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Structural, immunological, and antioxidant studies of β-glucan from edible mushroom *Entoloma lividoalbum*

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ABSTRACT

A water soluble β-glucan having molecular weight ~2×10^5 Da was isolated from hot water extract of the fruit bodies of an edible mushroom *Entoloma lividoalbum* (Kühner & Romagn) Kubička. This polysaccharide (ELPS) contains (1 → 3,6)-β-D-Glc*p*, (1 → 3)-β-D-Glc*p*, (1 → 6)-β-D-Glc*p*, and terminal β-D-Glc*p* moieties in a molar ratio of nearly 1:1:3:1. Chemical and spectroscopic analysis showed that the backbone of glucan consists of three (1 → 6)-β-D-glucopyranosyl and two (1 → 3)-β-D-glucopyranosyl residues, out of which one (1 → 3)-β-D-glucopyranosyl moiety was branched at *O*-6 with a terminal β-D-glucopyranosyl residue. This β-glucan exhibited macrophage, splenocyte, and thymocyte stimulations. It possesses promising antioxidant activities as evidenced from its hydroxyl and superoxide radical scavenging activities and reducing properties.

1. Introduction

Mushroom polysaccharides are potent renewable source for the development of several drugs. PSK from *Coriolus (Trametes) versicolor* (Cui & Chisti, 2003), lentinan from *Lentinus edodes* (Taguchi et al., 1983) and sonifilan (SPG) from *Schizophyllum commune* (Fujimoto et al., 1983) have been recognized as anticancer drugs throughout the world (Moradali, Mostafavi, Ghods, & Hedjaroude, 2007). Mushroom polysaccharides have drawn the attention of chemists and immunobiologists for their immunomodulating (Wasser & Weis, 1999), antioxidant (Maity, Samanta, et al., 2014; Patra et al., 2013), and antitumor activities (Wasser, 2002). Mushrooms have a long traditional history in curing various life threatening diseases. Polysaccharides derived from these medicinal mushrooms have been explored for several years and found to possess immunomodulatory and anticancer properties. The anticancer activity of these polysaccharides is mediated mostly through the activation of immune cells such as B cells, T cells, macrophages and NK cells (Nandi et al., 2013; Wasser, 2002). It has been reported that *in vivo* administration of β-glucans (Kogan, 2000) can enhance immune reactions and up regulate the resistance of host against tumor cells. Furthermore, administration of glucans to macrophages activated by LPS can lead to increased production of cytokines like interleukin-1 and TNF-α which subsequently induce lymphocyte differentiation and proliferation to enhance immune responses (Adachi, Okazaki, Ohno, & Yadomae, 1994; Chihara, 1992). It is also noteworthy to mention the antioxidant activity of the polysaccharide which depends on monosaccharide composition and their different arrangements during polymerization (Tsiapali et al., 2001). Antioxidants prevent the chain reactions by hydrogen donation and interrupting the process of oxidation. As a result, stable free radicals cannot survive or propagate further oxidation (Aruoma, 1999; Wade, Jackson, Highton, & Van Rij, 1987). Different types of reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide and hydroxyl radicals are closely involved in various human diseases, such as cerebral ischemia, diabetes, alzheimer, inflammation, rheumatoid arthritis, atherosclerosis and cancer, as well as aging processes (Halliwell & Gutteridge, 1989). Antioxidants play key role to prevent the generation of ROS or scavenge them and minimize oxidative tissue damage. Hence, ROS induced oxidative cell damage can be prevented by supplementation of naturally occurring biomolecules like polysaccharide, which is one of the most acceptable techniques for modern therapy.

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Edible and non-toxic mushroom *Entoloma lividoalbum* (Kühner & Romagn.) Kubička normally grows on Sikkim Himalayan region, India during July to August (Das, 2010). It was reported that this mushroom contains minerals and nutrients such as calcium, aluminum and iron (Maity, Samanta, et al., 2014). The antimicrobial activity (Rai, Sen, & Acharya, 2013), antioxidant properties (Maity, Samanta, et al., 2014), and protective role in human lymphocytes (Maity, Nandi, et al., 2014) of *E. lividoalbum* were reported. From these points of views *E. lividoalbum* may therefore be useful as a medicinal fungus with various immunostimulating and other protective effects. Two water soluble polysaccharides, PS-I (Maity, Samanta, et al., 2014) and PS-II (Maity, Nandi, et al., 2014) were isolated from the alkaline extract of the mushroom, characterized and reported. In the present investigation another water soluble polysaccharide (ELPS) has been isolated from the aqueous extract of this mushroom and characterized as β-glucan. Similar type of β-glucan was isolated by Bhanja et al. (2012) but differing in the number of sugar moieties and linkages in the skeleton chain. The water insoluble β-glucan (PS-II) isolated by Bhanja et al. (2012) contained (1 → 3)-linked three glucose moieties in the skeleton chain with branching at C-6 by another glucose residue, where as the present material is a water soluble β-glucan (ELPS) consisting of five (1 → 3)-, (1 → 6)-linked glucose moieties in the skeleton chain with branching at C-6 by another one. In the present investigation detailed structural characterization, immunological studies and antioxidant properties of the ELPS have been carried out and reported herein.

2. Materials and methods

2.1. Isolation and purification of the polysaccharide

Fresh fruit bodies of the mushroom *E. lividoalbum* (Kühner & Romagn.) Kubička (700 g) were collected from Sikkim Himalayan region, India, gently washed with water, cut into pieces and boiled at 100 °C with distilled water for 10 h, cooled, centrifuged, supernatant was precipitated in EtOH (1:5) to get crude polysaccharide (900 mg) and purified using the procedure as described in previous publication (Maity, Samanta, et al., 2014). The crude polysaccharide (25 mg) was purified by gel-permeation chromatography (GPC) on column (90 cm × 2.1 cm) of Sepharose 6B using distilled water as the eluent with a flow rate of 0.5 mL min^{-1}. A total of 90 test tubes were collected and monitored by the phenol-sulfuric acid method (York, Darvill, McNeil, Stevenson, & Albersheim, 1986) at 490 nm using Shimadzu UV–vis spectrophotometer, model-1601. A single homogeneous fraction (test tube 20–32) was collected and freeze-dried, yielding 15 mg pure polysaccharide. This experiment was repeated eight times and 110 mg of pure polysaccharide was collected and preserved for further analysis.

2.2. General methods

The average molecular weight of the ELPS was measured as reported earlier (Maity, Nandi, et al., 2014). The optical rotation was measured on a Jasco Polarimeter model P-1020 at 30 °C. For monosaccharide analysis, the ELPS (3 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 (Maity, Nandi, et al., 2014). The absolute configuration of monosaccharide was determined by the method of Gerwig, Kamerling, and Vliegenthart (1978). Periodate oxidation, Smith degradation, and methylation experiments were carried out by the method described previously (Maity, Nandi, et al., 2014). 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) for monosaccharide analysis. All GLC analyses were performed at 170 °C. GLC–MS analysis was performed on 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. Finally NMR experiments (Dueñas-Chaso et al., 1997; Hård, Zadelhoff, Moonen, Kamerling, & Vliegenthart, 1992) were carried out by a Bruker Avance DPX-500 spectrometer at 30 °C as reported in our previous paper (Maity, Nandi, et al., 2014).

2.3. Immunostimulating properties

Limulus Amebocyte Lysate (LAL) assay was performed to confirm the presence of any endotoxin in the ELPS by the procedure as described previously (Nandi et al., 2013). Macrophage, splenocyte and thymocyte proliferation assays were determined by adopting the procedures as described previously (Bhanja et al., 2012; Nandi et al., 2013). The formulas to calculate the SPI and TPI are as follows:

$$\text{Splenocyte Proliferation Index (SPI)} = \frac{\text{O.D. value of glucan treated splenocytes}}{\text{O.D. value of negative control (PBS)}} \times 1$$

$$\text{Thymocyte Proliferation Index (TPI)} = \frac{\text{O.D. value of glucan treated thymocytes}}{\text{O.D. value of negative control (PBS)}} \times 1$$

The full form of O.D. is "Optical Density" or absorbance value of the dissolved formazon crystals during MTT assay, which gets reduced after interacting with the oxidoreductase enzymes present only in viable cells.

2.4. Antioxidant properties

2.4.1. Hydroxyl 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 (100–800 μg/mL) of ELPS, $FeCl_3$ (100 mM), EDTA (104 μM), ascorbate (100 μM) and H_2O_2 (1 mM). Following incubation 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) were added to the reaction mixture, which was then heated in a boiling water bath for 15 min. After cooling, absorbance was measured at 535 nm, with butylated hydroxytoluene (BHT) as positive control (Halliwell, Gutteridge, & Aruoma, 1987). The hydroxyl radical Scavenging activity of ELPS was calculated by the following equation:

$$\text{Scavenging activity (\%)} = \frac{A_0 - A_1}{A_0} \times 100$$

where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of sample. The effect of hydroxyl radical Scavenging activity was expressed as IC_{50}: the amount of the sample needed to inhibit hydroxyl radical concentration by 50%.

2.4.2. Superoxide radical scavenging activity

The method by Martinez, Marcelo, Marco, and Moacyr (2001) for determination of the superoxide radical was followed with modification in the riboflavin-light-nitroblue tetrazolium (NBT) system. Each 3 mL reaction mixture contained 50 mM sodium phosphate