *C. zedoaria* were adequately accepted as a rich source of medicines. Two polysaccharides (PS), water soluble and water insoluble, were isolated from the hot water extract of rhizomes of *C. zedoaria* of which the detailed structural characterization of water soluble polysaccharide is carried out in this chapter. The water soluble PS was fractionated through S-6B column yielding only one fraction. The apparent molecular weight of the polysaccharide was estimated from a calibration curve prepared with standard dextrans as $1.88\times10^2$ kDa and it showed specific rotation of $[\alpha]_D^{25.5}$ + 134.8 (*c* 0.54, water). The polysaccharide (4.0 mg) on acid hydrolysis with 2 M $CF_3CO_2H$ (2 mL) followed by paper chromatographic analysis showed the presence of rhamnose, glucose, galactose, galacturonic acid and arabinose. GLC analysis of alditol acetates of the sugars showed the presence of L-rhamnose, D-glucose, D-galactose and L-arabinose in a molar ratio of 1:1:1:1, but carboxyl-reduced PS on hydrolysis followed by GLC examination showed the presence of above sugars now in a molar ratio of 1:1:2:1. This result confirmed the presence of D-galacturonic acid in the polysaccharide. The absolute configuration of the polysaccharide was determined by the method of Gerwig et al. taking intact polysaccharide and carboxyl-reduced polysaccharide into consideration and confirmed by NMR-experiment.

The polysaccharide was methylated using Ciucanu and Kerek method followed by hydrolysis and alditol acetate preparation. The alditol acetates of the methylated polysaccharide were analyzed and identified by GLC-MS experiment, which revealed the presence of 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl-L-rhamnitol, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol, 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methyl-D-galactitol and 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methyl-L-arabinitol in a molar ratio of nearly 1:1:1:1. These result indicated the presence of terminal rhamnopyranosyl, (1→6)-linked glucopyranosyl, (1→3,4)-linked galactopyranosyl and (1→2)-linked arabinopyranosyl moieties. The alditol acetates of the methylated, carboxyl reduced PS showed the above peaks in a molar ratio of 1:1:2:1 which indicated the presence of (1→3,4)-linked Gal*p*A. But the carboxyl reduced methylated PS showed the presence of 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-galactitol along with the above peaks in a molar ratio of 1:1:1:1:1 indicating

that Gal*p*A was present as (1→4) linked and the 3-position of galacturonic acid was linked with OAc group, which was confirmed by NMR experiment. The periodate oxidation was carried out with the polysaccharide. The periodate-oxidized, $NaBH_4$-reduced material obtained from the polysaccharide, upon hydrolysis with TFA followed by GLC analysis showed the presence of D-galactose only. A part of periodate-oxidized PS on hydrolysis showed the presence of galacturonic acid in addition to the above sugar in the paper chromatographic examination. The GLC-MS analysis of the alditol acetates of the periodate-oxidized, carboxyl-reduced methylated polysaccharide showed the presence of 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methyl-D-galactitol and 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-galactitol. These result confirmed that galactopyranosyl and galacturonic acid were present as (1→3,4)-linked and (1→4)-linked moieties, respectively.

The 500 MHz $^1$H NMR spectrum of the polysaccharide recorded at 27 °C showed five signals in the anomeric region. The anomeric signals were observed at δ 5.38, 5.22, 5.12, 5.07 and 5.04. The integral value of the first signal (δ 5.38) was almost double than that of others (δ 5.22, 5.12, 5.07, and 5.04). These observations indicated that all the signals corresponded to one proton except the signal at δ 5.38, which corresponded to two protons. The $^{13}$C-NMR spectrum (125 MHz) at 27 °C exposed four anomeric signals at δ 103.5, 100.6, 100.1 and 100.0. All the $^1$H and $^{13}$C signals were assigned using DQF-COSY, TOCSY, and HMQC NMR experiments.

The resonance at δ 5.12 was correlated to δ 72.1 (C-3 signal of **D**) observed from the H-1/C-1 correlation in HMQC experiment indicating that it was not for anomeric proton, but for H-3 of residue **D** whose C-3 position was attached by OAc group for which H-3 proton has been shifted downfield towards anomeric region. The other five anomeric proton signals were corresponded to five sugar residues which were designated as **A**−**E** according to their decreasing anomeric chemical shifts in $^1$H-NMR spectrum. The anomeric configuration of the sugar residues was determined from $J_{\text{H-1,H-2}}$ and $J_{\text{C-1,H-1}}$ coupling constant values. From the above experimental data the residues [**A**–**E**] were found to have following linkages;

Residue **A:** (1→6)-linked α-D-glucopyranose

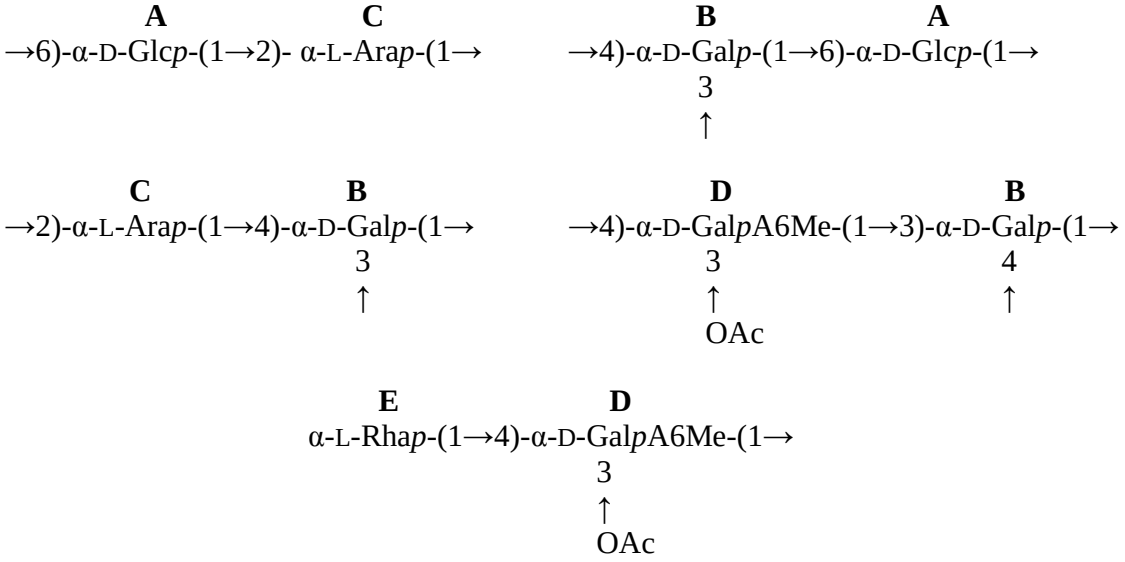
Residue **B:** (1→3,4)-linked α-D-galactopyranose

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

Residue **D:** methyl ester of (1→4)-linked 3-*O*-acetyl-α-D-galacturonic acid

Residue **E:** terminal α-L-rhamnopyranose

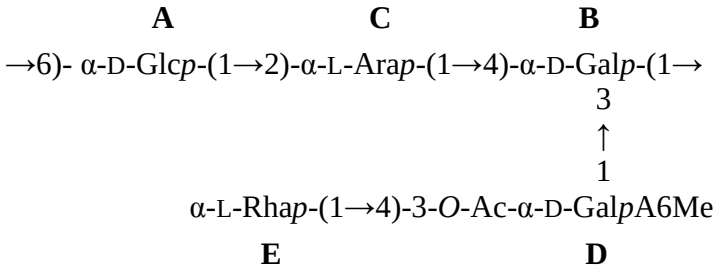
The sequences of glycosyl residues in PS were determined on the basis of NOESY experiment, followed by confirmation with HMBC experiment. Residue **A** had inter-residue NOE contacts from H-1 to H-2 of residue **C**, residue **B** had NOE contacts from H-1 to H-6a and H-6b of **A**, residue **C** had NOE contacts from H-1 to H-4 of **B**, residue **D** had NOE contacts from H-1 to H-3 of **B** and similarly residue **E** had NOE contacts from H-1 to H-4 of **D**. Hence, the following sequences were established as;




The sequences of these linkages were further confirmed by the HMBC experiment. Cross-peaks were found between H-1 (δ 5.38) of residue **A** and C-2 (δ 78.5) of residue **C** (**A** H-1, **C** C-2); and between C-1 (δ 100.0) of residue **A** and H-2 (δ 3.92) of residue **C** (**A** C-1, **C** H-2). The cross-peaks between H-1 (δ 5.38) of residue **B** and C-6 (δ 68.9) of residue **A** (**B** H-1, **A** C-6); and between C-1 (δ 100.1) of residue **B** and H-6a (δ 3.62) and H-6b (δ 3.98) of residue **A** (**B** C-1, **A** H-6a; **B** C-1, **A** H-6b) were observed. The cross-peaks were also found between H-1 (δ 5.22) of residue **C** and C-4 (δ 81.7) of residue **B**

(**C** H-1, **B** C-4); and between C-1 (δ 100.6) of residue **C** and H-4 (δ 4.2) of residue **B** (**C** C-1, **B** H-4). Again cross-peak were observed between H-1 (δ 5.07) of residue **D** and C-3 (δ 78.9) of residue **B** (**D** H-1, **B** C-3); and between C-1 (δ 100.6) of residue **D** and H-3 (δ 3.93) of residue **B** (**D** C-1, **B** H-3). The 3-position of residue **D** attached with acetyl group was confirmed by the presence of cross-peak **D** H-3, **D** OAc (carbonyl carbon). Finally cross-peak between H-1 (δ 5.04) of residue **E** and C-4 (δ 77.0) of residue **D** (**E** H-1, **D** C-4); and between C-1 (δ 103.5) of residue **E** and H-4 (δ 3.67) of residue **D** (**E** C-1, **D** H-4) were observed.

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




## *Chapter-IV*

Among different types of mushrooms of the genus *Agaricus*, *Agaricus blazei*, *Agaricus bisporus*, *Agaricus bitorquis* are reported as commonly available edible mushrooms and among them *A. blazei* and *A. bisporus* are reported to possess anti-tumor polysaccharides. But no work relating to polysaccharides of *A. bitorquis* is reported in literature. In West Bengal, India *A. bitorquis* is cultivated during the rainy season and local people consume them as delicious vegetable. The fruiting bodies of the mushroom were boiled with water for several hours. The solid residue was filtered and the liquid was then centrifuged. The supernatant was collected and precipitated with 1:10 (v/v) ethanol and kept overnight at 4 °C. The precipitated material was dissolved in water and reprecipitated with ethanol. Exhaustive dialysis of the precipitated material was carried out with DEAE cellulose bag to remove small carbohydrate molecules. It was then

freeze-dried to obtain crude polysaccharide. The crude polysaccharide (30 mg) was then purified by gel permeation chromatography. One homogeneous fraction (test tubes 24-36) was collected and freeze-dried, yielding 12 mg of material. The purification process was carried out in several lots. The pure polysaccharide (ABPS) has a specific rotation of $[\alpha]_D^{25}$ –31.6 (*c* 0.6, water) and the molecular weight of the polysaccharide was estimated ~$1.8\times10^5$ Da from a calibration curve prepared with a standard dextrans. The structure of the repeating unit of the polysaccharide was carried out on the basis of total hydrolysis, methylation analysis studies by GLC-MS analysis and then NMR studies ($^1$H, $^{13}$C, TOCSY, DQF-COSY, NOESY, ROESY, HMQC, and HMBC).

The polysaccharide was hydrolyzed with 2 M trifluoroacetic acid (TFA) and the alditol acetate on analysis through GLC using columns A (3% ECNSS-M) and B (1 % OV-225) indicate the presence of only glucose. The absolute configuration of glucose was determined as D by the method of Gerwig et al. The absorption at 900 $cm^{-1}$ in the IR spectrum indicates that ABPS has β-glucopyranosidic linkages. Now to find the mode of linkage the glucan was methylated using the method of Ciucanu and Kerek and then Purdie and Irvine followed by hydrolysis and conversion into alditol acetates. The alditol acetate was analyzed by GLC using columns A and B and GLC-MS using an HP-5 fused silica capillary column and found to contain 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol only. This indicates the presence of only (1→6)-linked D-glucopyranosyl moiety in the glucan.

The 500 MHz $^1$H NMR spectrum of PS at 27 $^o$C showed one anomeric signal at 4.50 ppm and the coupling constant values ($J_{H\text{-}1,H\text{-}2}$ ~ 8.5 Hz and $J_{H\text{-}1,C\text{-}1}$ ~ 160 Hz) suggested that it is β-linked. The proton chemical shifts from H-1 to H-6 were assigned from the DQF-COSY and TOCSY spectra.

The 125 MHz $^{13}$C spectrum of PS at 27 $^\circ$C exhibits one anomeric carbon signals at 103.4 ppm and this anomeric carbon signal was assigned for the β-linked residue and this assignment was also corroborated by HMQC experiment. All the carbon signals of the glucopyranoside residue were assigned with the help of HMQC spectrum. The carbon signals at 73.5, 76.0, 69.9, 75.3 and 69.2 ppm correspond to the C-2, C-3, C-4, C-5 and

C-6 respectively of the glucopyranoside residue. The C-6 signal of the glucopyranoside residue at 69.2 ppm is shifted to 7.4 ppm downfield compared to the standard methyl glycosides due to the α-glycosilation effect.

The sequence of glycosyl residues of the polysaccharide was confirmed from HMBC experiment. Long-range $^{13}C$-$^{1}H$ correlations were obtained from the HMBC spectrum. The cross peaks of both anomeric protons and carbons of each of the sugar moieties were examined and both inter and intra residual connectivities were observed from the HMBC experiment. For explanation of HMBC experiment two units of glucose (**A** and **A´**) are considered. Cross peaks were found between H-1 (4.50 ppm) of residue **A** and C-6 (103.4 ppm) of residue **A´** (**A** H-1, **A´** C-6) and vice versa and C-1 (103.4 ppm) of residue **A** and H-6a (4.20 ppm) and H-6b (3.84 ppm) of residue **A´** (**A** C-1, **A´** H-6a, **A** C-1, **A´** H-6b) and vice versa.

Thus the appearance of these cross peaks clearly supports the presence of the following repeating unit in the polysaccharide isolated from *Agaricus bitorquis*.

→6)-β-D-Glc*p*-(1→

## *Chapter-V*

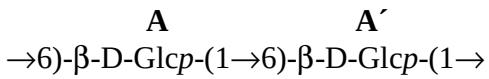
Mushrooms are nutritionally beneficial and especially, polysaccharides are important for possessing immunomodulatory and antitumor activity. Attempts are being made to produce new hybrid mushrooms by protoplast fusion or backcross mating of different mushroom strains to enhance their properties and the main object of this work is to compare the polysaccharide composition of this hybrid mushroom with their parents as well as other hybrid mushrooms. The present mushroom obtained through backcross mating between *PfloVv12* and *Volvariella volvacea*, is a temperature tolerant species. *PfloVv12* strain was initially prepared from the parent mushroom *Pleurotus florida* and *Volvariella volvacea*. The polysaccharide was isolated from the hot aqueous-extract of fresh fruit-bodies of hybrid mushroom. It yield 660 mg of crude water soluble polysaccharide and on fractionation of this water-soluble polysaccharide through

Sepharose 6B column three fractions were obtained (PS-I, 7mg, PS-II, 6mg and PS-III, 9.5mg). Separation was carried out in several lots, and each fraction was again purified through Sepharose 6B column to obtain only one peak. The molecular weight of PS-I was estimated as $1.88\times10^5$ Da, PS-II $1.32\times10^5$ Da, and PS-III $0.92\times10^5$ Da from a calibration curve prepared with standard dextran. The specific rotation of PS-I, PS-II and PS-III were determined as $[\alpha]_D$ -26.46 (c 0.08, water, 22 °C), $[\alpha]_D$ +21.95 (c 0.09, water, 21.8 °C) and $[\alpha]_D$ +35.45 (c 0.08, water, 22 °C) respectively.

**Characterization of PS-I:** PS-I on hydrolysis with 2M trifluoroacetic acid followed by alditol acetate preparation and analysis through GLC using column A (3 % ECNSS-M) and B (1 % OV-225) indicated the presence of only glucose. The absolute configuration of the sugar was determined by the Gerwig et al. and found that the glucose residues in PS-I have D configuration. The PS-I was then methylated by Ciucanu and Kerek, and then Purdie method followed by hydrolysis and alditol acetate preparation. The alditol acetates were then further analysed by GLC-MS using ZB-5MS capillary column. The PS-I showed the presence of only 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol. This result showed that in PS-I (1→6)-linked D-glucopyranosyl moiety was present.

The 500 MHz $^1$H NMR spectrum of PS-I at 27 °C showed one anomeric signal at 4.50 ppm and the 125 MHz $^{13}$C spectrum of PS-I at 27 °C also exhibits one anomeric carbon signals at 103.2 ppm. The coupling constant values ($J_{H\text{-}1,H\text{-}2}$ ~8.5Hz and $J_{H\text{-}1,C\text{-}1}$ ~160Hz) suggested that it was β-linked and the large $^3J_{2,3}$ and $^3J_{3,4}$ coupling constants (~9.0 Hz) indicated that it had *gluco* configuration. The proton chemical shifts from H-1 to H-6 were assigned from the DQF-COSY and TOCSY spectra. All the carbon signals of the glucopyranoside residue were assigned with the help of HMQC spectrum. All the carbon signals were nearly to the standard values except the C-6 signal (67.2 ppm) which showed 5.4 ppm downfield shift indicating that the glucose unit was linked at this position. Hence it was (1→6)-linked glucopyranosyl residue. The appearance of inter-residual contacts between H-1 of one residue and both H-6a and H-6b of another residue in NOESY spectrum also supported the (1→6)-linkage in the glucopyranosyl residue. The sequence of the glycosyl residues in the polysaccharide in PS-I was confirmed by the

HMBC experiment and to explain the HMBC results two glucose units were considered as **A** and **A´**. The presence of cross-peaks in the HMBC spectrum between H-1 of residue **A** and C-6 of residue **A´** and vice versa and cross-peaks between C-1 of residue **A** and H-6a and H-6b of residue **A´** and vice versa clearly supported the following repeating unit in the polysaccharide.




**Characterization of PS-II and PS-III:** Acid hydrolysis followed by GLC analysis of PS-II and PS-III showed the presence of glucose, galactose and mannose. The absolute configuration of glucose, galactose and manose in both PS-II and PS-III have only D configuration. The GLC-MS analysis of the alditol acetates of methylated polysaccharide of both PS-II and PS-III revealed the presence of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-mannitol, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-galactitol and 1,2,5,6-tetra-*O*-acetyl-3,4-di-*O*-methyl-D-glucitol in a molar ratio of nearly 1:1:1. These results indicated the presence of non-reducing end D-manopyranosyl, (1→6)-linked D-galactopyranosyl and (1→2,6)-linked D-glucopyranosyl moieties in both the polysaccharides. A further linkage confirmation was carried out by periodate oxidation experiment. The GLC analysis of the alditol acetate derived from periodate oxidation, reduction followed by methylation of the fractions showed no sugar peak. These results indicated that all the sugar moieties are consumed during periodate oxidation.

The 500 MHz $^{1}$H NMR spectrum of PS-II at 50 °C showed three signals in the anomeric region at 5.13, 4.99 and 4.80 ppm in a ratio of nearly 1:1:1. In the 125 MHz $^{13}$C-NMR spectrum at 50 °C three anomeric signals appeared at 102.1, 99.1 and 98.6 ppm. All the $^{1}$H and $^{13}$C signals were assigned using DQF-COSY, TOCSY, HMQC, and HMBC NMR experiments. Coupling constants were measured from DQF-COSY spectrum. The three sugar moieties were designated as residues **A**, **B**, and **C** according to their decreasing chemical shifts in the $^{1}$H NMR spectrum.

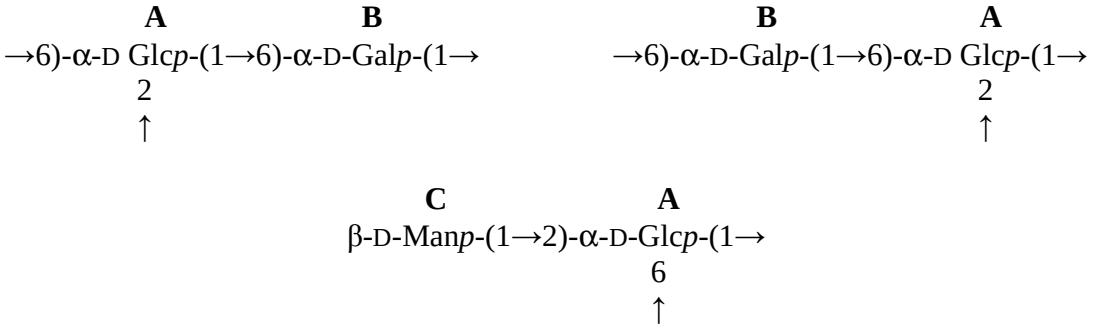
On the basis of proton and carbon chemical shifts and from proton-proton and C-1, H-1 coupling constants values all the sugar residues were assigned as follows;

Residue **A:** (1→2,6)-linked α-D-glucopyranose

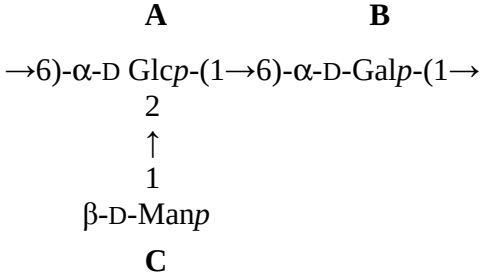
Residue **B:** (1→6)-linked α-D-galactopyranose

Residue **C:** nonreducing end β-D-mannopyranose

The sequences of glycosyl residues of PS-II were determined from NOESY as well as ROESY experiments and the following connectivities were observed;




Long-range $^{13}C–^{1}H$ correlation obtained from the HMBC spectrum corroborated the assigned repeating unit obtained from the NOESY experiment. From the HMBC experiment the cross-peaks of both anomeric protons and carbons of each of the sugar residues were examined, and intra- and inter-residual connectivities were assigned. Inter residual cross-peaks were observed between **A**H-1/**B**C-6; **A**C-1/**B**H-6a**;** **A**C-1/**B**H-6b; **B**H-1/**A**C-6; **B**C-1/**A**H-6a; **B**C-1/**A**H-6b; **C**H-1/**A**C-2; **C**C-1/**A**H-2. Thus, the appearance of these cross-peaks clearly supports the presence of the following repeating unit in the polysaccharide, PS-II;



In $^{1}H$ NMR (500 MHz) spectrum at 50 °C, PS-III showed three signals in the anomeric region at 5.12, 4.98 and 4.78 ppm in a ratio of nearly 1:1:1 and in the $^{13}C$-NMR spectrum (125 MHz) at 50 °C showed also three anomeric signals appeared at 102.0, 99.1 and 98.7 ppm. From the NOESY and HMBC experiments it was confirmed that the PS-III contains the same repeating unit as the PS-II.