Carbohydrate Research 343 (2008) 3120–3122

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Note

Structural characterization of a water-soluble β-(1→6)-linked D-glucan isolated from the hot water extract of an edible mushroom, *Agaricus bitorquis*

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ARTICLE INFO

Article history:
Received 29 July 2008
Received in revised form 6 September 2008
Accepted 10 September 2008
Available online 24 September 2008

Keywords:
Agaricus bitorquis
Polysaccharide
Glucan
Structure
NMR spectroscopy

ABSTRACT

A water-soluble polysaccharide, isolated from the hot aqueous extract of an edible mushroom, *Agaricus bitorquis*, was found to consist of D-glucose only. On the basis of total hydrolysis, methylation analysis, and NMR studies (^{1}H, ^{13}C, TOCSY, DQF-COSY, NOESY, ROESY, HMQC, and HMBC), the structure of the repeating unit was established as

→6)-β-D-Glc*p*-(1→

In recent years, mushrooms have become attractive as functional food and as source of new drugs. Many investigators have isolated and identified antitumor polysaccharides from mushrooms such as lentinan from *Lentinus edodes*,[1–3] a protein–polysaccharide complex from the fruiting body of *Agaricus blazei*,[4–6] and schizophyllan from *Schizophyllum commune*.[7] These polysaccharides are poorly water-soluble linear[4,8–12] and branched[1,2,13,14] β-glucans containing (1→3)- and (1→6)-linked glycosidic bonds, which are well known for their immunomodulatory[15,16] and antitumor[17,18] properties. The antitumor activity of these polysaccharides is caused by the enhancement of the immune response, which involves macrophage activation.[19,20]

Among mushrooms of the genus *Agaricus*, *A. blazei*, *A. bisporus*, and *A. bitorquis* are commonly available and edible. *A. blazei*[4–6] and *A. bisporus*[21] are furthermore reported to contain antitumor polysaccharides. A completely different polysaccharide from that of its fruiting body was obtained from the culture of *A. blazei* in liquid medium, and this was shown to consist of a main chain of β-(1→2)-linked D-mannopyranosyl residues with β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl substituents as side chains.[22] Up to now, polysaccharides of *A. bitorquis* have not been reported in the literature. In the present study, a water-soluble polysaccharide was extracted from *A. bitorquis*, and its characterization as a linear β-(1→6)-linked glucan is reported.

The pure polysaccharide (ABPS) has a specific rotation of $[\alpha]_D^{25}$ −31.6 (*c* 0.6, water), and the molecular weight of the polysaccharide was estimated to be ~1.8×10^5 Da from a calibration curve prepared with a standard dextran.[23] The polysaccharide was hydrolyzed with 2 M trifluoroacetic acid (TFA) and the alditol acetate on analysis through GLC using columns A (3% ECNSS-M) and B (1% OV-225) indicated the presence of only glucose. The absolute configuration of glucose, as determined by the method of Gerwig et al.,[24] was D. The absorption at 900 cm^{-1} in the IR spectrum indicated that ABPS had β-glucopyranosidic linkages.[25] The mode of linkage of the glucan was obtained after methylation using the methods of Ciucanu and Kerek[26] and then Purdie and Irvine[27] followed by hydrolysis and conversion into a single alditol acetate. The latter was analyzed by GLC using columns A and B and by GLC–MS using an HP-5 fused silica capillary column and proved to be 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol. This indicates the presence of only (1→6)-linked D-glucopyranosyl constituents in the glucan.

The 500 MHz ^{1}H NMR spectrum (Fig. 1) of PS at 27 °C showed one anomeric signal at 4.50 ppm. The coupling constant values ($J_{H\text{-}1,H\text{-}2} \sim 8.5$ Hz and $J_{H\text{-}1,C\text{-}1} \sim 160$ Hz) suggested that it is β-linked. The proton chemical shifts (Table 1) from H-1 to H-6 were assigned from the DQF-COSY and TOCSY spectra. The 125 MHz ^{13}C spectrum (Fig. 1) of PS at 27 °C exhibited one anomeric carbon signals at 103.4 ppm, which was assigned to a β-linked residue. This assignment was also corroborated by an HMQC experiment. All the carbon signals (Table 1) of the glucopyranoside residues were assigned with the help of an HMQC spectrum. The carbon signals

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doi:10.1016/j.carres.2008.09.019

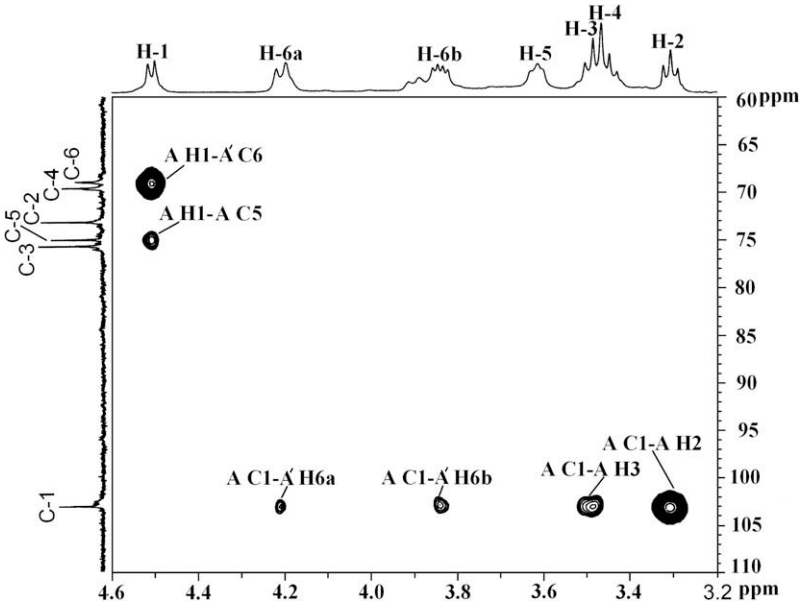

Figure 1. HMBC spectrum of the polysaccharide isolated from *Agaricus bitorquis* recorded with a delay time of 80 ms.

at 73.5, 76.0, 69.9, 75.3, and 69.2 ppm correspond to C-2, C-3, C-4, C-5, and C-6, respectively, of the glucopyranoside residue. The C-6 signal at 69.2 ppm is shifted to 7.4 ppm downfield compared to the standard methyl glycosides[28,29] due to the α-glycosylation effect.

The sequence of glycosyl residues in the polysaccharide was confirmed from an HMBC experiment. Long-range ^{13}C-^{1}H correlations were obtained from the HMBC spectrum (Fig. 1). Cross peaks of both anomeric protons and carbons of each of the sugar moieties were examined and both inter- and intraresidual connectivities were observed (Table 2). For explanation of the HMBC results, two units of glucose (**A** and **A′**) are considered. Cross peaks were found between H-1 (4.50 ppm) of residue **A** and C-6 (103.4 ppm) of residue **A′** (**A** H-1, **A′** C-6) and vice versa and C-1 (103.4 ppm) of residue **A** and H-6a (4.20 ppm) and H-6 b (3.84 ppm) of residue **A′** (**A** C-1, **A′** H-6a, **A** C-1, **A′** H-6 b) and vice versa.

Table 1
^{1}H NMR[a] and ^{13}C NMR[b] chemical shifts for the polysaccharide Isolated from *Agaricus bitorquis* in D_2O at 27 °C

Glycosyl residue	H-1/ C-1	H-2/ C-2	H-3/ C-3	H-4/ C-4	H-5/ C-5	H-6a, H-6b/C-6
→ 6)-β-D-Glc*p*-(1→	4.50 103.4	3.31 73.5	3.49 76.0	3.47 69.9	3.61 75.3	4.20,[c] 3.84[d] 69.2

[a] Values of the ^{13}C chemical shifts were recorded with reference to acetone as internal standard and fixed at δ 31.05 ppm at 27 °C.
[b] Values of the ^{1}H chemical shifts were recorded with respect to the HOD signal fixed at δ 4.73 ppm at 27 °C.
[c,d] Interchangeable.

Table 2
The significant $^{3}J_{H,C}$ connectivities observed in an HMBC spectrum for the anomeric protons/carbons of the sugar residues of the polysaccharide of *Agaricus bitorquis*

Residue	Sugar linkage	H-1/C-1 δ_H/δ_C	Observed connectivities		
			δ_H/δ_C	Residue	Atom
A	→ 6)-β-D-Glc*p*-(1→	4.50	69.2	**A′**	C-6
			75.3	**A**	C-5
		103.4	4.20	**A′**	H-6a
			3.84	**A′**	H-6b
			3.48	**A**	H-3
			3.31	**A**	H-2

Thus, the appearance of these cross peaks clearly supports the following repeating unit for the polysaccharide isolated from *Agaricus bitorquis*.

→6)-β-D-Glc*p*-(1→

1. Experimental

1.1. General methods

Optical rotation was measured on a Jasco Polarimeter model P-1020 at 25 °C. All GLC–MS experiments were carried out in a Hewlett–Packard 5970 MSD instrument using HP-5 fused silica capillary column. The program was isothermal at 150 °C; hold time 2 min, with a temperature gradient of 4 °C min^{-1} up to a final temperature of 200 °C. The molecular weight of the polysaccharide was determined as described previously.[30–34] For monosaccharide analysis, the polysaccharide sample (2.5 mg) was hydrolyzed with 2 M CF_3COOH (2 mL) and the analysis was carried out as described earlier.[30–34] The absolute configuration of the monosaccharide constituent was assigned according to Gerwig et al.[24] For methylation analysis, the polysaccharide was methylated according to Ciucanu and Kerek.[26] NMR experiments were carried out as reported in our previous papers.[30–34,36,37]

1.2. Isolation and purification of the polysaccharide

The fresh fruiting bodies of the mushroom, *Agaricus bitorquis* (1 kg), were collected from the local market and washed thoroughly with water. The material was then boiled with distilled water for 6 h, and the pure polysaccharide was isolated as reported in our previous papers.[30–35] The crude polysaccharide (30 mg) was purified by gel permeation chromatography. One homogeneous fraction (test tubes 24–36) was collected and freeze-dried, yielding 12 mg of material. The purification process was carried out in several lots.

Acknowledgments

The authors are grateful to Professor S. Roy, Director, IICB, Dr. A. K. Sen (Jr.), IICB and Dr. S. Lahiri, IACS, Kolkatta, for providing instrumental facilities. Mr. Barun Majumder of Bose Institute,

Kolkata, is acknowledged for preparing NMR spectra. DST, Govt of India is acknowledged for sanctioning a project (Ref. No.: SR/S1/OC-52/2006 dated 19/02/2007). One of the authors (C.K.N.) is grateful to UGC, New Delhi for offering junior research fellowship.

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