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Water-insoluble glucans from the edible fungus *Ramaria botrytis*

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ABSTRACT

Two water-insoluble glucans were isolated from the fruiting bodies of an edible fungus, *Ramaria botrytis* by extracting with sodium hydroxide. Structural characterizations of these glucans were investigated on the basis of total hydrolysis, methylation analysis, periodate oxidation, Smith degradation, and NMR experiments (^{1}H, ^{13}C, TOCSY, DQF-COSY, and HSQC) and the structures of the repeating units were established as:

PS-I →3)-α-D-Glc*p*-(1→

PS-II →3)-β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→
6
↑
1
β-D-Glc*p*

1. Introduction

Numerous bioactive polysaccharides have been isolated from various fungi (Kozarski et al., 2011; Smiderle et al., 2008; Srivastava & Kulshreshtha, 1989; Wasser, 2002) of which glucans are the most abundant forms of polysaccharides found inside the cell wall of the fruit bodies of fungi (Chan, Chan, & Sze, 2009). Several glucans have been reported from various sources using different procedures (Bohn & BeMiller, 1995; Kollar et al., 1997). The glucans in mushrooms are present mostly as linear β-(1→3)-(Misaki & Kakuta, 1995; Ojha, Chandra, Ghosh, & Islam, 2010), β-(1→6)-(Nandan et al., 2008; Sarkar et al., 2012) and non linear form with β-(1→3) backbone branched at O-6 (Mizuno et al., 1990; Rout, Mondal, Chakraborty, & Islam, 2008), and β-(1→6) backbone branched at O-3 (Maji et al., 2012; Sen et al., 2013). The β-D-glucans are biologically important for their outstanding ability to enhance and stimulate the immune systems of human (Blascheck, Kasbauer, Kraus, & Franz, 1992; Kiho, Sakushima, Wang, Nagai, & Ukai, 1991; Kulicke, Lettau, & Thielking, 1997) and are thus regarded as typical biological response modifiers (BRMs). Currently, three major β-D-glucans

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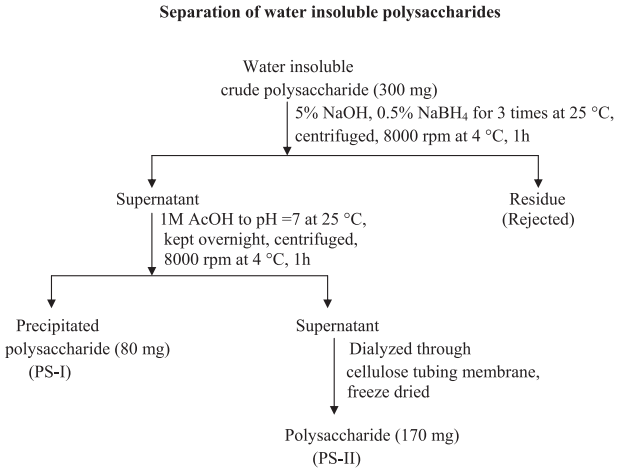
like krestin isolated from *Trametes versicolor*, lentinan from *Lentinus edodes*, and schizophyllan from the *Schizophyllium commune* (Fang et al., 2012; Zhang, Cui, Cheung, & Wang, 2007) are clinically used as BRMs for cancer therapy. The β-D-glucans do not attack cancer cells directly but stimulate natural killer cells (NK-cells), T-cells, B-cells and macrophage dependent immune systems and there by cancer cells are targeted and destroyed without side effect (Fang et al., 2012; Zhang et al., 2007); the possible mechanism of action has been demonstrated by Chihara (1981). They are also used as dietary substances that can reduce the plasma cholesterol and enhance the hematopoietic response (Borchers, Keen, & Gershwin, 2004; Hamano et al., 1999). In vitro experiment shows that β-D-glucan can directly stimulate leukocytes and exhibit cytotoxic, and antimicrobial activity (Smiderle et al., 2008). In addition, they exhibit anti-inflammatory, antioxidative, and analgesic activities (Smiderle et al., 2008). Except for β-D-glucans, another kind of glucans having α-linkages has been identified in various mushrooms. Compared with the intensive investigations of β-D-glucans, the biological activity of (1→3)-α-D-glucans has been reported rarely in the literature (Wiater et al., 2011). Some groups have studied the structure and solution properties of (1→3)-α-D-glucans from *Lentinus edodes* (Zhang & Cheung, 2002) and *Poria cocos* mycelia (Jin et al., 2004). Some bioactive and medicinal properties of (1→3)-α-D-glucan derivatives have also been reported (Wang, Deng, Li, & Tan, 2007). A carboxymethylated derivative of (1→3)-α-D-glucan from the fruiting bodies of *Amanita muscaria* exhibits antitumor activities (Kiho et al., 1994). Carboxymethylated fungal (1→3)-α-D-glucans expressed cytotoxic effects (Wiater et al., 2011).

Ramaria botrytis, an ectomycorrhizal fungus (Nandi et al., 2012) grows in symbiotic association with the roots of Chir pine (*Pinus roxburghii*) and Beech (*Fagus sylvatica*) trees in coniferous forest, throughout the world especially in the mountains of Eastern Asia, Europe, and North America. It is known as cauliflower coral. Several compounds such as amino acids, glycerophospholipids, phytochemicals (Barros, Venturini, Baptista, Estevinho & Ferreira, 2008; Yaoita, Satoh, & Kikuchi, 2007) and recent an immunoenhancing water soluble (1→6)-, (1→3)-β-D-glucan (Bhanja et al., 2013) were isolated from this mushroom and reported. In the present work two water insoluble glucans have been isolated from the alkali extract of the fruit bodies of *R. botrytis* and reported herein.

2. Materials and methods

2.1. Isolation, fractionation, and purification of crude polysaccharide

The fresh fruit bodies of the edible mushroom, *R. botrytis* (500 g) were collected from Darjeeling, Himalaya hill region. Crude polysaccharide from *R. botrytis* was extracted through hot water extraction followed by treating NaOH applying the method reported earlier (Bhanja et al., 2013) to produce two fractions. The water insoluble fraction was now separated using the following flow-sheet diagram;



2.2. Monosaccharide analysis

Both PS-I and PS-II (4.0 mg) were hydrolyzed with 2 M CF_3COOH (2 mL) in a round-bottom flask at 100 °C in a boiling water bath for 18 h. The excess acid was completely removed by co-distillation with water. Then, the hydrolyzed product of each polysaccharide was divided into two parts. One part was examined by paper chromatography (Hoffman, Lindberg, & Svensson, 1972) in *n*-BuOH–AcOH–H_2O (*v*/*v*/*v*, 4:1:5, upper phase; X) and AcOEt–pyridine–H_2O (*v*/*v*/*v*, 8:2:1; lower phase; Y). Another part was reduced with $NaBH_4$, followed by acidification with dilute CH_3COOH. It was then co-distilled with pure CH_3OH to remove excess boric acid. The reduced sugars were acetylated with 1:1 pyridine-acetic anhydride in a boiling water bath for 1 h to prepare the alditol acetates, which were analyzed by GLC. Quantification was carried out from the peak area, using response factors from standard monosaccharides using inositol as standard.

2.3. Absolute configuration of monosaccharide

The method used was based on Gerwig, Kamerling, and Vliegenthart (1978). Both polysaccharides (2.5 mg) were hydrolyzed with CF_3COOH, and then the acid was removed by co-distillation with distilled water. A solution of 250 μL of 0.625 M HCl solution treated with *R*-(−)-2-butanol was added and heated at 80 °C for 16 h. Then the reactants were evaporated and TMS-derivatives were prepared with *N*,*O*-bis (trimethylsilyl) trifluroacetamide (BSTFA). The products were analyzed by GLC using a capillary column SPB-1 (30 m × 0.26 mm) with a temperature program (3 °C/min) from 150 to 210 °C. The resulting 2,3,4,6-tetra-*O*-TMS-(+)-2-butylglycosides obtained were identified by comparison with those prepared from the D and L enantiomers of different monosaccharide.

2.4. Methylation analysis

PS-I and PS-II (6.0 mg) were methylated using the method described by Ciucanu and Kerek (1984). The methylated products were isolated by making a partition between $CHCl_3$ and H_2O (5:2, *v*/*v*). The organic layer containing products was washed with water for several times by taking 4 mL of water