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Short Communication

A WATER-INSOLUBLE β-D-GLUCAN FROM HOT AQUEOUS EXTRACT OF AN EDIBLE MUSHROOM, *CALOCYBE INDICA* VAR. APK2: ISOLATION AND CHARACTERIZATION

Eshita Kar Mandal*[1], Soumitra Mandal[2]
[1]Sevayatan Sikshan Mahavidyalaya, Sevayatan, Jhargram, West Bengal, India
[2]Department of Chemistry, Fakir Chand College, Diamond Harbour, South 24-Parganas, West Bengal, India
**Corresponding author: eshitakarmandal@gmail.com*

ABSTRACT

Glucans, specially β-glucans are used as immunomodulating as well as anti-cancer agents. Hence, structural studies of the glucans are the matter of immense interest. A water-insoluble polysaccharide (M_W ~2.25×10^5 Da), isolated from the hot aqueous extract of an edible mushroom, *Calocybe indica* var. APK2, was found to consist of D-glucose only. On the basis of total hydrolysis, methylation analysis, periodate oxidation, and NMR studies (^{13}C, DEPT-135), the structure of the repeating unit of the polysaccharide was established.

Keywords: *Calocybe indica* var. APK2; Polysaccharide; Glucan; Structure; NMR analysis

1. INTRODUCTION

Mushrooms are a nutritionally functional food and have traditionally been used as folk medicine. Mushroom polysaccharides have attracted the attention of chemist and immunobiologists for their immunoenhancing and anti-tumor properties [1-4]. Mushrom polysaccharides mostly destroy cancer cells through immunomodulation [5]. Hence, structural studies of the polysaccharides, specially mushroom polysaccharides are the matter of great interest for the researchers in recent days. Several linear [6, 7] and branched glucans [8, 9] and heteroglycans [10, 11] of higher basidiomycetes exert strong immunostimulating and anti-tumor activity.

Calocybe indica var. APK2 was collected [12] from Tamil Nadu Agricultural University, Coimbatore, India. An immunostimulating (1→4)-, (1→6)-branched glucan [13] from hot aqueous extract and two new water-soluble (1→6)-, (1→4)-α, β-glucan and water-insoluble (1→3)-, (1→4)-β-glucan [14] from alkaline extract of the parent mushroom *Calocybe indica* were found. An immunoenhancing cytotoxic heteroglycan [1] was isolated along with a water-insoluble β-glucan also from hot aqueous extract of fruit bodies of *C. indica* var. APK2. Structural characterization of that water-insoluble β-glucan from hot aqueous extract of *C. indica* var. APK2 has been presented herein.

2. EXPERIMENTAL

Fresh fruiting bodies of an edible mushroom *Calocybe indica* var. APK2 (500 g) was crushed, boiled for 7 h in distilled water, kept overnight at 4 °C and filtered. The crude water-soluble (wt. 330 mg) and water-insoluble (wt. 100 mg) polysaccharide were isolated by the method applied as reported earlier [1, 13-16]. The water-soluble crude polysaccharide (30 mg) was purified by gel-permeation chromatography on a Sepharose-6B column using water as eluent and one homogeneous fraction was obtained, freeze dried, yielding 13 mg of material [1]. But, in case of water-insoluble crude polysaccharide the eluent was 4% NaCl. At first, the water-insoluble crude polysaccharide was dissolved in 4% NaOH and then neutralized by dil. HCl under cold condition and freez-dried. After freez-drying, the freshly prepared water-insoluble crude polysaccharide (15 mg) was passed through Sepharose S-6B column which was previously saturated with 4% NaCl solution, one homogeneous fraction was obtained, freeze dried, yielding 8 mg of material. The purification process was carried out for several lots and further purified and collected.

For monosaccharide analysis, the polysaccharide sample (3.0 mg) was hydrolyzed with 2 M CF_3COOH (2 mL), and the analysis was carried out as described earlier [13, 14]. The molecular weight of the polysaccharide was determined as reported earlier [13, 14, 17]. Paper chromatographic studies were performed on Whatmann Nos. 1 and 3 mm sheets. Solvent systems used were (X) BuOH:HOAc:H_2O (v/v/v, 4:1:5, upper phase) and (Y) EtOAc:pyridine:H_2O (v/v/v, 8:2:1). Silver nitrate in acetone (1.2%), methanol in sodium hydroxide solution, and 5% sodium thiosulphate solution were used as spray reagents [18]. The absolute configuration of the monosaccharide constituent was assigned according to Gerwig et al [19]. The polysaccharide was methylated according to Ciucanu and Kerek method [20]. Periodate oxidation was performed as described in earlier reports [14, 21, 22]. A gas-liquid chromatograph Hewlett-Packard Model 5730 A was used, having a flame ionization detector and glass columns (1.8m x 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). All GLC analyses were performed at 170 °C. Gas-liquid chromatography-mass spectrometric (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. NMR experiments were carried out at 27 °C as reported earlier [13, 14, 23, 24].

3. RESULTS

The molecular weight [17] of the polysaccharide was estimated from a calibration curve prepared with standard dextrans as $\sim 2.25\times10^5$ Da. The polysaccharide was hydrolyzed by 2 M trifluoroacetic acid (TFA), and the alditol acetates of the hydrolyzate were analyzed by GLC using columns A (3% ECNSS M) and B (1% OV-225).The analysis showed the presence of glucose, only. Paper chromatographic analysis [18] of the hydrolyzate further confirmed the presence of glucose. The absolute configuration of the monosaccharide was determined as D configuration by the method of Gerwig et al [19]. For assigning the mode of linkages, the glucan was methylated by the method of Ciucanu and Kerek [20], followed by hydrolysis and alditol acetate preparation [25]. The alditol acetates were analyzed through GLC using columns A and B, and also by GLC-MS analysis was performed on Shimadzu GC-MS Model QP2010 Plus automatic system, using ZB-5MS capillary column (30 m × 0.25 mm), revealed the presence of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol, 1, 3, 4,5-tetra-*O*-acetyl-2,6-di-*O*-methyl-D-glucitol, and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol in a ratio of nearly 2:1:1 (Table 1). These results indicated the presence of (1→3)-linked, (1→3,4)-linked, and terminal D-glucosyl moieties in the glucan. For further linkage information of sugar moieties, the periodate oxidation experiment [21, 22] was carried out with the glucan. GLC-MS analysis of the alditol acetates of the periodate-oxidized, $NaBH_4$ reduced, methylated polysaccharide showed the presence of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol and 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methyl-D-glucitol in a ratio of nearly 2:1. This result indicated that (1→3)-linked and (1→3, 4)-linked residues were retained, while the non-reducing terminal D-glucosyl moiety was destroyed during oxidation.

Table 1: GLC and GLC–MS data for the alditol acetates derived from the methylated polysaccharide isolated from *Calocybe indica* var. APK2

Methylated sugars	t_R [a]	t_R [b]	Characteristic Fragments [c] (*m/z*)	Molar ratio	Mode of linkage
2,3,4,6-Me_4-Glc*p*	1.00	1.00	43, 45, 87, **101**, 117, 129, 145, 161	1.0	Glc*p*-(1→
2,6-Me_2-Glc*p*	3.83	3.38	43, 45, 58, 87, 101, **117**, 129, 143	1.0	→3,4)-Glc*p*-(1→
2,4,6-Me_3-Glc*p*	1.95	1.82	43, 45, 87, 101, **117**, 129, 161	2.0	→3)-Glc*p*-(1→

[a] *Retention time with respect to that of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol on a 3% ECNSSM column on Gaschrom-Q at 170 °C,*

[b] *Retention time with respect to that of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol on a 1% OV-225 column on Gaschrom-Q at 170 °C,*

[c] *Equipped with a HP-5-fused-silica capillary column using a temperature program from 150 °C (2 min) to 200 °C (5 min) at 2 °C min^{-1}.*

In the 125 MHz ^{13}C (Fig. 1, Table 2) NMR spectrum at 27 °C, the anomeric carbon signals at δ 103.4 and 103.8 ppm were the clear evidence of β-conformation of the all D-glycosyl residues. DEPT-135 (Fig. not shown) NMR spectra showed the absence of C-6 linkage in the glucan. The non-reducing terminal D-glucosyl, (1→3,4)-, and (1→3)-linked moieties were designated as **A**, **B**, and **C**, respectively. The anomeric carbon chemical shift of the residue **A** was observed at the position of δ 103.4. The other carbon chemical shifts correspond nearly to the standard values of standard methyl glycosides [26, 27], and it was assigned as non-reducing terminal D-glucosyl sugar moiety.

Residue **B** showed downfield shift of C-3 at δ 86.8 and C-4 at δ 77.7 compared to standard values of methyl glycosides indicating that it was (1→3,4)-linked D-glucosyl moiety. Since, residue **B** is the most rigid part of the backbone of the glucan, its C-3 (δ 86.8) appeared at the upfield region in comparison to the C-3 values of other (1→3)-linked residues.

The (1→3)-linked residues were designated as **C**. The downfield shift at C-3 (δ 87.2) position indicated that the residues were (1→3)-linked D-glucosyl moiety. Hence, the glucan is a branched (1→3)-, (1→4)-β-D-glucan consisting of a tetrasaccharide segment with one non-reducing terminal β-glucosyl residue and three internal β-glucosyl residues, of which one is 3,4-disubstituted and two are 3-mono substituted. Therefore, based on all the above chemical and spectroscopic evidence, the possible structure of the tetrasaccharide repeating unit of the water-insoluble β-glucan was established as

C **B** **C**
→3)-β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→
4
↑
1
β-D-Glc*p*
A

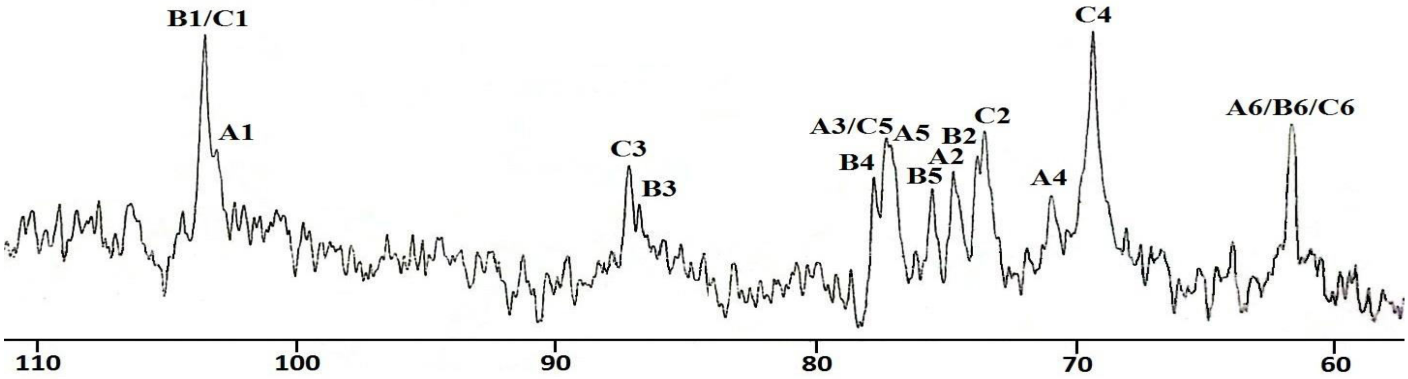

Fig. 1: ^{13}C NMR (125 MHz) spectrum of a water-insoluble polysaccharide isolated from *Calocybe indica* var. APK2 at 27°C in Me$_2$SO-*d*$_6$.

Table 2: The chemical shifts in the ^{13}C NMR^d spectrum of a water-insoluble polysaccharide isolated from *Calocybe indica* var. APK2 in Me$_2$SO-d$_6$ at 27 ^{0}C

Sugar Residue	C-1	C-2	C-3	C-4	C-5	C-6
β-D-Glc*p* (lit.[26])	104.0	74.1	76.8	70.6	76.8	61.8
β-D-Glc*p*-(1→**A**	103.4	74.6	77.2	70.9	77.0	61.7
→3,4)-β-D-Glc*p*-(1→**B**	103.8	73.7	86.8	77.7	75.4	61.7
→3)-β-D-Glc*p*-(1→**C**	103.8	73.4	87.2	69.3	77.2	61.7

[d] *Values of chemical shifts were recorded with reference to acetone as internal standard and fixed at δ 31.05 ppm. at 27 °C.*

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