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Structural investigation of water-soluble polysaccharides extracted from the fruit bodies of *Coprinus comatus*

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ABSTRACT

Water-soluble polysaccharide material, extracted from the stipes of the fruit bodies of *Coprinus comatus* by hot water, was fractionated by sequential weak anion-exchange and size-exclusion chromatography. The relevant fractions were subjected to structural analysis, including (D/L) monosaccharide/methylation analysis and 1D/2D NMR spectroscopy. Besides the disaccharide α,α-trehalose [α-D-Glc*p*-(1↔1)-α-D-Glc*p*], high-molecular-mass α-D-glucans (the most abundant component) consisting of [→4)-α-D-Glc*p*-(1→]$_n$ backbones with ~10% branching at C-6 by terminal α-D-Glc*p*-(1→6)- or α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)- units, lower-molecular-mass linear β-D-glucans consisting of [→6)-β-D-Glc*p*-(1→]$_m$ sequences, and a lower-molecular-mass pentasaccharide-repeating α-L-fuco-α-D-galactan, {→6)-α-D-Gal*p*-(1→6)-[α-L-Fuc*p*-(1→2)-]α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→}$_p$, were found to be present.

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1. Introduction

Coprinus comatus, the shaggy ink cap, lawyer's wig, or shaggy mane, is a common mushroom often seen growing on lawns, along gravel roads, and waste areas. Already for many years the Chinese population appreciates these species, looking like chicken drumsticks, for their high nutritional value and their delicious taste. Nowadays, the mushrooms are widely cultivated in China and their output exceeds 380 thousand tons per year (CEFA, 2008).

In the past decades the incidence of diabetes, particularly type 2 diabetes which accounts for >90% of the cases, has increased quickly (151 million in 2000 to 285 million in 2010) (IDF, 2009). Some mushroom species have been reported to possess hypoglycemic activity in diabetic (*ob*/*ob*) mice (Cho et al., 2007) and patients with type 2 diabetes (Konno et al., 2001). With respect to *C. comatus*, several studies have demonstrated that its consumption helps in regulating blood glucose concentrations (Bailey, Turner, Jakeman, & Hayes, 1984; Yu et al., 2009).

In our recent study of the fruit bodies of *C. comatus*, a comparison of an ethanol extract, an alkali-soluble polysaccharide fraction, a protein fraction, a crude fiber fraction from the stipes, and a water-soluble polysaccharide fraction revealed that the latter fraction gave the best glucose-lowering activity. In alloxan-induced diabetic mice it decreased the blood glucose to a level similar to that of normal mice after 28 days (Li, Lu, & Suo, 2010). Furthermore, it was shown that the cap and stipe of *C. comatus* have anti-oxidant properties (Li, Lu, Suo, Nan, & Li, 2010).

Here, we report a structural analysis of the water-soluble polysaccharide fraction from the fruit bodies of *C. comatus*. As the stipe of the fruit bodies contains more polysaccharide material than the cap (47.8% versus 21.9%) (Li, Lu, & Suo, 2010), the stipe polysaccharides were targeted in this study.

2. Experimental

2.1. Materials

Fresh fruit bodies of *C. comatus* were obtained from Xinxiang City, Henan Province, China. The stipe and the cap of the fruit bodies were separated and cut half, then air-dried in an oven (beginning

Abbreviations: DP, degree of polymerization; EI-MS, electron-impact mass spectrometry; GLC, gas-liquid chromatography; HSQC, heteronuclear single quantum coherence; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; MLEV, composite pulse devised by M. Levitt; NMR, nuclear magnetic resonance; NOESY, nuclear overhauser enhancement spectroscopy; TLC, thin-layer chromatography; TOCSY, total correlation spectroscopy; UV, ultraviolet.

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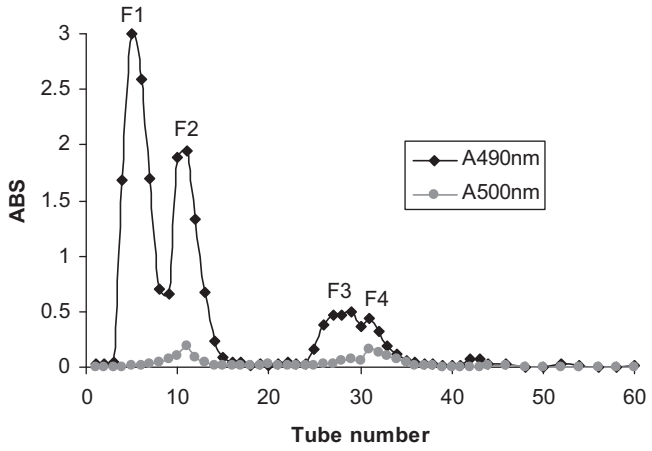


Fig. 1. Chromatogram of the crude water-soluble polysaccharide material prepared from the stipes of the fruit bodies of *Coprinus comatus* on a DEAE-Sepharose CL-6B column (3.5 cm × 30 cm). Fractions F1 and F2 were eluted with 0.2 M sodium phosphate buffer, pH 6; fractions F3 and F4 were eluted with a linear gradient of 0.3–1.5 M NaCl in 0.2 M sodium phosphate buffer, pH 6; flow rate 2 mL/min; monitored by a phenol–H_2SO_4 assay at 490 nm (carbohydrate) and a Folin–phenol assay at 500 nm (protein).

at 30 °C, then increasing 5 °C every 3 h until 45 °C). The dried stipes were ground to powder (40 mesh).

2.2. Extraction of water-soluble polysaccharide material

Stipe powder of *C. comatus* (100 g) was extracted three times with 1 L 95% ethanol under reflux for 2 h to remove lipid, and the residue was extracted three times with 2 L distilled water for 2 h at 80 °C with intermediate centrifugation (2000 × *g*, 15 min). After concentrating the collected aqueous supernatants to 400 mL (reduced pressure at 40 °C), a precipitation was performed with 3 volumes of 95% ethanol. The precipitate was washed with ethanol and acetone, and then dried at 40 °C, yielding 12.65 g crude polysaccharide material.

2.3. Fractionation of water-soluble polysaccharide material

Crude polysaccharide material (500 mg) was dissolved in 100 mL 0.2 M sodium phosphate buffer (pH 6.0), and after centrifugation the solution was applied to a DEAE-Sepharose CL-6B column (3.5 cm × 30 cm). Two fractions (F1 and F2) were eluted with 0.2 M sodium phosphate buffer (pH 6.0), and another two fractions (F3 and F4) were eluted by a linear gradient of 0.3–1.5 M NaCl in 0.2 M sodium phosphate buffer (pH 6.0) (Fig. 1). The elution was performed at a flow rate of 2 mL/min, 10 mL/tube, and monitored by a phenol–H_2SO_4 assay at 490 nm and a Folin–phenol assay at 500 nm. The four isolated fractions were dialyzed against distilled water, and then lyophilized. Fraction F1 was further fractionated on a Sepharose CL-6B column (1.6 cm × 100 cm), eluted with 0.1 M NaCl at a flow rate of 20 mL/h, 15 min/tube, and monitored by a phenol–H_2SO_4 assay at 490 nm and a Folin–phenol assay at 500 nm, yielding the carbohydrate-positive fraction F1-1. Fraction F2 was fractionated on the same column, now eluted with distilled water, yielding a carbohydrate-positive fraction F2-2, which was further purified on a Bio-Gel P-2 column (1.1 cm × 48 cm), eluted with 10 mM ammonium bicarbonate at a flow rate of 10 mL/h. Fractions F3 and F4 were further fractionated on a Sepharose CL-4B column (2.8 cm × 50 cm), eluted with 10 mM ammonium bicarbonate at a flow rate of 50 mL/h, and monitored by UV absorption at 206 nm and by a phenol–H_2SO_4 assay at 490 nm, yielding three carbohydrate-positive fractions, encoded F3-1, F3-2, and F4-3, respectively.

2.4. Monosaccharide analysis

Samples were subjected to methanolysis (1 M methanolic HCl, 24 h, 85 °C; internal standard mannitol). The resulting mixture of methyl glycosides was trimethylsilylated (1:1:5 hexamethyldisilazane–trimethylchlorosilane–pyridine; 30 min, room temperature), then quantitatively analyzed by GLC on an EC-1 column (30 m × 0.32 mm; Alltech, Deerfield, IL), using a Chrompack CP9002 gas chromatograph with flame-ionization detector (temperature program 140–240 °C, 4 °C/min) (Kamerling & Vliegenthart, 1989). The absolute configurations of the monosaccharides were determined by GLC analysis of the trimethylsilylated (−)-2-butyl glycosides under the same conditions (Gerwig, Kamerling, & Vliegenthart, 1978).

2.5. MALDI-TOF mass spectrometry

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS), in positive-ion mode, was performed on an AXIMA™ instrument (Shimadzu Kratos Inc., Manchester, UK), equipped with a nitrogen laser (337 nm, 3 ns pulse width), using aqueous 10% 2,5-dihydroxybenzoic acid as a matrix.

2.6. Methylation analysis

Samples were permethylated using CH_3I and solid NaOH in DMSO as described (Needs & Selvendran, 1993), then hydrolyzed with 2 M TFA (2 h, 120 °C), to give the partially methylated monosaccharides, which were reduced with $NaBD_4$ (2 h, room temperature). After neutralization with HOAc and removal of boric acid by co-evaporation with methanol, followed by acetylation with 1:1 acetic anhydride-pyridine (30 min, 120 °C), the mixtures of partially methylated alditol acetates were analyzed by combined gas-liquid chromatography electron-impact mass spectrometry (GLC-EI-MS) on an EC-1 column (30 m × 0.25 mm) using a GCMS-QP2010 Plus instrument (Shimadzu Kratos Inc.) using a temperature gradient of 140–250 °C at 8 °C/min (Kamerling & Vliegenthart, 1989).

2.7. NMR spectroscopy

Resolution-enhanced 1D/2D 500-MHz NMR spectra were recorded in D_2O on a Varian Inova 500 spectrometer (NMR Center, University of Groningen) or on a Bruker DRX-500 spectrometer (Bijvoet Center, Department of NMR spectroscopy, Utrecht University) at a probe temperature of 300 K. Before analysis, samples were exchanged twice in D_2O (99.9 atom% D, Cambridge Isotope Laboratories, Inc., Andover, MA) with intermediate lyophilization, and then dissolved in 0.6 mL D_2O. Suppression of the HOD signal was achieved by applying a WEFT pulse sequence for one-dimensional experiments and by a pre-saturation of 1 s during the relaxation delay in two-dimensional experiments. The TOCSY spectra were recorded using an MLEV-17 mixing sequence with spin-lock times of 20–200 ms. The NOESY spectra were recorded using standard Bruker XWINNMR software with a mixing time of 200 ms. The carrier frequency was set at the downfield edge of the spectrum in order to minimize TOCSY transfer during spin-locking. Natural abundance ^1H–^{13}C HSQC experiments (^1H frequency 500.0821 MHz, ^{13}C frequency 125.7552 MHz) were recorded without decoupling during acquisition of the ^1H FID. Resolution enhancement of the spectra was performed by a Lorentzian-to-Gaussian transformation for one-dimensional spectra or by multiplication with a squared-bell function phase shifted

by π/(2.3) for two-dimensional spectra, and when necessary, a fifth order polynomial baseline correction was performed. Chemical shifts (δ) are expressed in ppm by reference to internal acetone (δ 2.225 for ^{1}H and δ 31.08 for ^{13}C).

3. Results

3.1. Chemical composition of the stipes of C. comatus

The chemical composition of the powdered stipes of the fruit bodies of *C. comatus* comprises: moisture, 7.1%; ash, 11.4%; lipid, 4.1%; polysaccharide, 47.8%; reducing sugar, 2.5%; crude protein, 12.7%; soluble protein, 7.2%; amino acids, 0.6%; nucleic acids, 3.9%, and crude fiber, 7.2% (Li, Lu, & Suo, 2010).

3.2. Fractionation of the crude water-soluble polysaccharide material prepared from the stipes of C. comatus

After delipidation of the stipe powder with ethanol, a water-soluble polysaccharide fraction was prepared by hot water extraction (80 °C) and subsequent precipitation with ethanol. The yield of the isolated crude polysaccharide material was 12.6% (w/w) and its carbohydrate content was 70.5% (w/w) based on a phenol–H_2SO_4 assay. The remaining 29.5% was predominantly protein.

Using DEAE-Sepharose CL-6B weak anion-exchange chromatography, the crude polysaccharide material (500 mg) was separated into four fractions, i.e. two neutral fractions, denoted F1 and F2, and two charged fractions, denoted F3 and F4 (Fig. 1). After dialysis against distilled water and lyophilization, the yields (w/w) of F1, F2, F3, and F4 were 21.7%, 15.1%, 4.3%, and 2.3%, respectively.

The fractions F1 and F2 were each further separated by gel-filtration on Sepharose CL-6B, affording the more pure, carbohydrate-positive fractions F1-1 and, after an extra separation on Bio-Gel P-2, F2-2, respectively. The strongly overlapping charged fractions F3 and F4 were each further separated by gel-filtration on Sepharose CL-4B, yielding for F3 two main carbohydrate-positive fractions F3-1 and F3-2, and for F4 one main carbohydrate-positive fraction F4-3, respectively. As it will be shown below, no charged carbohydrate components were found in fractions F3-1, F3-2, and F4-3; the original charged fractions F3 and F4 also contained protein material, which may have been associated in some way with these polysaccharides. These five carbohydrate-containing fractions were investigated in detail by NMR spectroscopy.

3.3. Fraction F1-1

Fraction F1-1 was protein-free according to a Folin–phenol assay and UV tests at 260 and 280 nm. Monosaccharide analysis, including absolute configuration determination, revealed the presence of D-glucose only (Table 1). Methylation analysis showed that the F1-1 polysaccharide material was composed of terminal (10.8%), 4-mono-substituted (76.0%), 6-mono-substituted (3.9%), and 4,6-di-disubstituted (9.3%) Glc residues (Table 2), suggesting the presence of a glucan built up mainly from (1→4)-linked residues, with about 10% branching at C-6 and a minor amount of (1→6)-linked residues.

Table 1
Monosaccharide analysis (mol%) of water-soluble (poly)saccharide fractions isolated from the stipes of the fruit bodies of *C. comatus*.

Fraction	D-Glc	D-Gal	L-Fuc	D-Man	D[a]-GlcA
F1-1	100	0	0	0	0
F2-2	100	0	0	0	0
F3-1	100	0	0	0	0
F3-2	34.3	51.7	11.3	2.6	<0.5
F4-3	89.9	2.8	0.5	2.1	1.5

[a] Tentatively assigned, due to low abundance.

Table 2
Methylation analysis of water-soluble (poly)saccharide fractions isolated from the stipes of the fruit bodies of *C. comatus*.

Structural feature	Molar percentages				
	F1-1	F2-2	F3-1	F3-2	F4-3
Glc*p*(1→	10.8	100	10.5	3.8	6.2
→4)Glc*p*(1→	76.0		76.5	4.5	4.8
→6)Glc*p*(1→	3.9		3.2	15.3	84.4
→4,6)Glc*p*(1→	9.3		9.8	3.2	0.6
Gal*p*(1→				2.5	
→6)Gal*p*(1→				47.2	2.5
→2,6)Gal*p*(1→				10.3	
Man*p*(1→				2.3	1.5
Fuc*p*(1→				11.0	

The ^{1}H NMR spectrum of fraction F1-1 (Fig. 2a), and detailed inspection of TOCSY spectra with different mixing times (spectra not shown), revealed two overlapping α-anomeric signals at δ 5.40/5.39 (residues **A** and **B**), and one signal at δ 4.96 (residue **C**), corresponding with the occurrence of -(1→4)-α-D-Glc*p*-(1→4)-, -(1→4,6)-α-D-Glc*p*-(1→4)-, and terminal α-D-Glc*p*-(1→6)- elements, respectively (Dobruchowska et al., 2012; Van Leeuwen et al., 2008). The assignments of the protons and carbons (via HSQC measurements) of the residues are listed in Table 3. The peak area ratio of **A** + **B**:**C** was 87:13, which corresponds roughly with the methylation analysis. Due to its low abundance, the 6-mono-substituted glucose residue (methylation analysis, 3.9%) could not clearly be detected in the ^{1}H NMR analysis, but could stem from a -(1→6)-α-D-Glc*p*-(1→6)-**D** unit (having overlapping signals H-1 at δ 4.97, H-2 at δ 3.58, and H-3 at δ 3.70 with the **C** unit).

Considering the various analytical data, it is proposed that F1-1 contains an α-D-glucan, having a backbone of →4)-α-D-Glc*p*-(1→ units with approximately 10% branching at C-6 by terminal

Table 3
^{1}H and ^{13}C NMR chemical shifts[a] (δ, D_2O, 300 K) of glucose residues of the α-D-glucan, present in fraction F1-1.

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
A -(1→4)-α-D-Glc*p*-(1→4)-	5.40 100.7	3.62 72.6	3.95 74.3	3.64 78.2	3.84 72.2	3.87; 3.81 61.5
B -(1→4,6)-α-D-Glc*p*-(1→4)-	5.39 100.5	3.64 72.6	4.02 74.4	3.65 78.2	3.98 72.1	4.00; 3.79 66.5
C α-D-Glc*p*-(1→6)-	4.96 98.8	3.55 72.4	3.70 74.3	3.43 70.6	3.73 73.3	3.85; 3.76 61.7
D -(1→6)-α-D-Glc*p*-(1→6)-	4.97 98.8	3.58 72.4	3.70 74.3	n.d.[b] n.d.	n.d. n.d.	n.d. n.d.

[a] In ppm relative to the signal of internal acetone (δ 2.225 for ^{1}H and δ 31.07 for ^{13}C).
[b] Not determined, due to low abundance.

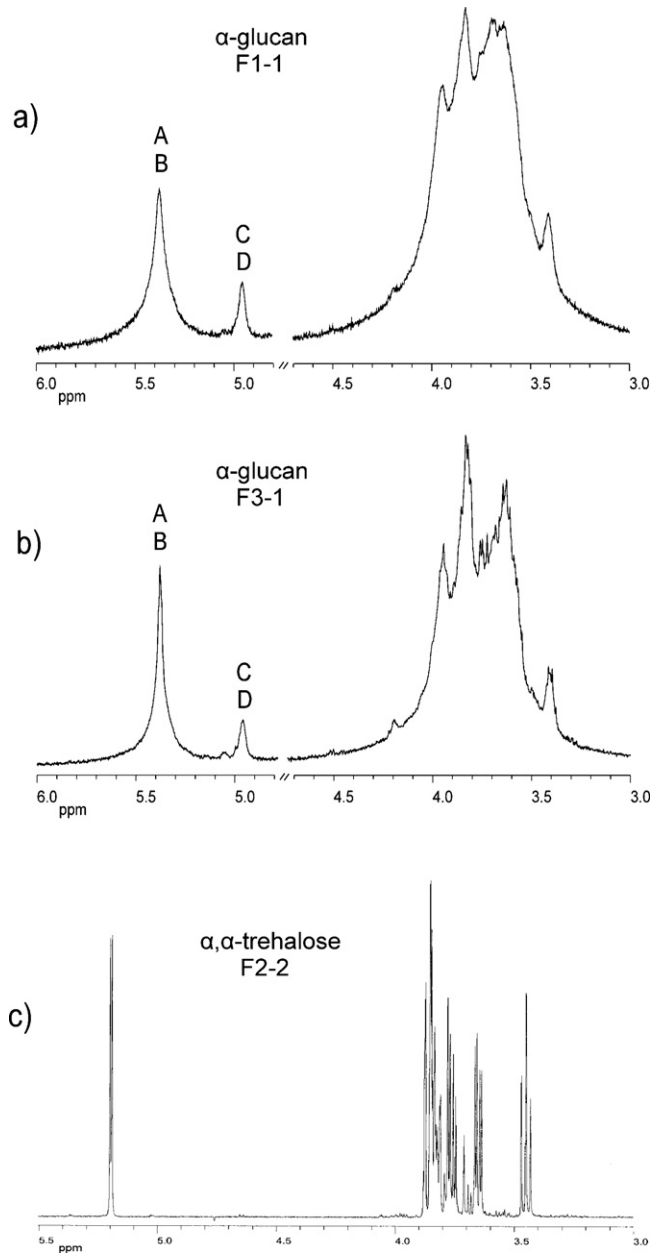


Fig. 2. 1D 500-MHz ^{1}H NMR spectra of fraction F1-1 (a) and fraction F3-1 (b), both containing an (1→4)-α-D-glucan with ~10% (1→6) branching, and fraction F2-2 (α,α-trehalose) (c) in D_2O at 300 K.

α-D-Glc*p*-(1→6)- (~7%) or α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)- (~3%) units. Although a choice between a branched repeating saccharide unit and a backbone with non-regularly distributed side chains could not be made, for the sake of clarity the α-D-glucan will be presented as a repeating saccharide unit, as follows:

C α-D-Glc*p*
1
↓
6
D α-D-Glc*p* (+/-)
1
↓
6

{→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1[→4)-α-D-Glc*p*-(1]$_4$→4)-α-D-Glc*p*-(1→}$_n$
A **A** **B** **A** **A** **A**

Terminal α-D-Glc*p*-(1→4)- elements (H-1, δ 5.39; H-2, δ 3.59; H-3, δ 3.70; H-4, δ 3.42) were not detected in the ^{1}H NMR analysis, probably due to the high molecular mass of the glucan (1267 kDa; data not shown) (Dobruchowska et al., 2012; Van Leeuwen et al., 2008).

3.4. Fraction F2-2

The only monosaccharide component in the purified fraction F2-2 was D-glucose (Table 1), whereas thin-layer chromatography (TLC) showed the presence of a compound with the same retention time as maltose (data not shown). MALDI-TOF-MS analysis ($[M+Na]^+$, 365 Da) indicated a molecular weight of 342 Da in accordance with a disaccharide, and methylation analysis revealed the presence of only terminal Glc units (Table 2). The 1D ^{1}H NMR spectrum of F2-2 (Fig. 2c) turned out to be identical with that of α,α-trehalose, α-D-Glc*p*-(1↔1)-α-D-Glc*p* (H-1, δ 5.200, $^3J_{1,2}$ = 4 Hz).

3.5. Fraction F3-1

Monosaccharide analysis of fraction F3-1 showed the presence of D-glucose only (Table 1). Methylation analysis (Table 2) and ^{1}H NMR analysis (Fig. 2b) indicated an (1→4)-α-D-glucan structure with about 10% →6-branching, similar to the structure established for fraction F1-1.

3.6. Fraction F3-2

Monosaccharide analysis, including D/L determination, of fraction F3-2 mainly revealed the presence of D-glucose, D-galactose, and L-fucose, together with a small amount of D-mannose and a trace amount of glucuronic acid (Table 1). Methylation analysis (Table 2) revealed four main substitution features: →6)Gal*p*(1→ (47.2%), →2,6)Gal*p*(1→ (10.3%), Fuc*p*(1→ (11.0%), and →6)Glc*p*(1→ (15.3%). Furthermore, smaller amounts of terminal Glc*p*, Gal*p*, and Man*p* residues were detected, as well as 4-mono- and 4,6-di-substituted Glc units. Due to the applied methylation analysis protocol, the substitution pattern of the trace amount of glucuronic acid could not be detected.

Based on the subsequent TOCSY assignments using different mixing times (e.g. Fig. 3, 200 ms), the ^{1}H NMR spectrum (Fig. 3) of fraction F3-2 contained six anomeric signals at δ 5.08 (residue **A**), δ 5.06 (residue **B**), δ 5.02 (residue **C**), δ 5.00 (residue **D**), δ 4.98 (residue **E**), and δ 4.532 (residue **F**), respectively. The **A** to **E** signals showed $^3J_{1,2}$ < 3 Hz, indicating α-anomeric configurations. However, residue **F** has a β-anomeric configuration based on its chemical shift and $^3J_{1,2}$ = 8 Hz. In the HSQC spectrum (Fig. 3), six anomeric carbon signals were present, i.e. at δ 103.7 (residue **F**), 102.1 (residue **A**), and 98.6 (residues **B**, **C**, **D**, and **E**). Terminal Glc*p*, Gal*p*, and Man*p* residues, as well as 4-mono- and 4,6-di-substituted Glc units, as found by methylation analysis, occurred at too low amounts (<3.5%) to be detected in the NMR analysis.

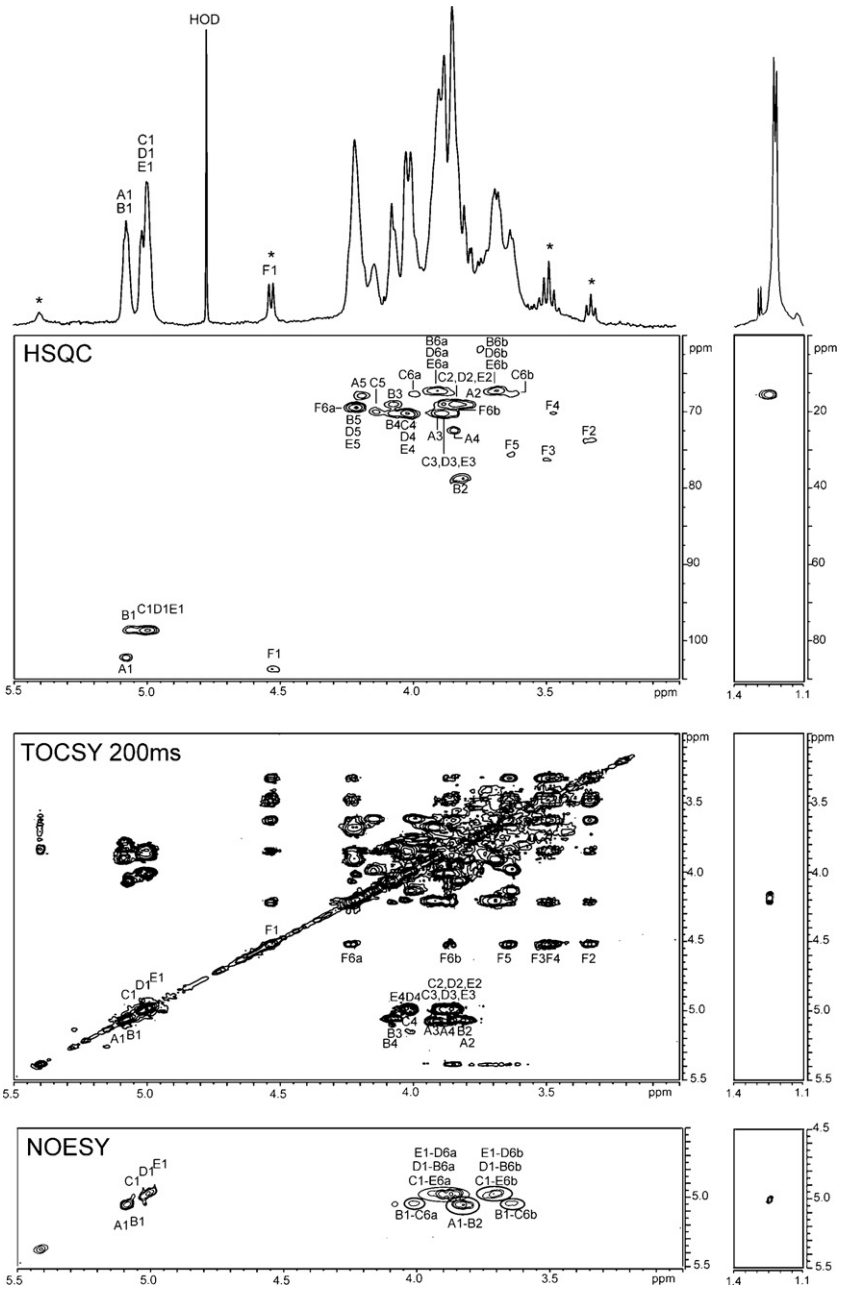


Fig. 3. 1D 500-MHz ^{1}H NMR, HSQC, TOCSY (200 ms), and NOESY spectra of fraction F3-2 in D_2O at 300 K. The main compound is an α-L-fuco-α-D-galactan. Signals indicated with an asterix are stemming from an α-D-glucan and a β-D-glucan. For full details, see text.

The ^{1}H and ^{13}C chemical shifts of residues **A**–**E** are presented in Table 4. They were assigned using TOCSY (different mixing times) combined with HSQC spectra. The broad signal at $\delta \sim 5.07$ contained two H-1 protons stemming from a terminal α-L-Fuc*p* residue (**A**) and a 2,6-di-substituted α-D-Gal*p* residue (**B**). The complete scalar-coupling network of residue **A** was deduced from the TOCSY spectra at different mixing times between 10 and 200 ms. The methyl group of residue **A** was reflected by a high-field signal at δ 1.240 (Fig. 3). Although the TOCSY tracks of **A** H-1 and **B** H-1 were overlapping, the set of **B** H-1,2,3,4,5,6a,6b signals could be determined in connection with the HSQC spectrum. The downfield shifts of **B** C-2 (δ 78.5, $\Delta\delta$ 9.3; Me α-D-Gal*p*, $\delta_{C\text{-}2}$ 69.2) and **B** C-6 (δ 67.3, $\Delta\delta$ 5.1; Me α-D-Gal*p*, $\delta_{C\text{-}6}$ 62.2) (Bock & Thøgersen, 1982) supported the 2,6-di-substitution of the Gal*p* **B** residue. The broad signal at $\delta \sim 5.00$ contained three H-1 protons, belonging to 6-mono-substituted α-D-Gal*p* residues (**C**, **D**, and **E**). Due to the equatorial orientation of H-4, the TOCSY **C**, **D**, and **E** H-1 tracks showed only three cross-peaks H-2, H-3, and H-4. The values for H-5 and H-6a,6b followed from the HSQC spectrum. The downfield shifts of **D** C-6/**E** C-6 (δ 67.3, $\Delta\delta$ 5.1) and of **C** C-6 (δ 67.7, $\Delta\delta$ 5.5) are in accordance with 6-substituted α-D-Gal*p* residues (Bock & Thøgersen, 1982).

Inspection of the 2D NOESY spectrum (Fig. 3) showed on the **A** H-1 track a NOE connectivity with **B** H-2 in agreement with an **A**(1→2)**B** linkage. Inter-residue NOE cross-peaks between **D** H-1 and **B** H-6a/b supported the **D**(1→6)**B** linkage, indicating the branching position of residue **B**. The NOE cross-peaks between **B** H-1 and **C** H-6a/b support the **B**(1→6)**C** linkage, whereas

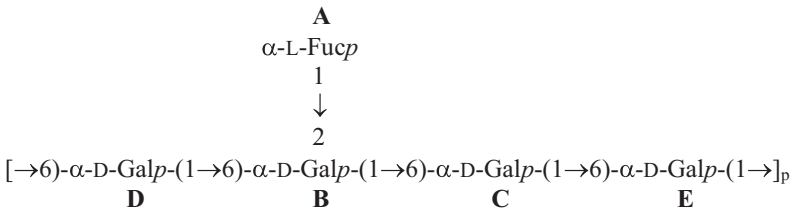
Table 4
^{1}H and ^{13}C chemical shifts[a] (δ, D_2O, 300 K) of monosaccharide residues of the α-L-fuco-α-D-galactan, present in fraction F3-2.

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; CH_3 C-6
A α-L-Fuc*p*-(1→2)-	5.08 102.1	3.80 69.2	3.92 70.3	3.85 72.5	4.19 67.9	1.24 16.3
B -(1→2,6)-α-D-Gal*p*-(1→6)-	5.06 98.6	3.83 78.5	4.07 69.0	4.08 70.2	4.21 69.5	3.91; 3.69 67.3
C -(1→6)-α-D-Gal*p*-(1→6)-	5.02 98.6	3.85 69.0	3.88 70.3	4.02 70.2	4.14 70.0	4.00; 3.62 67.7
D -(1→6)-α-D-Gal*p*-(1→6)-	5.00 98.6	3.85 69.0	3.88 70.3	4.02 70.2	4.21 69.5	3.91; 3.69 67.3
E -(1→6)-α-D-Gal*p*-(1→6)-	4.98 98.6	3.85 69.0	3.88 70.3	4.03 70.2	4.21 69.5	3.91; 3.69 67.3

[a] In ppm relative to the signal of internal acetone (δ 2.225 for ^{1}H and δ 31.07 for ^{13}C).

overlapping inter-residue NOE cross-peaks reflect the two remaining (1→6) linkages.

In conclusion, it is proposed that the major component in fraction F3-2 is an α-L-fuco-α-D-galactan, consisting of a pentasaccharide repeating unit with the following structure:



It should be noted that the ^{1}H NMR spectrum of F3-2 is similar to the spectrum published for a neutral fucogalactan isolated from the mycelium of *C. comatus* (Fan et al., 2006), although the chemical shift data show some deviations from our assignments. However, our values (Table 4) are more in agreement with the values obtained by a simulation of the structure using the CASPER database (www.casperold.organ.su.se).

The TOCSY pattern for the **F** H-1 signal at δ 4.532 (=residue **A** in Table 5), combined with the **F** C-6 signal at δ 69.5, is typical for -(1→6)-β-D-Glc*p*-(1→6)- residues and strongly suggest the additional presence of a (1→6)-β-D-glucan (minor component) (Hreggvidsson et al., 2011), also found, but more abundant, in fraction F4-3. Comparison of the NMR data of the fractions F3-2 and F4-3 (see below), including the TOCSY, HSQC, and NOESY data, showed that the Glc residues present in fraction F3-2 had no correlations with the Gal and Fuc residues, supporting separate components.

Furthermore, the presence of a minor amount of 4-linked α-D-Glc*p* residues (~4%) was indicated by the H-1 signal at δ 5.40. Combined with the 4-mono- and 4,6-di-substituted Glc residues, as observed in the methylation analysis, it supports the presence of α-D-glucan material, as already discussed for fractions F1-1 and F3-1.

3.7. Fraction F4-3

Monosaccharide analysis, including D/L determination, of fraction F4-3 showed that mainly D-glucose (89.9%) was present with minor amounts of D-galactose, L-fucose, D-mannose, and glucuronic acid (Table 1). Methylation analysis (Table 2) showed the following substitution patterns for glucose: →6)Glc*p*(1→ (84.4%), Glc*p*(1→ (6.2%), →4)Glc*p*(1→ (4.8%), and →4,6)Glc*p*(1→ (0.6%). Furthermore, small amounts of 6-mono-substituted Gal*p* and terminal Man*p* were identified.

Based on the subsequent TOCSY assignments using different mixing times (e.g. Fig. 4, 200 ms), the ^{1}H NMR spectrum showed overlapping major and minor β-anomeric signals at δ 4.532 (residue **A**, $^{3}J_{1,2}$ = 8 Hz) and δ 4.517 (residue **B**), respectively. The presence of a reducing Glc unit is reflected by the **R**α and **R**β H-1 signals at δ 5.225 and 4.645, respectively. Starting from the anomeric signals of **A**, **B**, **R**α, and **R**β in the 2D TOCSY spectrum, the chemical shifts of all non-anomeric protons for each residue could be determined (Table 5) (Hreggvidsson et al., 2011). Although the anomeric signals of **A** and **B** strongly overlap, their specific H-2,3,4,5,6a,6b signal sets could be found making use of the **A** H-6a (δ 4.226) track. Taking into account the ^{13}C chemical shift data, derived from the HSQC spectrum (not shown), the downfield shift of **A** C-6 (δ 69.5, $\Delta\delta$ 7.7; Me β-D-Glc*p*, $\delta_{C\text{-}6}$ 61.8) (Bock & Thøgersen, 1982) demonstrated this residue to be 6-substituted. The ^{1}H chemical shift sets of **A** and **B** are characteristic for internal -(1→6)-β-D-Glc*p*-(1→6)- units and a terminal β-D-Glc*p*-(1→6)- residue, whereas the ^{1}H NMR data of **R**α and **R**β reflect a -(1→6)-D-Glc*p* reducing end (Fujimoto, Hattori, Uno, Murata, & Usui, 2009; Kono, Kawano, Tajima, Erata, & Takai, 1999). The non-reducing terminal position of **B** is also supported by its ^{13}C chemical shifts (**B** C-6, δ 61.5). In the NOESY spectrum (not shown), the inter-residual cross-peaks between **A** H-1 and **R**α/β H-6ab, between **A** H-1 and **A** H-6ab, and between **B** H-1 and **A** H-6ab indicated the sequences **A**(1→6)**R**α/β, **A**(1→6)**A**, and **B**(1→6)**A**, in agreement with a relatively small linear (1→6)-β-D-glucan. Integration of the anomeric signals (**A**+**B**:**R**α/β) revealed a degree of polymerization of about DP16, in close agreement with the methylation analysis. Assuming that the 4- and 4,6-linked Glc residues (methylation analysis) belong to the α-D-glucan material (see F1-1, F3-1, and F3-2), the following structure is proposed for the β-D-glucan:

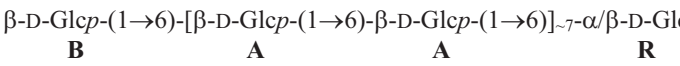


Table 5
^{1}H and ^{13}C chemical shifts[a] (δ, D_2O, 300 K) of glucose residues of the linear (1→6)-β-D-glucan (DP ~16), present in fraction F4-3.

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
A -(1→6)-β-D-Glc*p*-(1→6)-	4.53	3.33	3.50	3.47	3.63	4.22; 3.86
	103.7	73.8	76.3	70.2	75.6	69.5
B β-D-Glc*p*-(1→6)-	4.52	3.33	3.50	3.40	3.46	3.93; 3.73
	103.6	73.8	76.3	70.3	76.5	61.5
Rα -(1→6)-α-D-Glc*p*	5.23	3.55	3.70	3.50	4.00	4.13; 3.90
	92.8	72.4	73.7	70.6	71.4	69.6
Rβ -(1→6)-β-D-Glc*p*	4.65	3.25	3.49	3.47	3.62	4.18; 3.86
	96.7	75.1	76.7	70.6	75.8	70.8

[a] In ppm relative to the signal of internal acetone (δ 2.225 for ^{1}H and δ 31.07 for ^{13}C).

4. Concluding remarks

Five fractions, containing water-soluble (poly)saccharides, were chromatographically isolated from a hot water extract from the stipes of *C. comatus* fruit bodies. One fraction contained the disaccharide α,α-trehalose. It remains unclear why α,α-trehalose was present in the water-soluble polysaccharide material and was not removed by dialysis; probably it co-precipitated/associated with some protein material in the ethanol precipitation step. It is well known that trehalose occurs naturally in insects, plants, fungi, and bacteria, and its major dietary source is mushrooms. Trehalose is implicated in anhydrobiosis – the ability of plants and animals to withstand prolonged periods of desiccation (Aletor, 1995). The other four fractions contained polysaccharide materials, which were analyzed by monosaccharide and linkage analyses and NMR spectroscopy.

First, the most abundant polysaccharide [about 25% (w/w) of the water-soluble material], was identified as an α-D-glucan, built up from a main chain of →4)-α-D-Glc*p*(1→ residues with about 10% branching at C-6 by terminal α-D-Glc*p*-(1→6)- or α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)- units. Similar α-D-glucan structures have been observed earlier for the mushrooms *Agaricus bisporus* and *Agaricus brasiliensis* (Smiderle et al., 2011).

Secondly, a linear β-D-glucan, composed of →6)-β-D-Glc*p*-(1→ residues, was established [<7% (w/w) of the water-soluble material]. Similar β-D-glucan structures have been published earlier for the mushrooms *Agaricus blazei* Murill (Chung, Cho, & Kim, 2005) and *Calocybe indica* (Mandal et al., 2010).

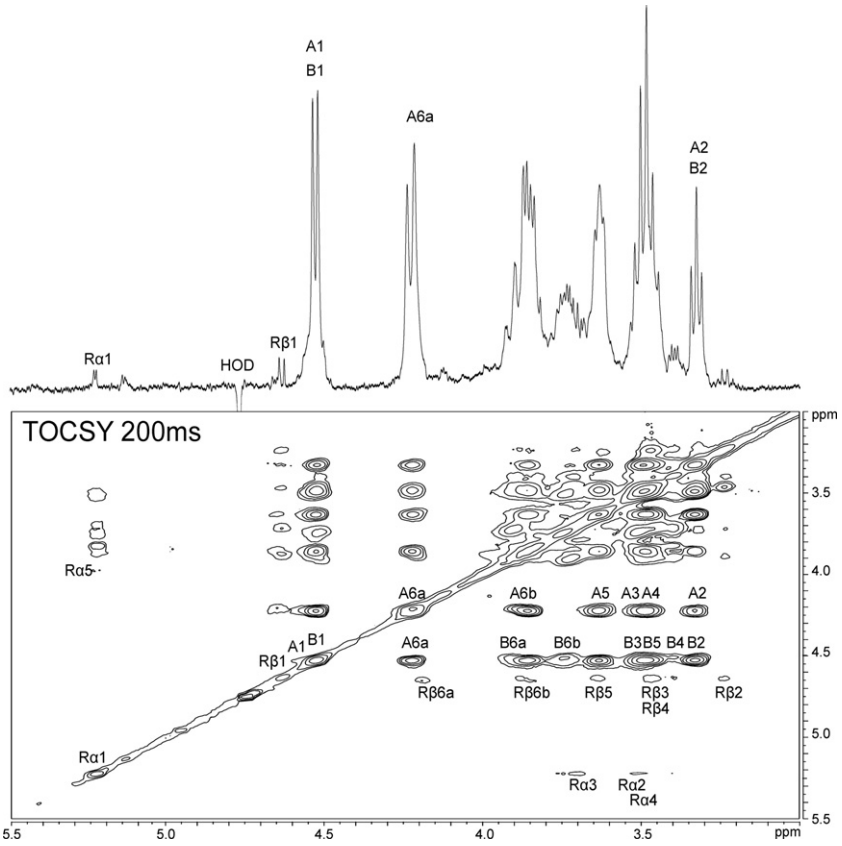


Fig. 4. 1D 500-MHz ^{1}H NMR and TOCSY (200 ms) spectra of fraction F4-3 in D_2O at 300 K. The main compound is a β-D-glucan. For full details, see text.

Thirdly, the structure of an α-L-fuco-α-D-galactan was determined consisting of pentasaccharide repeating units,{→6)-α-D-Gal*p*-(1→6)-[α-L-Fuc*p*-(1→2)-]α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→}$_p$ [<7% (w/w) of the water-soluble material]. An identical fucogalactan has been isolated earlier from the mycelium of *C. comatus* (Fan et al., 2006). Several fungal heterogalactans have been reported previously, including fucogalactans from *Ganoderma applanatum* (Usui, Iwasaki, & Mizuno, 1981), *Agaricus brasilliensis* and *A. bisporus* var. *hortensis* (Komura et al., 2010), mannogalactan from *Pleurotus pulmonarius* (Smiderle et al., 2008), mannofucogalactans from *Fomitella fraxinea* Imaz. (Cho, Koshino, Yu, & Yoo, 1998), *Flammulina velutipes* Sing. (Mukumoto & Yamaguchi, 1977), *Polyporus pinicola* (Fraser, Karácsonyi, & Lindberg, 1967), fucomannogalactan from *Laetiporus sulphureus* Murr (Alquini, Carbonero, Rosado, Cosentino, & Iacomini, 2004), and glucofucogalactan from *Hericium erinaceus Pers* (Wang, Luo, & Liang, 2004). These structures consist mainly of a backbone of (1→6)-linked α-D-Gal*p* residues and are substituted at C-2 by either fucose and/or mannose. For *C. comatus*, this could explain the presence of small amounts of mannose. Some fractions also contained D-glucuronic acid, of which the origin is still unknown and further investigation is needed. Additionally, the correlation between structure and hypoglycemic activity of the water-soluble polysaccharides will be further investigated in future work.

Acknowledgements

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