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Characterization and lectin microarray of an immunomodulatory heteroglucan from *Pleurotus ostreatus* mycelia

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ABSTRACT

Glucans isolated from various mushroom and mycelia sources are interestingly being studied nowadays as a potent therapeutic agent. The present work was focused on the isolation, characterization and immunomodulatory study of a novel water soluble glucan from the pure mycelia of *Pleurotus ostreatus*. The extracted glucan was found to have a high molecular weight of $\sim 2.7 \times 10^6$ Da and mainly comprised of glucose, mannose and fucose in a ratio of 3:2:1 with both β and α linkages. Presence of terminal or interior glucose, mannose and fucose residues was also revealed using a high throughput miniaturized platform of lectin microarray. The heteroglucan folded into a triple helical conformation and exhibited enhanced immune cell activation and anti-tumor potential in tumor bearing mice model. Thus, potential biological functions incorporated in these glucan molecules acts in accord with its structural property and exploration of such structure–function relationship will unveil its diverse mechanism of action.

1. Introduction

Polysaccharides or glucans derived from mushroom species have been widely recognized as 'immunomodulators' (biological response modifiers, immunopotentiators and immunostimulants) as they are able to stimulate the host immune system and exert antitumor or antimicrobial activities along with other medicinal effects (Lindequist, Niedermeyer, & Julich, 2005; Wasser, 2002). The form of polysaccharides found to be associated in fungal cell walls mainly comprise of homo or heteroglucans having 1 → 3 linear β-glycosidic chain with 1 → 6 side branches or simple linear 1 → 3 α-glycosidic linkage (Wasser, 2002). It has been suggested that higher degree of structural variability and conformational complexity of these polymeric molecules may be responsible for potent host immune response generation (Bohn & BeMiller, 1995). As per literature, fungal derived polysaccharides are mostly isolated from mushroom fruit bodies or their mycelial forms as intracellular glucans with few exceptions of mycelia culture broth being used for exopolysaccharide isolation (Zhang, Cui, Cheung, & Wang, 2007). Interestingly, these carbohydrate molecules from various mushroom sources such as fruiting bodies of *Agaricus bisporus*, *A. brasiliensis*, *Phellinus linteus* and spores of *Ganoderma lucidum* served as potent immunostimulant (Kozarski et al., 2011). Till date, only six mushroom derived polysaccharides namely lentinan, schizophyllan, active hexose correlated compounds (AHCC), Maitake-D fraction, Polysaccharide K and Polysaccharide P from *Lentinus edodes*, *Schizophyllum commune*, mixture of mushroom, *Grifola frondosa* respectively, are commercially available as drugs (Lull, Wichers, & Savelkoul, 2005). This directs the need to further exploration of other mushroom and mycelia species for their immunomodulatory potential so as to enrich the glycobiological world for therapeutic purpose.

Commercially cultivated mushroom species of the genus *Pleurotus* has now gained immense importance due to its immunomodulatory, antimicrobial and antioxidative properties (Bobek & Galbavy, 2001; Shamtsyan et al., 2004). Extracts from the sclerotia and mycelia of *Pleurotus tuber-regium* has been shown to have anti tumor effects on S-180 in mice model as well as inhibit growth of HL-60 leukemia cancer cell line *in vitro* (Zhang, Zhang, Cheung, & Ooi, 2004). In another mushroom, α- and β-glucan from *Pleurotus pulmonarius* inhibited the viability of numerous colon cancer cell lines expressing varying quantities of galectin-3 *in vitro* (Lavi et al., 2010). *Pleurotus ostreatus*, an edible mushroom (commercially known as oyster mushroom) has been studied and found to have medicinal and potential prebiotic effects. Oyster mushroom glucans isolated by hot water and alkaline method

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were tested on nine probiotic strains of *Lactobacillus*, *Bifidobacterium* and *Enterococcus* and showed to stimulate growth of these colon microorganisms (Synytsya et al., 2009). Biological immune responses to glucans have been reported to be in part non-specific and determined by size, chemical structure, triple helical conformation, degree of side branching, solubility and gel forming ability (Bohn & BeMiller, 1995). However, the exact structure–function relationship still remains a dilemma and needs deeper investigation in the structural and cellular activity level.

P. ostreatus fruiting bodies have the probability of being highly susceptible to temperature and seasonal variations. Owing to limited supply and geographical diversity of these mushroom species, cultivation of its mycelial form in controlled conditions may thereby reduce structural variability caused by physiological entities. Therefore, studies based on immunomodulation and anti-cancer effects of glucans extracted from mycelia are supposed to have more reliable findings. Till date, our group has established mycelial culture of the fungus known as *P. ostreatus* by liquid broth culture fermentation and water-soluble proteoglycans isolated from the mycelia were elucidated for its structural and biological activities (Sarangi, Ghosh, Bhutia, Mallick, & Maiti, 2006). However, alkali soluble intracellular glucans from the mycelia of *P. ostreatus* have not been elucidated. Lectin microarray technology utilizing the lectin binding affinity of saccharides is an exquisite strategy to study the structural backbone of a particular glucan molecule. Lectins interact with carbohydrates mostly through hydrogen bonds, metal coordination, van der Waals and hydrophobic interactions (Weis & Drickamer, 1996). Generally, lectins bind to their target through multiple binding sites, which increases its specificity. The present work was focused on 2% KOH alkali based isolation of intracellular glucans from *P. ostreatus* mycelia, its characterization physiochemically as well as by lectin array method and determination of conformational and immunomodulatory property.

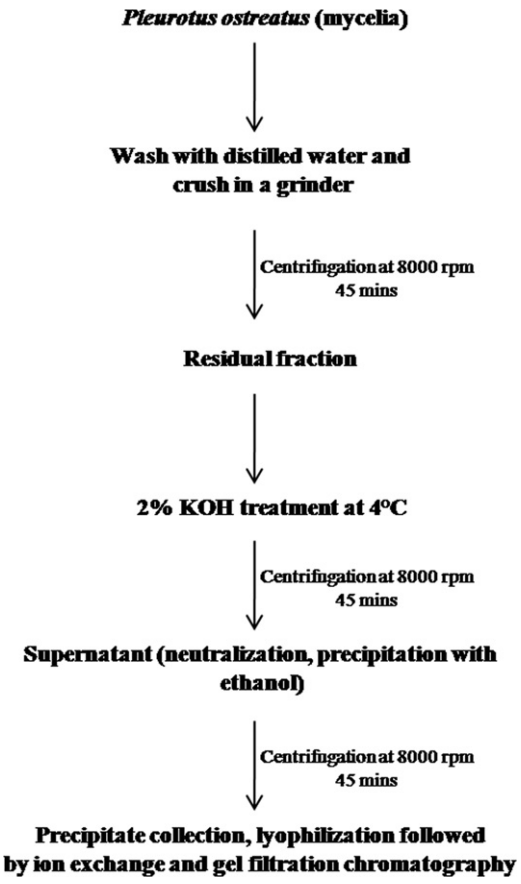

Fig. 1. Schematic representation of isolation and purification of intracellular glucan from *P. ostreatus* fungal mycelium.

2. Materials and methods

2.1. Mycelial culture conditions and biomass production

The mycelium of *P. ostreatus* was obtained as a kind gift from Rural Development Center, IIT Kharagpur. Mycelial cultures were maintained in broth potato dextrose media (24 g/l, pH 6.5) for large scale biomass production. Liquid media of about 125 ml was poured into each 500 ml conical flasks, sealed with cotton plug and autoclaved in 1.5 atmospheric pressure and 121 °C for 15 min. Solid mycelia cultures of size 2–3 cm^2 were then cut in sterile conditions, dispensed in each culture flask and maintained as static culture in an incubator at 25 °C for 21 days in dark. Mycelia bodies collected after 21 days of post seeding were stored in frozen conditions until processed. Mycelia were harvested in stationary phase of growth to obtain maximum carbohydrate yield.

2.2. Isolation and purification of polysaccharide

Intracellular polysaccharide was extracted from the mycelia of *P. ostreatus* as described by Mallick, Maiti, Bhutia, and Maiti (2010) with few modifications. Approximately 1–1.5 kg of mycelial biomass was washed with distilled water and crushed in a grinder. The latter was centrifuged at 8000 rpm for 45 min and the residual fraction processed for 2% KOH based polysaccharide isolation. Polysaccharide obtained after alkali treatment overnight was finally neutralized and ethanol precipitated to get a dry pellet which was initially lyophilized and later resuspended in 20 mM Tris buffer (pH 8.0). At the end of this preliminary isolation, the alkali extracted polysaccharide was further purified by passing through DEAE-Sephadex (Sigma) ion-exchange chromatography column (100 mm × 10 mm) at 0.5 ml/min flow rate. The flow through of the ion-exchange column was collected as the purified glucan and dialyzed against de-ionized water using 12 kDa cut off membrane (Sigma Aldrich). The sample was then concentrated by lyophilization to obtain a white powdery substance and stored at −20 °C for experimental use. Schematic representation of the isolation procedure has been described in Fig. 1. Approximately 1.50 g pellet/kg mycelial biomass was obtained in this procedure. The overall yield of the purified polysaccharide after anion-exchange chromatography was 30% of the dried pellet obtained after alkali extraction.

Carbohydrate and protein content of the purified glucan was determined by spectrophotometric measurement using phenol-sulphuric acid (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) and Lowry's (Lowry, Rosebrough, Farr, & Randall, 1951) method respectively. Very minute level of protein was detected in the purified sample with carbohydrate content of the polysaccharide retaining greater than 90–95% in repeated isolation. The polysaccharide from the mycelia was found to be water soluble in nature.

2.3. Molecular weight determination by gel permeation chromatography

The molecular weight of the polysaccharide was determined on a Sepharose CL-6B column (12 mm × 500 mm; column volume 55 ml) attached to an automated AKTA prime plus system which monitors the flow rate of the equilibrating column buffer as well as sample buffer and is equipped with a fraction collector. The buffer used for column equilibration was 20 mM Tris buffer with 150 mM NaCl at pH 8.0 and samples for injection to the column was

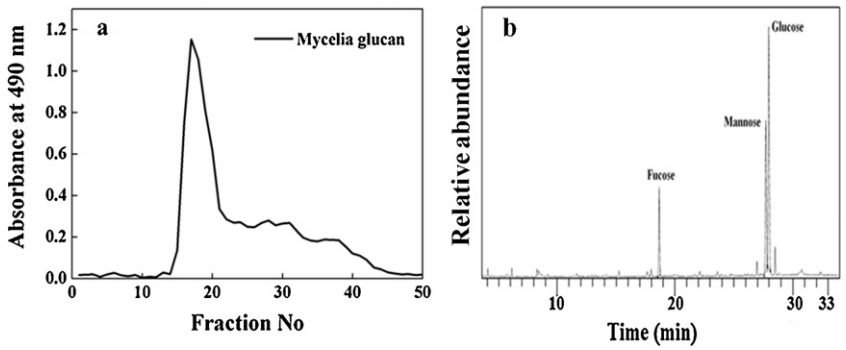

Fig. 2. (a) Gel permeation chromatography of alkali extracted glucan. (b) Sugar composition of isolated glucan from *P. ostreatus* mycelia determined by gas liquid chromatography (GLC) technique.

suspended in the same equilibrating buffer. Buffer solutions used in gel permeation columns was filtered through a 0.22 μm filter (Millipore) and degassed prior to use. The polysaccharide sample (10 mg/ml) was injected into the column along with buffer solution at a flow rate of 0.4 ml/min and 1 ml fractions collected was checked for carbohydrate content by phenol sulphuric acid method. Molecular weight of the polysaccharide or glucan was estimated from standard reference curve plotted using known molecular weight dextrans such as Blue dextran (2000 kDa) and FITC-Dextrans of 250, 70 and 10 kDa (Sigma Aldrich). (Dong & Fang, 2001; Farina, Sineriz, Molina, & Perotti, 2001). Fractions were collected and phenol-sulphuric acid test results showed a single symmetrical peak indicating that the glucan was homogenous in nature and with a high molecular weight of $\sim 2.7 \times 10^6$ Da respectively (Fig. 2a).

2.4. Chemical characterization

Monosaccharide composition was determined by hydrolyzing the polysaccharides with 2 M CF_3COOH (2 mL) at 100 °C for 18 h, followed by reduction with sodium borohydride (9 mg). Acidification was then performed with dilute CH_3COOH, and thereafter co-distilled with pure CH_3OH to remove excess boric acid. Acetylation of the reduced polysaccharide was carried out with 1:1 pyridine-acetic anhydride in a boiling water bath for 1 h and the final derivatized alditol acetate was analyzed by gas liquid chromatography, GLC (Hewlett–Packard Model 5730 A) equipped with HP-5-fused silica capillary column, a flame ionization detector and glass columns (1.8 mm × 6 mm) (Rout, Mondal, Chakraborty, Pramanik, & Islam, 2005). Program was run in an isothermal state at 150 °C with a hold time of 2 min and a temperature gradient of 4 °C/min up to a final temperature of 200 °C.

Specific optical rotation (identification cell = 20, H_2O, $c = 0.1\%$, $\lambda = 589$ nm) of the polysaccharide sample was measured in an Anton paar MCP.200 Polarimeter at 25 ± 2 °C. Alkaline extracted polysaccharide was also characterized using a Fourier Transform Infra-Red Spectrophotometer (Perkin-Elmer Instruments, Spectrum One FT-IR Spectrometer). The dried polymer was grinded with Potassium bromide (KBr) powder at a ratio of 1:50 and pressed into pellets of halide salt or disc for FT-IR spectral measurement in the frequency range of 4000–400 cm^{-1}. For ^{13}C NMR study, polysaccharide (15 mg/ml) was dissolved in D_2O (0.5 ml) and centrifuged to remove undissolved particles. All spectra were recorded in a Bruker ACF400 NMR spectrometer.

2.5. Lectin microarray technology

Lectins which are recognized as specific carbohydrate binding proteins may be utilized as an important tool in elucidating the structure of labeled polysaccharides in solution. A stable carbohydrate labeling method is derivatization with anthranilic acid which finally gives a fluorescence property to the compound (emission wavelength at 410 nm). Anthranilic acid (30 mg/ml) was dissolved in 4% sodium acetate trihydrate (w/v) and boric acid (2% w/v) solution of methanol. Solid sodium cyanoborohydride was then added to the above mixture at a final concentration of 45 mg/ml to give the final labeling mixture. 200 μl of labeling mixture was then added to dry polysaccharide (2 mg), mixed thoroughly and heated constantly at 60 °C for atleast 3 h. Derivatized sample was cooled to room temperature and about 1 ml of acetonitrile/water (97:3, v/v) solution added. The final sample was vortexed and centrifuged two times to wash unbound anthranilic acid. Pellet obtained was later dissolved in phosphate buffer saline (PBS) and filtered to obtain a clear dissolved form of the labeled polysaccharide (Neville et al., 2004).

Lectin microarray based sugar analysis was performed on functionalized slides as described by Roy, Das, Maiti, and Chakraborty (2011). Slides were treated with piranha solution (H_2SO_4: H_2O_2 = 1:1 by volume) and amino-propyltrimethoxysilane (APTS) for surface oxidation and amine modification which was then further coated with glutaraldehyde to allow Schiff's base formation between aldehyde and amine group of lectins. Microarray fabrication was performed with the method reported by Park and Shin (2002). Five lectins namely Con A, PSA, UEA, AGA and HPA dissolved in 10 mM PBS (1 mg/ml) with an additional 10% glycerol solution (for enhancing the solution viscosity) served as the spotting lectin sample. Spotting of lectin solutions in functionalized slides were done in Omnigrid Microarray spotter (Genomic Solution, USA) with a minimum spot spacing of 270 μm. The final spotted slides were dried overnight in vacuum for 18–20 h. Each individual spot carried a volume of 0.6 nL. Microchannel molds made from a mixture of PDMS base to crosslinker (Sylgard 184, Dow Corning) in the ratio of 10:1 w/w after heating were then blended upon the lectin spotted slides. Anthranilic acid labeled polysaccharide solution was allowed to pass through the microchannels to allow the hybridization of specific carbohydrates recognized by the individual lectin spots. Mass transport and fluid flow was monitored using a syringe pump (PHD-22/2000, Harvard Bioscience, USA) at 50 μl/min rate for about 10 min followed by washing with ultrapure water to remove unbound samples. Hybridized molecules were detected by fluorescence imaging of microarray spots in an epifluorescence microscope (Olympus IX71) equipped with respective optical filters and a charge-coupled camera. Captured images were analyzed for intensity measurement using Matlab software. Degree of microspot intensity may be correlated with the percent composition of sugar residues in the isolated glucan. A dendrogram depicting the degree of relatedness of the hybridized sample with the lectin spot was also plotted using Cluster 3.0 and Java Tree View software.

2.6. Conformational analysis and particle size determination

Intrinsic viscosity [η] of polysaccharide (1 mg/ml) suspended in increasing NaOH concentration of 0–0.5 M was measured with a Vibro Viscometer SV 1A (A&D Company Limited, Japan) at 25 ± 0.5 °C. The kinetic energy correction was negligible. Huggins and Kraemer's equations were applied to estimate the intrinsic viscosity by extrapolating the regression line of the reduced viscosity *versus* concentration to zero value as follows:

$$\frac{\eta_{sp}}{c} = [\eta] + k'[\eta^2]c \quad \text{(i)}$$

$$\frac{\ln \eta_r}{c} = [\eta] + k''[\eta^2]c \quad \text{(ii)}$$

where k' and k'' are constants for a given sample at a particular condition in a given solvent; η_{sp}/c, the reduced specific viscosity; $(\ln \eta_r)/c$, the inherent viscosity and c is the concentration (Wang et al., 2004).

Helical and random coil conformity of polysaccharides were examined using congo red dye binding assay in increasing alkaline (Ogawa, Watanabe, Tsurugi, & Ono, 1972) and urea (Mao, Hsu, & Hwang, 2007) conditions. In brief, polysaccharide (1 mg/ml) was dissolved with varying concentrations of NaOH (0–0.5 M) in a stepwise increment of 0.05 M containing 24.4 μM of congo red solution. The absorption spectrum of the solutions was recorded in a Thermo Scientific Double beam UV Vis Spectrophotometer (Spectrascan UV 2700) from 400 to 700 nm range at room temperature. As control, the absorption wavelength of congo red solution alone in different NaOH concentrations were measured. Similarly, shift in λ_{max} value of polysaccharide from 0 to 5 M urea concentrations was also recorded.

The hydrodynamic size of polysaccharide molecule was estimated with a Brookhaven 90 plus particle size analyzer. Sample (1 mg/ml) was dissolved in ultrapure water and particle size recorded with a laser light scattering angle $\theta = 90^\circ$ at 27 °C placing the suspension in a polystyrene cuvette.

2.7. Immunomodulatory and cytotoxicity assays

2.7.1. Peritoneal macrophage isolation

Primary mouse peritoneal macrophages were isolated aseptically from the peritoneal cavity of Swiss albino mice, weighing 19–21 g and maintained in sterile conditions in the laboratory. The cells were grown in RPMI (Roswell Park Memorial Institute) medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml antibiotic (penicillin and streptomycin) and cultured at 37 °C in a humidified 5% CO_2 incubator.

2.7.2. Assay for nitric oxide production

Nitric oxide (NO) production was measured on peritoneal macrophages to determine the *in vitro* activation of stimulated cells. Briefly, isolated macrophages were adjusted to a density of 1×10^6 cells/ml of which about 180 μl was plated in a 96 well plate. Cells were allowed to adhere for few hours and positive control (LPS) as well as different concentrations of glucan treatment (10, 20, 50, 100, and 200 μg/ml) added to the respective wells. All glucan samples for testing have been passed through polymyxin B agarose column to ensure endotoxin free treatment. Treated cells were then incubated for 48 h at 37 °C in a 5% CO_2 incubator and NO production assayed by estimating the nitrite concentration in the cell supernatant with Griess reagent (1% sulfanilamide in 2.5% phosphoric acid, 0.1% napthylethyldiamine dihydrochloride in 2.5% phosphoric acid). Equal volumes of isolated supernatants and Griess reagent were mixed and incubated at room temperature for 10 min, following which absorbance was recorded at 540 nm (Sarangi et al., 2006).

2.7.3. Antitumor and immune enhancing assay

Swiss albino mice (20–24 g) used for *in vivo* studies were housed in proper light and dark conditions with nutritive food and water *ad labium*. Room temperature was maintained at 22 ± 2 °C. Ascites of Sarcoma 180 (S-180) were drawn aseptically from the peritoneal cavity of tumor bearing mice and washed thoroughly with phosphate buffer saline (PBS). Viability of the tumor cells was greater than 95% as evaluated by trypan blue exclusion method. Cells were counted, diluted with PBS and then injected subcutaneously (s.c.) into the left forelimb of experimental mice (1×10^6 cells/mice) for solid tumor formation. On tumor development, mice were finally divided into three groups consisting of six mice each. Treatment was administered at a dose of 10 and 20 mg/kg intraperitoneally on every alternate day for a period of 10 days. Volume of sample injected was 0.1 ml/20 g body weight. Model control groups received equal volume of PBS. Mice were weighed and sacrificed on the 11th day of treatment and solid tumors isolated and weighed. Tumor inhibition was examined using the following formula:

$$\text{Inhibitory percent}(\%) = \frac{T_{wt}C - T_{wt}T}{T_{wt}C} \times 100$$

where $T_{wt}C$ = tumor weight of control group and $T_{wt}T$ = tumor weight of treated group.

Lymphocyte proliferation of treated and control groups were assayed to examine the immunostimulatory property of glucan. Briefly, single cell suspension of splenocytes were isolated from all groups of mice in sterile conditions and contaminating RBCs were removed with a hypotonic RBC lysis buffer, washed with sterile PBS three times and resuspended in complete RPMI medium. Cells were counted and seeded at a desired concentration of 1×10^6 cells/ml in 96 well plates (180 μl/well) and incubated with or without LPS (2 μg/ml) and Con A (10 μg/ml). Cultures were set up in triplicates and maintained for 72 h at 37 °C in a humidified 5% CO_2 incubator. Proliferation was evaluated by standard MTT method using an ELISA plate reader at an absorbance of 595 nm (Mosmann, 1983) and presented as lymphocyte proliferation index (LPI).

2.8. Statistical analysis

All statistical data were represented as mean ± standard deviation (SD) with significance level evaluated using *t*-test statistics. *P*-value < 0.05 was considered to be of significant level.

3. Results and discussion

3.1. Compositional analysis by GLC

The monosaccharide composition of intracellular polysaccharide isolated from *P. ostreatus* mycelia was determined by gas–liquid chromatography (GLC) technique. Peaks obtained in the chromatogram revealed that the polysaccharide molecule is composed of major glucose units (50.54%), with minor proportions being taken by other sugar residues such as mannose (31.22%) and fucose (16.58%). The molar ratio of the individual sugars in the polysaccharide *i.e.*, glucose, mannose and fucose are therefore nearly 3:2:1, thereby indicating the molecule to be a heteroglucan (Fig. 2b).

3.2. Lectin microarray based characterization

Lectin microarray based interpretation of polysaccharide backbone may be considered as a new technique utilizing one biological data for the characterization of another unknown biological sample. Plant lectins are widely known for their specific sugar moiety binding specificities. The binding affinity of lectins remains higher for the terminal and hanging sugar moieties rather than masked

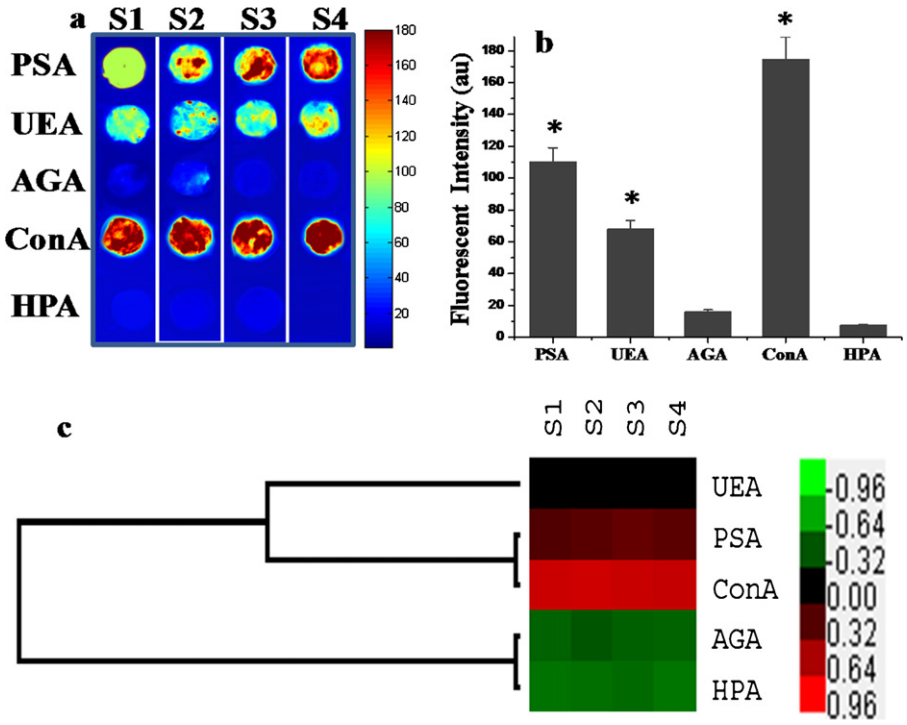

Fig. 3. Microarray based compositional analysis of glucan isolated from *P. ostreatus* mycelia. (a) Lectin–glucan hybridized microspots with intensity scale bar. (b) Fluorescence intensity (absorbance unit) bar plotted using Matlab software for the different hybridized lectin spots. $^{*}P < 0.05$. (c) Dendrogram of the hybridized arrays depicting the binding affinity of the heteroglucan into the pockets of the respective lectins. S1, S2, S3 and S4 represents replicates of the same glucan sample.

residues due to their higher availability (Ramos et al., 2001). Exposed sugars bind strongly while the interior sugar residues form bonds with lesser specificity to its corresponding lectins, but still overall contribute to the binding affinity. Fluorescence intensity obtained after hybridization of anthranilic acid labeled glucan to the respective immobilized lectin arrays was thus measured (Fig. 3a). Con A (*Canavalia ensiformis*) unanimously binds to any form of glucose and mannose while PSA (*Pisum sativum*) has higher affinity for the alpha form of the same sugar moieties. This was evidenced by the lesser fluorescence intensity in the PSA spots with respect to the high level intensity of Con A. The average intensity in UEA (*Ulex europaeus*) correlated with the presence of fucose sugar residue. No signal was obtained with lectins such as HPA (*Helix pometia*) and AGA (*Abrus precatorius*) affirming the absence of N-acetylgalactosamine and galactose moieties. Fluorescence intensity measurements using Matlab software approximately anticipated the percentage of individual sugar units present in the glucan molecule (Fig. 3b). Dendrogram of the hybridized array analyzed using Cluster 3.0 and Java tree also predicted a close linkage of the array between Con A and PSA, confirming the presence of a high amount of glucose and mannose followed by fucose as depicted by its close association with UEA (Fig. 3c). Distant linkage with AGA and HPA inferred the absence of the respective specific sugar moieties. Microarray based substantiation may therefore be considered to stand at par with other physical or chemical characterization methods of biopolymers and may be pertinent to provide cues of the diverse carbohydrate polymeric world.

3.3. Linkage elucidation from spectral analysis

Fourier transform infrared spectroscopic data has found intense use as a convenient method of identifying the functional groups associated with polysaccharide characterization. An intense band at 3404 cm^{-1} corresponds to the stretching vibration of hydroxyl groups of the alkaline extracted glucan as shown in Fig. 4a. The band at 2928 cm^{-1} is characteristic of C—H stretching vibration and the absorption peak at 1656 cm^{-1} attributes to bound water. Absorbance in the region of 1200–950 cm^{-1} is characteristic of every specific polysaccharide and peak positions determine their probable fingerprint (Mathlouthi & Koenig, 1986). Bands in the region of 1042 and 1074 cm^{-1} represents glucan moiety with C—O and C—C stretching vibrations in pyranoid rings thereby identifying a β-configuration of the sugar units with (1 → 3) and/or (1 → 6) linkage (Sandula, Kogan, Kacurakova, & Machova, 1999). Additionally, presence of a spectral band at 896 cm^{-1} is confirmative of the presence of β-glycosidic linkage in the molecule. Weak and low spectral bands around 856 cm^{-1} also suggest the probability of α-glycosidic linkage in the purified glucan at a meager ratio (Barker, Bourne, Stacey, & Whiffen, 1954). The IR spectral data thus suggest that the isolated polysaccharide heavily constitute of β-glucan linkages along with few α-linkages. A low positive specific optical rotation of about $[\alpha] = \pm 25^{\circ}$ ($c = 0.1, H_2O$) also additionally indicated the major presence of β-configuration in the sugar linkages.

The ^{13}C NMR spectrum (Fig. 4b) of the dissolved mycelia glucan recorded in D_2O at 27 °C showed anomeric carbon peak at 103.1 which was characteristic of β-linkage. A lower resonance signal at 99.1 ppm was indicative of an α-linked sugar pyranosyl moiety, suggesting that the repeating unit of the glucan mostly constituted of β-linkage along with α-linkage in a minor ratio (Bhunia et al., 2012; Mandal et al., 2011). Branching at C6 was confirmed by resonance signals at 68.7–69.5 ppm while a broad peak at 86.2 could be ascribed to C3 indicating the presence of β-1,3 and 1,6-linked sugar residues. The appearance of a signal at 16.5 ppm also strongly corresponded to L-fucopyranosyl residue in the heteroglucan chain (Mandal et al., 2011). The results obtained from ^{13}C NMR thus showed similar pattern of description as revealed by FT-IR analysis. Glucans with β-1,3 and 1,6 linkages are often considered to be biologically more desirable from medicinal perspective.

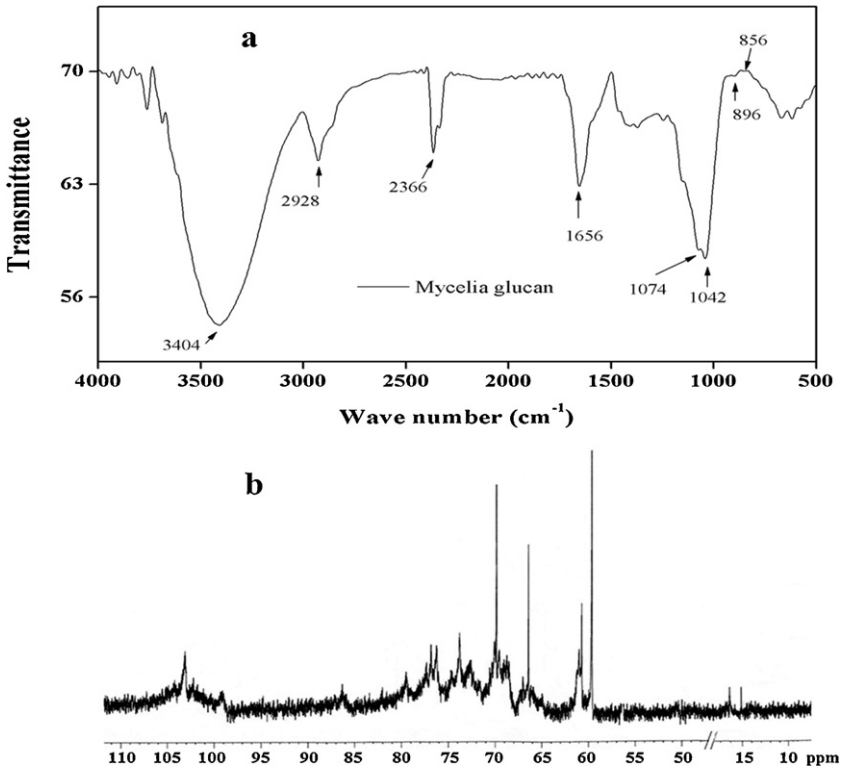

Fig. 4. (a) FT-IR and (b) ^{13}C NMR spectroscopy of *P. ostreatus* mycelia derived intracellular glucan.

3.4. Conformational studies in solution

Viscosity measurements were done to analyze higher order conformation of carbohydrate molecules which gets denatured in higher alkaline conditions (Wang et al., 2004). With an increase in NaOH concentration, inter and intra molecular hydrogen bonds present in the glucan starts breaking down resulting in a lower order conformity of the sample with less viscous nature. Fig. 5a shows the intrinsic viscosity against NaOH concentration plot of the glucan suggesting a gradual degradation of the highly ordered structure in increasing alkaline conditions with complete denaturation obtained at a concentration of 0.2 M.

Hydrodynamic diameter and poly dispersity index (PDI) of the heteroglucan molecule was determined in a particle size DLS instrument. It was found that the effective diameter of the compound in aqueous solution (1 mg/ml) was 105.2 nm with a PDI of 0.231 (Fig. 5b) indicating that the sample was homogenously distributed (PDI about 1 is referred to monodisperse molecules). Particle size and its distribution may be suggested to be related to its degree of biological activity as cells respond distinctly on recognition of an effective amount of any stimulatory molecule.

Interaction of the glucan with congo red at various alkaline concentrations (0–0.5 M) induces shift in the absorption maxima due to conformational deformity of the molecule at denaturing conditions (Mao et al., 2007). Chain conformation study using congo red concludes that the glucan molecule confronts to a helical order on binding to congo red which then degrades to random coils at 0.2 M NaOH concentration and retains the coil structure even at higher alkaline conditions (Fig. 5c). Similarly, urea also interacts with polar groups of biomolecules as well as promotes interaction of water with biopolymers by breaking intermolecular hydrogen bonds (Mao et al., 2007). In presence of urea (0–5 M), glucan conformation changes from a triple helical form to random coils rendering it highly water soluble. λ_{max} value increases with urea concentration in case of congo red solution but decreases in congo red glucan complex (Fig. 5d) thereby suggesting that the triple helical form of the polysaccharide is denatured due to the presence of urea. Helical conformity is one of the exquisite parameters in polysaccharide biology which is considered to be associated with its biological function such as immunomodulation.

3.5. NO production in peritoneal macrophage cells

Nitric oxide (NO) produced by macrophages has been known as potent effector molecules which target tumor cells for destruction. Hype in NO release by immune activating compounds signifies the immunostimulating potential of that sample. The amount of NO produced is determined by addition of Griess reagent. Fig. 6a demonstrates that the ability of glucan to stimulate peritoneal macrophages with respect to NO production increased in a dose-dependent manner. Maximum release was observed in 100 and 200 μg/ml concentration with 23.9 and 27.1 μM of NO produced respectively.

3.6. In vivo tumor inhibition and immunomodulation in treated tumor-bearing mice

The advent of polysaccharides as immunoenhancers has contributed largely in understanding its diverse biological applicability which may be extrapolated to therapeutical purposes. Cancer conditions are known to evade immune surveillance and thereby decline immune functions. Stimulation of the immune defense system in such conditions by immunomodulatory molecules serves as a prerequisite to counteract such disease conditions. In this context the isolated intracellular glucan from *P. ostreatus* fungal mycelia have been found to induce tumor inhibition by enhancing the immune cells function. Experiments performed in mice solid tumor model Sarcoma 180 (S-180) have shown that intraperitoneal treatment with different doses of the glucan in S-180 bearing mice reduced tumor growth. It was observed that about 37–51% tumor

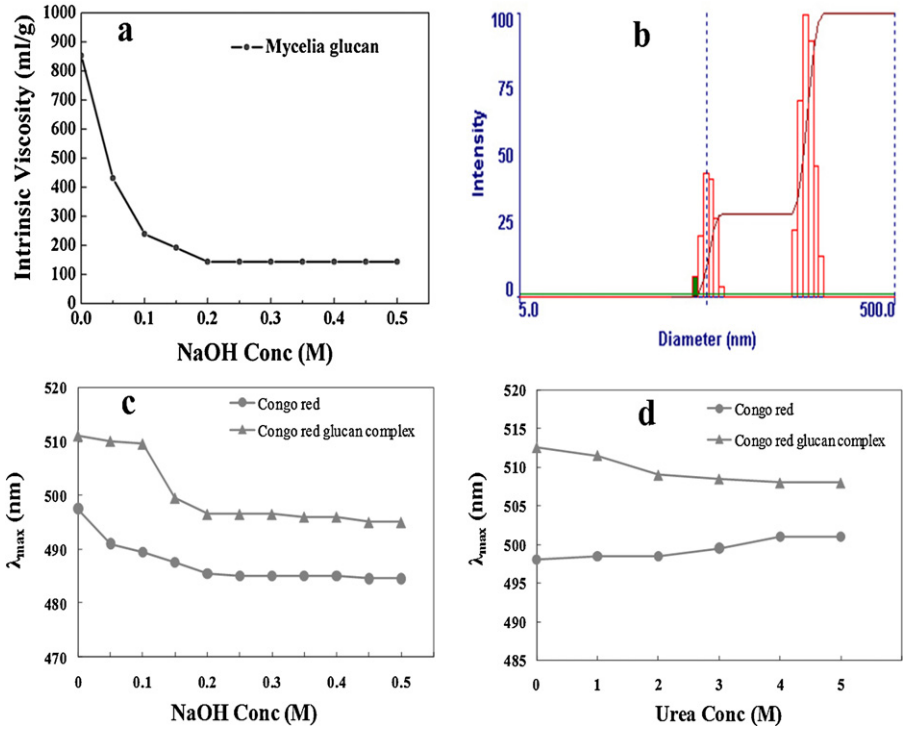

Fig. 5. (a) Gradual decrease in intrinsic viscosity [η], with an increase in the NaOH concentration of the heteroglucan solution (1 mg/ml) at 25 ± 0.5 °C. (b) Hydrodynamic diameter and PDI of the glucan molecule in aqueous solution (1 mg/ml). (c) Conformational analysis of the sample using congo red in alkaline and (d) urea concentrations with respect to shift in absorbance.

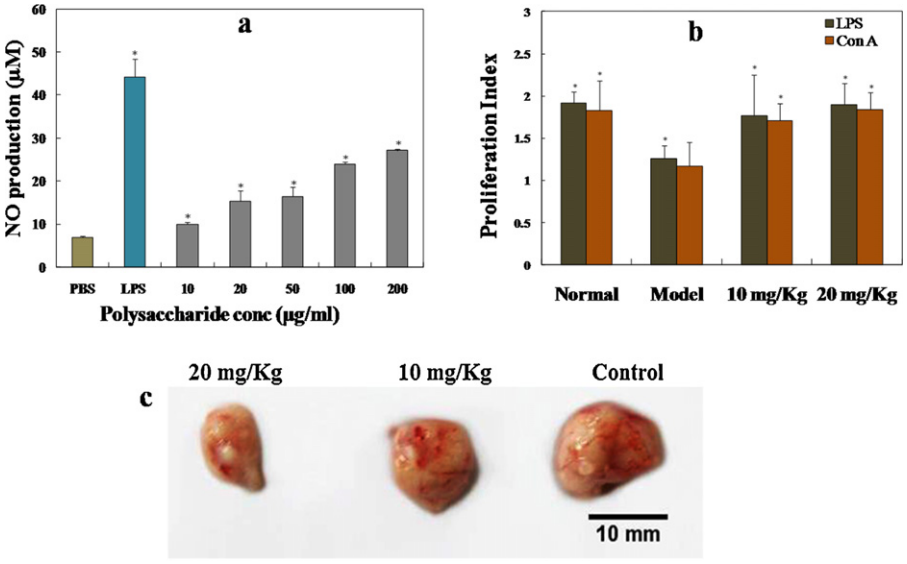

Fig. 6. (a) Nitric oxide (NO) production in peritoneal macrophages stimulated with different concentrations of the glucan. (b) Lymphocyte proliferation index of treated and untreated sarcoma 180 bearing mice groups on stimulation with mitogens such as LPS and Con A. Values are presented as mean ± SD of 10 different observations and significance level evaluated by comparing with the negative control using Student's *t*-test statistics: $^{*}P < 0.05$. (c) Decrease in tumor weight and size on treatment with different doses of the immunomodulatory polysaccharide.

Table 1
Tumor inhibitory effects due to glucan treatment on *in vivo* Sarcoma 180 mice tumor model.

Group	Polysaccharide (dose in mg/kg)	Tumor weight (in g)[a]	% Tumor inhibition	Body weight gain (%)
Control	–	0.92 ± 0.03	0.0	7
Treatment	10	0.58 ± 0.08	37.30[b]	4.4
Treatment	20	0.44 ± 0.04	51.89[b]	3

[a] Values are represented as mean ± SD ($n = 10$).
[b] Significance level from control: $P < 0.05$.

inhibition was caused on treatment with 10 and 20 mg/kg polysaccharide treatment with respect to the control (Table 1 and Fig. 6c). No direct cytotoxic effect on Sarcoma 180 cells was observed on glucan treatment in the *in vitro* system (data not shown) indicating that antitumor effect was immune system mediated. Body weight gain was slightly lesser in treated mice groups compared to the untreated group satisfying that polysaccharide treatment led to tumor reduction without causing much toxicity or side-effect to the animals. Tumor inhibition rate was thereby dose-dependent with higher dosage of 20 mg/kg treatment showing more beneficial effects than lesser dosage.

Proliferation assay of lymphocytes obtained from spleen of treated and untreated mice groups revealed the immunostimulatory potential of *P. ostreatus* mycelia derived heteroglucan. On stimulation with mitogens such as LPS and Con A, hype in immune cell proliferation was observed on polysaccharide treated groups with respect to the model control. Reduced immune cell proliferation in control may be subjected to decrease in immune surveillance which is a hallmark of cancer. However, immunomodulatory biomacromolecules such as glucan from mycelia sources may serve as a therapeutic agent in such cases thereby enhancing the surveillance mechanism and contributing to tumor inhibition. On treatment with glucan at 10 and 20 mg/kg to S-180 bearing mice, proliferation index (PI) of lymphocytes increased up to 1.77 and 1.9 on LPS stimulation with control group showing only 1.26 proliferations. Similarly Con A treatment also increased the proliferation index from 1.17 of control to 1.71 and 1.84 for 10 and 20 mg/kg treatment groups which was nearly equivalent to the proliferation index of normal healthy mice (Fig. 6b). This suggests the potential of immunomodulatory glucans in future therapeutic purposes with deeper clinical investigations.

4. Conclusion

Intracellular glucans isolated from *P. ostreatus* mycelia was composed of glucose, mannose and fucose with β and α-linkages and folded into a triple helical organization. Presence of glucose, mannose and fucose in the terminals or interior of the glucan molecule was also revealed by lectin microarray analysis suggesting the futuristic application of such lectin affinity in the structural determination of biomolecules. These polymer type molecules were also explicitly involved in enhancing immunoactivation to target tumor cell destruction. It may be thereby summoned that the functional property of the heteroglucan may be closely linked to its structural entities.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carbpol.2013.02.017.

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