Available online at www.sciencedirect.com
ScienceDirect
Carbohydrate Research 341 (2006) 2990–2993
Carbohydrate RESEARCH

Note

A water-insoluble (1→3)-β-D-glucan from the alkaline extract of an edible mushroom *Termitomyces eurhizus*

Indranil Chakraborty, Soumitra Mondal, Dilip Rout and Syed S. Islam*

Department of Chemistry and Chemical Technology, Vidyasagar University, West Midnapore 721 102, West Bengal, India

Received 1 August 2006; received in revised form 5 September 2006; accepted 12 September 2006
Available online 24 October 2006

Abstract—A water-insoluble glucan, TEINS has been isolated from the hot alkaline extract of an edible mushroom *Termitomyces eurhizus*. The total carbohydrate content of the polysaccharide fraction was found to be 98.4%, and it was found to contain only glucose as the monosaccharide constituent. On the basis of total acid hydrolysis, a methylation experiment, periodate oxidation and ^{13}C NMR experiment, the repeating unit of the polysaccharide was established as:

$$\rightarrow 3)\text{-}\beta\text{-}D\text{-}Glc\textit{p}\text{-}(1 \rightarrow$$

Keywords: *Termitomyces eurhizus*; Edible mushroom; Insoluble glucan; Structure; NMR spectroscopy

In recent years, mushroom polysaccharides have drawn the attention of both chemists and immunobiologists due to their multipurpose medicinal activities[1–3] that include immunomodulating and antitumour properties. But of all the polysaccharides isolated from mushroom origin, glucans are the most important due to their potent antitumour properties. Various linear (1→3)-β-glucans[4,5] and branched (1→3)(1→6)-linked β-glucans[6–8] isolated from different mushroom origins are well known.

Wild mushrooms of the genus *Termitomyces*, like *Termitomyces eurhizus*, *T. clypeatuso*, *T. striatus*, *T. robustus* and *T. microcarpus* have been identified as edible mushrooms with high nutritive value. Several value-added enzymes have been reported from the species *T. microcarpus*[9] and *T. clypeatus*.[10–14] The biological response[15] of *T. striatus* was determined using weanling rats. When the rats were fed with *T. striatus* that was dried at 60 °C for 48 h, the rats rapidly lost weight and showed pathological signs of toxicity by the second day, and all rats died by the fourth day of the experiment. With a diet of *T. striatus* that was dried at 90 °C for about 8 h, the rats marginally gained weight but all survived. *T. eurhizus* is a wild edible mushroom that grows in the laterite forest soil of South Bengal, and local people consume them as delicious vegetables. Two water-soluble polysaccharide fractions were isolated from the hot aqueous extract of the mushroom *T. eurhizus*[16] by our group and are reported in this journal. A water-insoluble glucan designated as TEINS has been isolated from the alkaline extract of *T. eurhizus*, and a detailed structural characterization of this material is reported herein. TEINS was obtained by extraction with 4% NaOH, followed by precipitation in ethanol and dialysis.

TEINS 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 signal of glucose, only. On paper chromatographic analysis of the hydrolyzate, only the spot for glucose was observed. The polysaccharide is therefore a glucan. The absorption at 890 cm^{-1} in the IR spectrum indicated that TEINS has β-glucopyranosidic linkages, which was further supported by its low $[\alpha]_{25}$ +5.09 (*c* 0.67, 4% NaOH). The total sugar content of this glucan was estimated by

* Corresponding author. Tel.: +91 9932629971 (mobile), +91 3222 268387 (R); fax: +91 3222 275329; e-mail: sirajul_1999@yahoo.com

0008-6215/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2006.09.009

Table 1. GLC and GLC–MS data for the alditol acetate derived from the methylated TEINS isolated from *Termitomyces eurhizus*

Methylated sugar	Retention time		Linkage type	Major fragments (*m*/*z*)
	t_R [a]	t_R [b]		
2,4,6-Me_3-Glc*p*	1.95	1.82	→3)-Glc*p*-(1→	45, 58, 71, 87, 99, 101, 117, 129, 143, 161, 201, 233

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

the phenol–sulfuric acid method[17] and was found to be 98.4%. The absolute D-configuration[18] of the monosaccharide was assigned by GLC examination of the (+)-2-butyl 2,3,4,6-tetra-*O*-TMS-glycoside. The glucan was then methylated using the method of Ciucanu and Kerek,[19] and then by the Purdie method,[20] followed by hydrolysis and alditol acetate preparation. The alditol acetates on analysis through GLC using columns A and B, and also by GLC–MS using an HP-5 fused-silica capillary column, revealed the presence of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol (*m*/*z*: 45, 58, 71, 87, 99, 101, 117, 129, 143, 161, 201, 233), only. This result indicates the presence of a (1→3)-linked D-glucopyranosyl moiety in the glucan. Further, GLC analysis of the alditol acetates of the periodate-oxidized, reduced, methylated polysaccharide showed the presence of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol. The retention of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol further confirms the linkage as determined by the methylation experiment (Table 1).

The 125-MHz ^{13}C NMR spectrum at 27 °C of the glucan showed six signals. The β configuration of the D-glucosyl residues was clearly evidenced by the presence of an anomeric peak at δ 103.27 ppm. The signal at δ 86.30 ppm was assigned to C-3 of a (1→3)-β-D-glucosyl residue. The downfield shift of C-3 by 9.17 ppm is due to the α-effect of glycosylation.[21] The ^{13}C NMR signals were tentatively assigned (Fig. 1) and are shown in Table 2, with the corresponding literature values.[22] From the above experimental evidence, it is thus concluded that TEINS is a glucan composed of a (1→3)-linked β-D-glucopyranosyl repeating unit.

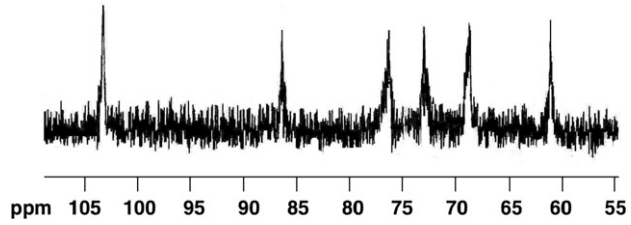

Figure 1. The ^{13}C NMR spectrum of TEINS isolated from *Termitomyces eurhizus* at 27 °C in Me_2SO-d_6.

Furthermore, the complex formation of TEINS with Congo Red was evaluated from the shift in the visible absorption maximum (λ_{max}) of Congo Red at various concentrations of NaOH, as reported in previous publications.[23,24] But on addition of NaOH at different low concentrations (0.05–0.20 M), there is no considerable shift of λ_{max}. It is therefore concluded that TEINS has a single helical structure.

1. Experimental

1.1. Isolation and purification

The fruit bodies of *T. eurhizus* (2 kg) were collected from the local forest and gently washed with water. After washing with distilled water and EtOH, the mushroom bodies were pulverized for extraction of polysaccharide by boiling with 4% NaOH for 30 min. The liquid was filtered and then kept overnight at 4 °C. It was then centrifuged at 4000 rpm at 8 °C for 1 h. The supernatant was collected and precipitated with 1:10 (v/v) ethanol at 25 °C. This was kept at 4 °C for 48 h, followed by centrifugation at 8 °C at 10,000 rpm for 1 h. The centrifugate was then washed with dehydrated ethanol and acetone several times. The precipitated material was extensively dialyzed against distilled water through a DEAE cellulose bag to remove excess alkali (tested with pH paper) and other low-molecular-weight material, and the material inside the dialysis bag was reprecipitated with ethanol. The precipitate was collected by centrifugation and freeze-dried (1.80 g), and structural investigations were carried out with this polysaccharide (TEINS).

1.2. Total acid hydrolysis

The sample, TEINS (2 mg) was hydrolyzed with 2 M CF_3CO_2H (1 mL) at 100 °C for 16 h in a boiling water bath. The hydrolyzate was then converted into its alditol acetate and analyzed by gas liquid chromatography (GLC) using a Hewlett–Packard model 5730 instrument equipped with a flame-ionization detector. The peak was

Table 2. The chemical shifts in the ^{13}C NMR spectrum of TEINS isolated from *Termitomyces eurhizus* in Me_2SO-d_6 at 27 °C

Residue	C-1	C-2	C-3	C-4	C-5	C-6
β-D-Glc*p* (lit.[22])	102.72	74.13	77.13	70.40	76.55	61.32
→3)-β-D-Glc*p*-(1→	103.27	73.89	86.30	68.72	76.22	61.12

identified and estimated with inositol as the internal standard. The alditol acetate was analyzed on a glass column (1.8 m × 6 mm) containing 3% ECNSS-M (A) and 1% OV-225 (B) on Gas Chrom Q (100–120 mesh) at 170 °C. Gas–liquid chromatography–mass spectrometric (GLC–MS) analysis was also performed on a Hewlett–Packard 5970A automatic GLC–MS system, using an HP-5 capillary column (0.25 m × 25 mm). 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. Quantitation was carried out from the peak area, using response factors from standard monosaccharides.

1.3. Determination of absolute configuration

The method used was based on the method of Gerwig et al.[18] After CF_3CO_2H hydrolysis of 1 mg of TEINS, the acid was removed by co-distillation with water. A solution of 250 μL of 0.625 M HCl in (+)-2-butanol was added to it, and the mixture was heated at 80 °C for 16 h. The reactants were then evaporated, and per-*O*-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 (+)-2-butyl 2,3,4,6-tetra-*O*-TMS-glycoside obtained was identified by comparison with those prepared from the D and L enantiomers of the monosaccharides.

1.4. Methylation analysis

TEINS was methylated using the method of Ciucanu and Kerek,[19] and the product was isolated by partition between $CHCl_3$ and H_2O. It was methylated again by the Purdie method.[20] The product showed no band in the region of 3600–3300 cm^{-1}. It was then hydrolyzed with 90% HCO_2H for 1 h. Excess HCO_2H was evaporated off by co-distillation with distilled water. The hydrolysate was then reduced with $NaBH_4$, and the alditol acetate was prepared as usual. The alditol acetate of the methylated sugar was analyzed by GLC (using columns A and B), and by GLC–MS using an HP-5 fused-silica capillary column using the temperature program as stated above.

1.5. Periodate oxidation study

TEINS was added to 0.1 M $NaIO_4$, and the mixture was kept at 4 °C for 48 h in the dark. The excess periodate was destroyed by ethylene glycol, and the solution was dialyzed against distilled water. It was then freeze dried. This material was divided into two portions. One portion was hydrolyzed by 2 M CF_3CO_2H for 16 h, and the alditol acetate was prepared. Another portion was methylated by the Ciucanu and Kerek method, and the alditol acetate of this methylated product was prepared. The alditol acetate was analyzed by GLC using columns A and B.

1.6. Paper chromatographic studies

Paper partition 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). The spray reagent used was alkaline silver nitrate solution.[25]

1.7. Optical rotation

Optical rotation was measured on a Perkin–Elmer model 241 MC spectropolarimeter at 25 °C.

1.8. GLC experiments

All gas–liquid chromatographies were performed on a Hewlett–Packard Model 5730A gas chromatograph having a flame-ionization detector and glass columns (1.8 m × 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.

1.9. GLC–MS experiments

All the GLC–MS experiments were carried out on a Hewlett–Packard 5970 MSD instrument using an 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.

1.10. FTIR

The IR spectrum was recorded with dried polysaccharide (1.1 mg) by a Jasco FTIR 6200 instrument using a solid-state ATR accessory.

1.11. NMR studies

The freeze-dried polysaccharide was kept over P_2O_5 in vacuum for several days and then deuterium exchanged three times, followed by lyophilization with D_2O. The ^{13}C spectrum of TEINS was then carried out with Me_2SO-d_6 at 27 °C in a Bruker Avance DPX 500 instrument using acetone as internal standard (δ 31.01 ppm).

1.12. Interaction with Congo Red

The interaction with Congo Red was evaluated from the shift in the visible absorption maximum of Congo Red

that was induced by the presence of polysaccharide at various concentrations of alkali. The solutions of TEINS (2 mg/mL) in 0.05–0.20 M NaOH containing 91×10^{-6} mol of Congo red were prepared. The absorption spectra were recorded from 400–700 nm at room temperature with a Shimadzu model UV1601 spectrophotometer.

Acknowledgements

The authors are thankful to Professor S. Lahiri, IACS, Jadavpur and Professor S. Roy, Director, IICB, Kolkata, for valuable suggestions and for use of the NMR facilities. One of the authors (I.C.) is grateful to the Lady Tata Memorial Trust (Mumbai) for providing a senior research scholarship.

References

1. Borchers, A. T.; Stern, J. S.; Hackman, R. M.; Keen, C. L.; Gershwin, M. E. *Proc. Soc. Exp. Biol. Med.* **1999**, *221*, 281–293.
2. Wasser, S. P.; Weis, A. L. *Crit. Rev. Immunol.* **1999**, *19*, 65–96.
3. Leung, Y. M.; Fung, K. P.; Choy, Y. M. *Immunopharmacology* **1997**, *35*, 255–263.
4. Sasaki, T.; Abiko, N.; Sugino, Y.; Nitta, K. *Cancer Res.* **1978**, *38*, 379–383.
5. Cihara, G.; Hamuro, J.; Maeda, Y. Y.; Arai, Y.; Fukuoka, F. *Nature* **1970**, *225*, 943–944.
6. Wasser, S. P.; Weis, L. A. *Int. J. Med. Mushrooms* **1999**, *1*, 31–62.
7. Kawagishi, H.; Inagaki, R.; Kanao, T.; Mizuno, T. *Carbohydr. Res.* **1989**, *186*, 267–273.
8. Daba, A. S.; Ezeronye, O. U. *Afr. J. Biotechnol.* **2003**, *2*, 672–678.
9. Skeeleton, G. S.; Matanganyidze, C. *Lett. Bot.* **1981**, *3*, 143–149.
10. Khowala, S.; Sengupta, S. *Enzyme Microb. Technol.* **1992**, *14*, 144–149.
11. Rouland, C.; Matoub, M.; Mora, P.; Petek, F. *Carbohydr. Res.* **1992**, *233*, 273–278.
12. Khowala, S.; Ghosh, A. K.; Sengupta, S. *Appl. Microbiol. Biotechnol.* **1992**, *37*, 287–292.
13. Sengupta, S.; Ghosh, A. K. *Biochim. Biophys. Acta* **1991**, *1076*, 215–220.
14. Ghosh, A. K.; Sengupta, S. *J. Biol. Sci.* **1987**, *11*, 275–285.
15. Adewusi, S. R. A.; Alofe, F. V.; Odeyemi, O.; Folabi, A. O.; Oke, O. L. *Plant Foods Human Nutr.* **1993**, *43*, 115–121.
16. Mondal, S.; Chakraborty, I.; Pramanik, M.; Rout, D.; Islam, S. S. *Carbohydr. Res.* **2004**, *339*, 1135–1140.
17. York, W. S.; Darvill, A. K.; McNeil, M.; Stevenson, T. T.; Albersheim, P. *Methods Enzymol.* **1985**, *118*, 33–40.
18. Gerwig, G. J.; Kamerling, J. P.; Vliegenthart, J. F. G. *Carbohydr. Res.* **1978**, *62*, 349–357.
19. Ciucanu, I.; Kerek, F. *Carbohydr. Res.* **1984**, *131*, 209–217.
20. Purdie, T.; Irvine, J. C. R. *J. Chem. Soc.* **1904**, *85*, 1049–1070.
21. Gruter, M.; Leeflang, B. R.; Kuiper, J.; Kamerling, J. P.; Vliegenthart, J. F. G. *Carbohydr. Res.* **1993**, *239*, 209–226.
22. Agarwal, P. K. *Phytochemistry* **1992**, *31*, 3307–3330.
23. Hara, C.; Kiho, T.; Tanaka, Y.; Ukai, S. *Carbohydr. Res.* **1982**, *110*, 77–87.
24. Kiho, T.; Kasturagawa, M.; Nagai, K.; Ukai, S. *Carbohydr. Res.* **1992**, *224*, 237–243.
25. Hoffman, J.; Lindberg, B.; Svensson, S. *Acta Chem. Scand.* **1972**, *26*, 661–666.