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A WATER-SOLUBLE β-D-GLUCAN FROM *BOLETUS ERYTHROPUS**

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Key Word Index—*Boletus erythropus*; Basidiomycetes; mushroom; water-soluble polysaccharides; β-D-glucan; HMQC NMR.

Abstract—The main component of a water extract of *Boletus erythropus* fruiting bodies is a M_r 10^6 glucan. The use of classical structural analysis and HMQC (heteronuclear multiple quantum coherence) NMR experiments indicates a (1→3) linked β-D-glucan structure with a single glucose residue attached to O-6 of the main chain and a branching frequency of 1/3. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Numerous mushroom polysaccharide extracts have been studied for their antitumour activities, based essentially on mouse transplanted *Sarcoma* 180 tests. These studies established a relationship between this activity and (1→3) linked β-D-glucan structures, particularly with O-6 substitutions, such as lentinan from *Lentinus edodes* [1] and schizophyllan from *Schizophyllum commune* [2]. In some cases, the necessity of triple-helix structures was demonstrated [3]. In order to extend these studies, we have examined polysaccharides from other families of mushrooms.

Amongst 10 000 species of mushrooms, about 60 species of *Boletaceae* with pores have been recorded; however, the antitumour properties of this family have not yet been studied, probably because of difficulties in growth.

Boletus erythropus was recently isolated in our laboratory. The water-soluble extract of this mushroom was selected for various tests on mice biological responses (to be published elsewhere). The present paper reports the isolation and chemical structure of the main component of the crude water-soluble polysaccharide extract from *B. erythropus* fruiting bodies. To our knowledge, this is the first paper reporting the use of HMQC experiments in the structural elucidation of this type of glucan polysaccharide.

RESULTS AND DISCUSSION

By repeated extraction with water and precipitation by alcohol, 150 mg of polysaccharide were obtained from 100 g of fresh fruiting bodies. Fractionation of the crude extract on DEAE Trisacryl M gave a major neutral fraction (N) (123 mg), a second fraction (A) (9.6 mg) eluted by the addition of 0.3 M NaCl to the buffer and a third fraction devoid of carbohydrate which was eluted with 2 M NaCl buffer, pH 7.2. Because of the small quantity, the A fraction was not fractionated further by gel filtration.

Gel filtration on a Sephacryl S400 HR column of the N fraction indicated the presence of a polysaccharide (N1, 93 mg) with a M_r of 10^6 and a smaller one (N2, 28 mg) (M_r 20 000). GC of the per(tri)methylsilylated monosaccharides obtained after hydrolysis of N2 showed the presence of fucose, mannose, glucose and galactose in a ratio of 1:1:1:2 (the complete structural determination of N2 will be published elsewhere). Analysis by GC-mass spectrometry of the methanolysed permethylated glucan N1 showed the presence of methyl 2,3,4,6-tetra-, 2,4,6-tri- and 4,6-di-*O*-methyl-glucosides (as acetates) in a molar ratio of 0.8:1.9:1, based on peak areas (Table 1). This indicated that the glucan has a highly branched structure, with branch points doubly substituted at positions 3 and 6.

1,6-Glycosidic linkages in polysaccharides are preferentially cleaved during acetolysis and the relative ratio for β(1→3) and β(1→6) linkages in disaccharides is 1:29 [4]. After reaction for 24 hr, gel filtration gave only two products (**a** and **b**). The ratio of glucose in **a** and **b** fractions was 2.7:1. Peaks **a** and **b** correspond to the void and total volume of the column, respectively.

*Dedicated to the memory of Prof. Bernard Fournet.

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Table 1. Methylation analysis of glucan N1 extracted from *Boletus erythropus*

	O-Methyl ethers					
	2,3,4,6*		2,4,6*		4,6*	
Parameters	α	β	α	β	α	β
Retention time (min)	15.3	13.9	22.8	20.7	28.3	26.1
Molar ratio†	0.8		1.9		1	

*As methyl-*O*-acetyl-*O*-methylglycosides.

†Values are given relative to one residue of 4,6-di-*O*-methyl-D-glucoside.

Methylation of **a** followed by methanolysis showed the presence of 1,3 (95%) and 1,6 (5%) glycosidic bonds. This is consistent with (1→3)-linked oligosaccharides resulting from almost complete cleavage of the branches attached by (1→6) linkages during methanolysis.

Simple high performance anion exchange chromatography of **b** revealed only one fraction identified as D-glucose, suggesting the presence of single D-glucosyl units as side-chains.

The above results indicate that the glucan from *B. erythropus* is a (1→3)-linked-glucan with single glucose residues attached on O-6 of the main chain and a branch-point occurs, on average, at each third glucose residue of the main chain.

The small quantity of glucan available and the low resolution of the 1D NMR spectrum obtained, led us to use HMQC experiments (Fig. 1). Table 2 summarizes the ^{13}C NMR signal assignments. These assignments are based on the reported values obtained for scleroglucan [5], H-3-B glucan [6] and HA-β glucan [7]. Table 2 also summarizes the ^{1} NMR signal assignments; these assignments are based on the reported values obtained for β-(1→3); β(1→6) macrocyclic glucan [8].

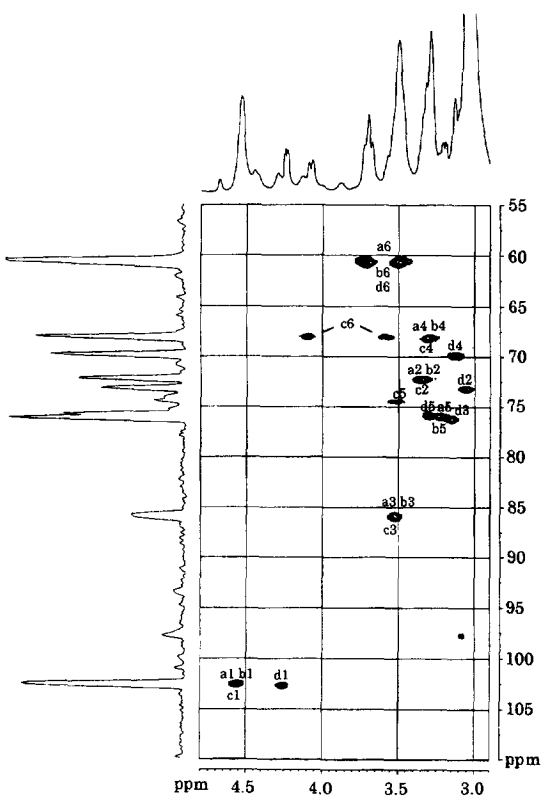

Fig. 1. HMQC NMR spectrum of the N1 glucan in DMSO-d_6 at 80°. A simple ^{1}H spectrum is given at the top.

Table 2. ^{1}H and ^{13}C NMR chemical shifts of glucan N1

Glucose unit	H_1	H_2	H_3	H_4	H_5	$H_{6,6'}$
a, b	4.55	3.35	3.52	3.30	3.21	3.48 3.71
c	4.55	3.35	3.52	3.30	3.52	3.58 4.11
d	4.26	3.05	3.14	3.11	3.30	3.48 3.71
	C_1	C_2	C_3	C_4	C_5	C_6
a, b	102.5	72.2	85.7	68.0	75.9	60.6
c	102.5	72.2	85.7	68.0	74.4	67.8
d	102.6	73.2	76.2	69.8	75.8	60.6

Letters **a**, **b**, **c** and **d** correspond to the glucose residues shown in Fig. 2.

The ^{1}H and ^{13}C chemical shifts in the anomeric region could be attributed to those of β-D-glucose residues. From the areas of signals of the ^{1}H NMR spectrum (Fig. 1) it was possible to estimate the relative ratios of β-(1→3) and β-(1→6) linkages as 3/1. From all the analyses, the structure of the N1 glucan was determined as that shown in Fig. 2.

Similar β-(1→3); β-(1→6) structures have been described in the polysaccharides from other fungi [9], with various M_r and branching frequencies. FPb glucan [10], isolated from the growth medium of *Piptoporus betulinus*, has the same structure, but its M_r was estimated at 6.5×10^5. The scleroglucan CS 11 differs by its M_r estimated at 5.7×10^6, according to Lecacheux *et al.* [11] and is classified as antitumoural [12], like numerous β-(1→3); β-(1→6)-D-glucans [13]. N1 thus appears to be a glucan with a classical branching frequency but with a M_r higher than most of the other known glucans from fungi.

EXPERIMENTAL

Extraction of polysaccharides. Fruiting bodies of *B. erythropus* Fries, collected in the southwest of France, were crushed in a mixer with H_2O (2 l kg^{-1}). The mixt. was centrifuged for 15 min at 10 000 *g* at 10°.

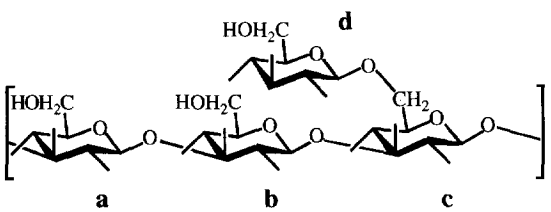

Fig. 2. Possible repeating unit in the N1 glucan.

Polysaccharide extractions with H_2O were repeated until there was no more pptn by 2 vols of EtOH (96%). The pooled ppts were dissolved in H_2O and the supernatant, after centrifugation, was dialysed against tap water (24 hr) and then against H_2O (2 × 24 hr) to give the crude extract.

Fractionation of polysaccharides. (1) *Ion-exchange chromatography*: The crude extract was passed through a DEAE Trisacryl M column (2.6 × 30 cm), in 20 mM Tris–HCl buffer, pH 8. It was sepd into a non-adsorbed polysaccharide fr. (N), an adsorbed polysaccharide fr. (A) (0.3M NaCl) and an adsorbed non-polysaccharide fr. (2M NaCl). (2) *Gel filtration chromatography*: The N fr. was fractionated by molecular size, on a S 400 HR column (2.6 × 90 cm), in 20 mM Tris–HCl buffer (pH 7.2) with 8 M urea to reduce interactions. The column was calibrated using standard dextran frs (2000, 670, 410, 150 and 50 kDa) under the same conditions.

Polysaccharide contents. Monosaccharides residues were converted into their Me glycosides (MeOH–HCl, 0.5 M, 80°, 24 hr), then into their TMSi derivatives [14] (pyridine/BSTFA, 2 hr) and examined by GC (OV-101 silicone capillary column, 25 m × 0.32 mm i.d., He pressure 0.4 bar. Temp. programme 120–240° at 2° min^{-1}).

Methylation analysis. Polysaccharide (500 μg) was methylated by the method of ref. [15]. Methylated polysaccharide was recovered in the organic phase after addition of $CHCl_3$ (0.5 ml × 3). The permethylated products were analysed by GC-MS, under the conditions described in ref. [16], as the acetylated partially methylated alditols obtained after hydrolysis (TFA, 4 M, 100°, 4 hr), reduction with $NaBH_4$, peracetylation, and as the acetylated partially methylated glucosides, obtained after methanolysis (MeOH–HCl, 0.5 M, 80°, 24 hr) of permethylated polysaccharide and peracetylation.

Acetolysis. Acetolysis of the glucan (1 mg) was performed according to ref. [17] in H_2SO_4 (10:10:1) (1 ml). After acetolysis, carbohydrates present in the residue were eluted from a column (1.6 × 65 cm) of BioRad Biogel-P2, with H_2O as eluent at a flow rate of 10 ml hr^{-1}. Frs of 2 ml were collected and assayed for total carbohydrate by the PhOH–H_2SO_4 method [18]. Polysaccharides excluded from the Biogel-P2 (fr. **a**) were methylated and the sugars eluted at the total vol. (fr. **b**) characterized by high performance anion-exchange chromatography on a CarboPAC PA-100 column in 0.045 M NaOH, equipped with a pulsed amperometric detector and at a flow rate of 1 ml min^{-1}.

NMR. Glucan (5 mg) was dissolved in 0.6 ml of DMSO-d_6. The ^1H spectrum was recorded with a Bruker AM 400 WB instrument at 80°. Standard Bruker software was used to obtain the HMQC spectrum [19]. Chemical shifts are reported relative to TMS using residual DMSO as a secondary ref. δ values of DMSO were taken as 2.50 ppm (^1H) and 39.6 ppm (^{13}C).

General analytical methods. Total carbohydrate content was measured by the anthrone–H_2SO_4 method [20]. Total protein content was measured by the method of ref. [21].

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