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A glucan from an ectomycorrhizal edible mushroom *Tricholoma crassum* (Berk.) Sacc.: isolation, characterization, and biological studies

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ABSTRACT

A water soluble glucan of average molecular weight ~1.74×10^5 Da was isolated from hot aqueous extract of the fruiting bodies of an ectomycorrhizal edible mushroom *Tricholoma crassum* (Berk.) Sacc. The structure of this glucan was elucidated on the basis of total hydrolysis, methylation analysis, Smith degradation, and 1D/2D NMR studies. Based on the above experiments the repeating unit of the glucan was established as:

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

This glucan showed macrophage activation in vitro by NO production in a dose dependent manner and strong splenocyte and thymocyte immunostimulation in mouse cell culture medium. It also exhibited good inhibition activity toward lipid peroxidation.

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1. Introduction

Tricholoma crassum (Berk.) Sacc., family Tricholomataceae[1] is an ectomycorrhizal fungus.[2,3] It grows in symbiotic relationship with the roots of coniferous trees in forest soil once in a year during rainy season throughout the world. It helps the plants in extracting nutrients especially phosphorous and attributes bio-control of root diseases. In return, this fungus gets sugars, amino acids, and other substances from the host plant for their growth and development. It is edible and non-toxic.[3,4] Only a few edible species of this family like *Tricholoma mutsutake* and *T. crassum* are found in the Asia (Japan, Thailand, and Sri-Lanka) while several other species *Tricholoma albobrunneum*, *Tricholoma flavovirens*, *Tricholoma paneolum*, *Tricholoma equestre*, *Tricholoma terreum*, and *Tricholoma georgii* are mostly found in Europe and America. The nutritional value of *T. crassum* is promising with 10.02 g carbohydrates, 0.287 g fats, 18.58 g protein, 2.71 mg calcium, and 3.35 mg iron per 100 g of fresh weight.[5] Because of its high nutritional value, the method of cultivation of this mushroom has been developed for commercialization and reported.[4] The fibrinolytic activity[6] using fruit bodies and synthesis of silver nanoparticles[7] with the extract of mycelium of *T. crassum* (Berk.) Sacc. were reported. Recently, the structural characterization of the hetero polysaccharide and its immunological activity from the alkaline extract of this mushroom *T. crassum* (Berk.) Sacc. were carried out by our group and reported.[8] But no works relating to the polysaccharides obtained from the aqueous extract of this mushroom are in literature. Hence, the structural characterization, immunological, and lipid peroxidation properties of the polysaccharide isolated from the aqueous extract of the fruit bodies of *Tricholoma crassum* (Berk.) Sacc. are reported herein first time.

2. Results

2.1. Isolation, purification, and chemical analysis of the polysaccharide

The hot aqueous-extract of fresh fruit-bodies (600 g) of *T. crassum* (Berk.) Sacc. was cooled, filtered, and precipitated in

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1:5 (v/v) alcohols. The residue was dialyzed, centrifuged, and freeze dried to yield 1.2 g of crude polysaccharide, which was further purified by gel-permeation chromatography to get pure polysaccharide (PS). The PS showed a specific rotation of $[\alpha]_D^{30.7}$ −9.6 (*c* 0.104, H_2O). The molecular weight[9] of this PS was estimated from a calibration curve prepared with standard dextrans as $\sim 1.74 \times 10^5$ Da. GLC analyses of alditol acetates of neutral sugar showed the presence of glucose unit only in the polysaccharide. The absolute configuration of the monosaccharide was determined by the method of Gerwig et al.,[10] which showed that glucose is present in polysaccharide with the D-configuration. The PS was methylated using the Ciucanu and Kerek method[11] followed by hydrolysis and alditol acetate conversion. The GLC–MS analysis of the alditol acetates of the methylated product showed the presence of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol, and 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol. These results indicated that terminal glucopyranosyl, (1→3)-linked glucopyranosyl, (1→6)-linked glucopyranosyl, and (1→3,6)-linked glucopyranosyl residues were present in the PS in the molar ratio of nearly 1:1:2:1. These linkages were further confirmed by periodate oxidation experiment. GLC analysis of alditol acetates of the periodate oxidized,[12,13] $NaBH_4$-reduced PS was found to contain only glucose unit, and periodate oxidized, reduced, methylated[14] PS showed the presence of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol and 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol in a molar ratio of nearly 1:1. These results clearly indicated that (1→6)-linked and terminal glucopyranosyl moieties were consumed during oxidation while (1→3)-linked glucopyranosyl and (1→3,6)-linked glucopyranosyl residues remained unaltered. Hence, these observations confirmed the mode of linkages of these monosaccharide components present in the PS.

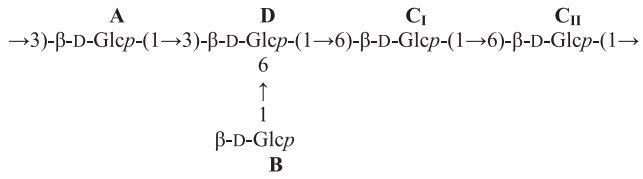
2.2. NMR and structural analysis of the polysaccharide

The ^{1}H NMR spectrum (500 MHz; Fig. 1) of the PS at 30 °C contained four anomeric signals at δ 4.78, 4.54, 4.52, and 4.51 ppm in a molar ratio of nearly 1:1:2:1. They were designated as **A**, **B**, **C**, and **D** according to their decreasing anomeric proton chemical shifts. In the ^{13}C NMR spectrum (125 MHz; Fig. 2) at the same temperature four signals were found in the anomeric region at δ 103.0, 102.9, 102.8, and 102.6 ppm in a molar ratio of nearly 2:1:1:1. On the basis of HSQC spectrum (Fig. 3) the signals at δ 103.0, 102.9, 102.8, and 102.6 ppm were assigned to anomeric carbons of residue **C**, **D**, **B**, and **A** respectively. All the ^{1}H and ^{13}C signals (Table 1) were assigned from DQF-COSY, TOCSY, and HSQC experiments. The proton coupling constants were measured from DQF-COSY experiment.

All the residues from **A**–**D** were established as β-anomer from coupling constant values $J_{H\text{-}1,H\text{-}2}$ ~8.5 Hz, and $J_{C\text{-}1,H\text{-}1}$ ~160 Hz. Again, the large coupling constant values $J_{H\text{-}2,H\text{-}3}$ ~10.0 Hz and $J_{H\text{-}3,H\text{-}4}$ ~9.5 Hz of all residues from **A**–**D** revealed that they were glucopyranosyl moiety (Glcp). In residue **A**, the downfield shift of C-3 (δ 84.4) with respect to standard value of methyl glycosides[15,16] indicated that it was (1→3)-linked β-D-Glcp. All the chemical shifts of residue **B** were nearly analogous with the standard values of methyl glycoside[15,16] of β-D-glucose. This observation clearly indicated that the residue **B** was non-reducing end β-D-Glcp. Two **C** residues ($\mathbf{C_I}$ and $\mathbf{C_{II}}$) were same in all chemical shift values excluding the chemical shift values of C-6. The different downfield shifts of C-6 (δ 69.0 and 68.9) of two **C** residues supported the presence of (1→6)-linking in β-D-Glcp with different chemical environments. Among two **C** residues, one residue ($\mathbf{C_I}$) was present in the adjacent position of the rigid part **D** and other residue ($\mathbf{C_{II}}$) was away from it. Between $\mathbf{C_I}$ and $\mathbf{C_{II}}$ residues, C-6 of $\mathbf{C_I}$ residue appeared slightly downfield in comparison to $\mathbf{C_{II}}$ residue due to the neighboring effect[17,18] of rigid part **D** of the backbone. In residue **D**, the chemical shift values of C-3 (δ 84.2) and C-6 (δ 68.8) shifting toward downfield regions, indicated the presence of (1→3,6)-linked β-D-Glcp. But the C-3 value of residue **D** resonated at logically upfield compared to the other (1→3)-linked **A** residue due to its presence at the most rigid part (residue **D**) of the backbone. Consequently, the C-6 value of residue **D** also resonated at fairly upfield compared to the (1→6)-linked other residues ($\mathbf{C_I}$ and $\mathbf{C_{II}}$) for the same reason. The linking at C-6 of the residues **C** and **D** was further confirmed from DEPT-135 spectrum (Fig. 4).

The sequence of glucosyl moieties was determined from ROESY (Fig. 5, Table 2) as well as NOESY (not shown) experiment. The inter-residual contacts **A**H-1/**D**H-3; **B**H-1/**D**H-6a, **D**H-6b; $\mathbf{C_I}$H-1/$\mathbf{C_{II}}$H-6a, $\mathbf{C_{II}}$H-6b; $\mathbf{C_{II}}$H-1/**A**H-3; and **D**H-1/$\mathbf{C_I}$H-6a, $\mathbf{C_I}$H-6b along with some other intra-residual contacts were also observed (Fig. 5). The above ROESY connectivities established the following sequences: **A** (1→3) **D**; **B** (1→6) **D**; $\mathbf{C_I}$ (1→6) $\mathbf{C_{II}}$; $\mathbf{C_{II}}$ (1→3) **A**; and **D** (1→6) $\mathbf{C_I}$.

Long range ^{13}C–^{1}H correlation of the HMBC experiment (Fig. 6, Table 3) further established the ROESY connectives. The cross-peaks of both anomeric protons and carbons of each of the sugar residues were examined and connectivities were determined from the HMBC experiment. Inter residual cross-peaks **A**H-1/**D**C-3; **A**C-1/**D**H-3; **B**H-1/**D**C-6; **B**C-1/**D**H-6a, **D**H-6b; $\mathbf{C_I}$H-1/$\mathbf{C_{II}}$C-6; $\mathbf{C_I}$C-1/$\mathbf{C_{II}}$H-6a, $\mathbf{C_{II}}$H-6b; $\mathbf{C_{II}}$H-1/**A**C-3; $\mathbf{C_{II}}$C-1/**A**H-3; **D**H-1/$\mathbf{C_I}$C-6; **D**C-1/$\mathbf{C_I}$H-6a, $\mathbf{C_I}$H-6b with other intra-residual peaks were observed (Fig. 6). Thus, the HMBC and ROESY connectivities clearly supported the presence of the pentasaccharide repeating unit in the glucan isolated from the edible mushroom *T. crassum* (Berk.) Sacc. as;



Controlled Smith degradation was carried out on the PS and the product was analyzed by ^{13}C NMR spectroscopy (Fig. 7, Table 4) to confirm further the sequence of the sugar residues present in the repeating unit. The ^{13}C NMR spectrum of Smith-degraded polysaccharide showed two anomeric carbon signals at δ 102.8

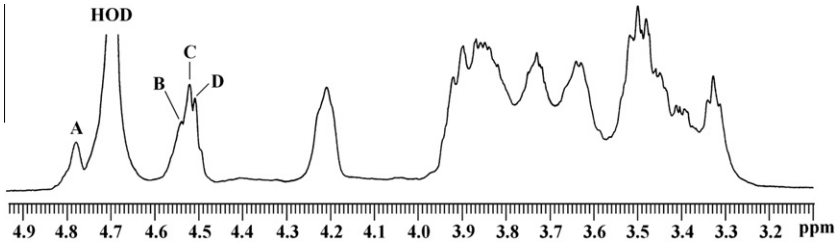


Figure 1. ^{1}H NMR spectrum (500 MHz, D_2O, 30 °C) of a glucan isolated from an edible mushroom *Tricholoma crassum* (Berk.) Sacc.

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