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

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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 (1D) monosaccharide/methylation analysis and 1D/2D NMR spectroscopy. Besides the disaccharide α:α-trehalose [α-β:α-Glc-(1→1):α-β-Glc], high-molecular-mass α-d-glucans (the most abundant component) consisting of [→4]-α-d-Glc-(1→1) backbone with ~10% branching at C-6 by terminal α-d-Glc-(1→6)- or α-d-Glc-(1→6)-α-d-Glc-(1→6)- units, lower-molecular-mass linear β-d-glucans consisting of [→6]-β-d-Glc-(1→1) sequences, and a lower-molecular-mass pentasaccharide-repeating α-l-fuco-α-d-galactan. [→6]-α-d-Glc-(1→6):[α-l-Fucp-(1→2):α-β-Galp-(1→6)-α-d-Galp-(1→6)-α-d-Galp-(1→1)]n, 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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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$_2$SO$_4$ 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$_2$SO$_4$ 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-4B column (2.8 cm × 50 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$_2$SO$_4$ 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$_3$I 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-EL-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$_2$O 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$_2$O (99.9 atom% D, Cambridge Isotope Laboratories, Inc., Andover, MA) with intermediate lyophilization, and then dissolved in 0.6 mL D$_2$O. Suppression of the HOD signal was achieved by applying a WFT 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 $^1$H–$^{13}$C HSQC experiments ($^1$H frequency 500.0821 MHz, $^{13}$C frequency 125.7552 MHz) were recorded without decoupling during acquisition of the $^1$H 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.
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, 0.8%; 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–H₂SO₄ 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.

The 1H 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-Glcp-(1→4)−, (1→4,6)-α-d-Glcp-(1→4)− and terminal α-d-Glcp-(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 1H NMR analysis, but could stem from α-(1→6)-α-d-Glcp-(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 (1→4)-α-d-Glcp-(1→6) units with approximately 10% branching at C-6 by terminal

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Monosaccharide analysis (mol%) of water-soluble (poly) saccharide fractions isolated from the stipes of the fruit bodies of C. comatus.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction</td>
<td>d-Glc</td>
</tr>
<tr>
<td>F1-1</td>
<td>100</td>
</tr>
<tr>
<td>F2-2</td>
<td>100</td>
</tr>
<tr>
<td>F3-1</td>
<td>100</td>
</tr>
<tr>
<td>F3-2</td>
<td>34.3</td>
</tr>
<tr>
<td>F4-3</td>
<td>89.9</td>
</tr>
</tbody>
</table>

* Tentatively assigned, due to low abundance.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Methylation analysis of water-soluble (poly)saccharide fractions isolated from the stipes of the fruit bodies of C. comatus.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structural feature</td>
<td>Molar percentages</td>
</tr>
<tr>
<td>F1-1</td>
<td>F2-2</td>
</tr>
<tr>
<td>Glc(1→)</td>
<td>10.8</td>
</tr>
<tr>
<td>→4Glc(1→)</td>
<td>76.0</td>
</tr>
<tr>
<td>→6Glc(1→)</td>
<td>3.9</td>
</tr>
<tr>
<td>→4,6Glc(1→)</td>
<td>9.3</td>
</tr>
<tr>
<td>Galp(1→)</td>
<td>2.5</td>
</tr>
<tr>
<td>→6Galp(1→)</td>
<td>10.3</td>
</tr>
<tr>
<td>Manp(1→)</td>
<td>2.3</td>
</tr>
<tr>
<td>Fucp(1→)</td>
<td>11.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 3</th>
<th>1H and 13C NMR chemical shifts* (δ, D2O, 300 K) of glucose residues of the α-d-glucan, present in fraction F1-1.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Residue</td>
<td>H-1</td>
</tr>
<tr>
<td>A (1→4)-α-d-Glcp-(1→4)-</td>
<td>5.40</td>
</tr>
<tr>
<td>B (1→4,6)-α-d-Glcp-(1→4)-</td>
<td>5.39</td>
</tr>
<tr>
<td>C α-d-Glcp-(1→6)-</td>
<td>4.96</td>
</tr>
</tbody>
</table>

* In ppm relative to the signal of internal acetone (δ 2.225 for 1H and δ 31.07 for 13C).

* Not determined, due to low abundance.
Terminal α-D-Glc-(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 1H 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 1H NMR spectrum of F2-2 (Fig. 2c) turned out to be identical with that of α,α-trehalose, α-D-Glc-(1→1)–α-D-Glc(H-1, δ 5.200, 2J1,2 = 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 1H 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 n/a 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)Galp(1→ (47.2%), →2,6)Galp(1→ (10.3%), Fucp(1→ (11.0%), and →6)Glcp(1→ (15.3%). Furthermore, smaller amounts of terminal Glcp, Galp, and Manp 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 1H NMR spectrum (Fig. 3) of fraction F3-2 contained six anomic 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 1J1,2 < 3 Hz, indicating α-anomeric configurations. However, residue F has a β-anomeric configuration based on its chemical shift and 3J1,2 = 8 Hz. In the HSQC spectrum (Fig. 3), six anomic 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 Glcp, Galp, and Manp 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.

\[
\begin{align*}
\text{C} & \quad \alpha-D\text{-Glc} \\
& \quad 1 \downarrow \\
& \quad 6 \\
\text{D} & \quad \alpha-D\text{-Glc} \quad (+/-) \\
& \quad 1 \downarrow \\
& \quad 6 \\
\end{align*}
\]

\[
\begin{align*}
\text{A} & \quad \alpha-D\text{-Glc-(1→4)} \quad \text{A} \\
\text{B} & \quad \alpha-D\text{-Glc-(1→4)} \quad \text{A} \\
\text{A} & \quad \alpha-D\text{-Glc-(1→4)} \quad \text{A} \\
\text{A} & \quad \alpha-D\text{-Glc-(1→4)} \quad \text{A} \\
\end{align*}
\]
Fig. 3. 1D 500-MHz $^1$H NMR, HSQC, TOCSY (200 ms), and NOESY spectra of fraction F3-2 in D$_2$O 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-Fucp residue (A) and a 2,6-di-substituted α-d-Galp 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 $\delta$ 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 ($\delta$ 78.5, $\Delta\delta$ 9.3; Me α-d-Galp, $\delta_{C-2}$ 69.2) and B C-6 ($\delta$ 67.3, $\Delta\delta$ 5.1; Me α-d-Galp, $\delta_{C-6}$ 62.2) (Bock & Thøgersen, 1982) supported the 2,6-di-substitution of the Galp B residue. The broad signal at $\delta \sim 5.00$ contained three H-1 protons, belonging to 6-mono-substituted α-d-Galp 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 ($\delta$ 67.3, $\Delta\delta$ 5.1) and of C C-6 ($\delta$ 67.7, $\Delta\delta$ 5.5) are in accordance with 6-substituted α-d-Galp 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...
overlapping inter-residue NOE cross-peaks reflect the two remaining (1→2) linkages.

In conclusion, it is proposed that the major component in fraction F3-2 is an α-1→fuc-α-1→galactan, consisting of a pentasaccharide repeating unit with the following structure:

\[
\begin{align*}
A & \rightarrow \alpha-L-Fucp \rightarrow 1 \\
B & \rightarrow \alpha-D-Galp-(1→6)\alpha-D-Galp-(1→6)\alpha-D-Galp-(1→6)\alpha-D-Galp-(1→6)
\end{align*}
\]

It should be noted that the 1H 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 CASPERD database (www.casperd.org/iorgan.sue.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-GlcP-(1→6) residues and strongly suggest the additional presence of a (1→6)-β-D-glucon (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-GlcP 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-glucon 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)-GlcP(1→ (84.4%), GlcP(1→ (6.2%), →4)-GlcP(1→ (4.8%), and →4,6)-GlcP(1→ (0.6%).

Furthermore, small amounts of 6-mono-substituted Galp and terminal Manp were identified. According to the subsequent TOCSY assignments using different mixing times (e.g., Fig. 4, 200 ms), the 1H NMR spectrum showed overlapping major and minor β-anomeric signals at δ 4.532 (residue A, 3H, J = 8 Hz) and δ 4.517 (residue B), respectively. The presence of a reducing Glc unit is reflected by the Rα and Rβ H-1 signals at δ 5.225 and 4.645, respectively. Starting from the anomeric signals of A, B, Rα, and Rβ 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-2,3,4,5,6,α,β signal sets could be found making use of the A H-6a (δ 4.226) track. Taking into account the 13C chemical shift data, derived from the HSQC spectrum (not shown), the downfield shift of A C-6 (δ 69.5, Δδ 7.7; Me β-D-GlcP, δC 61.8) (Bock & Thogersen, 1982) demonstrated this residue to be 6-substituted. The 1H chemical shift sets of A and B are characteristic for (1→6)-β-D-Glp-(1→6)- units and a terminal β-D-GlcP-(1→6)-residue, whereas the 1H NMR data of Rα and Rβ reflect a - (1→6)-O-GlcP 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 13C chemical shifts (B C-6, δ 61.5). In the NOESY spectrum (not shown), the inter-residual cross-peaks between A H-1 and Rα/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)Rα/β, A(1→6)A, and B(1→6)A, in agreement with a relatively small linear (1→6)-β-D-glucon structure. Integration of the anomeric signals (A+B:Rα/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-glucon material (see F1-1, F3-1, and F3-2), the following structure is proposed for the β-D-glucon:

\[
\begin{align*}
\beta-D-GlcP-(1→6)[\beta-D-GlcP-(1→6)]\beta-D-GlcP-(1→6)\rightarrow \alpha/\beta-D-GlcP
\end{align*}
\]
Table 5

$^1$H and $^{13}$C chemical shifts (δ, D$_2$O, 300 K) of glucose residues of the linear (1→6)-β-D-glucan (DP = 16), present in fraction F4-3.

<table>
<thead>
<tr>
<th>Residue</th>
<th>H-1</th>
<th>C-1</th>
<th>H-2</th>
<th>C-2</th>
<th>H-3</th>
<th>C-3</th>
<th>H-4</th>
<th>C-4</th>
<th>H-5</th>
<th>C-5</th>
<th>H-6a; C-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (1→6)-β-D-Glc- (1→6)-</td>
<td>4.53</td>
<td>3.33</td>
<td>3.50</td>
<td>3.47</td>
<td>3.63</td>
<td>4.22; 3.86</td>
<td>103.7</td>
<td>73.8</td>
<td>76.3</td>
<td>70.2</td>
<td>75.6; 69.5</td>
</tr>
<tr>
<td>B β-D-Glc- (1→6)-</td>
<td>4.52</td>
<td>3.33</td>
<td>3.50</td>
<td>3.40</td>
<td>3.46</td>
<td>3.93; 3.73</td>
<td>103.6</td>
<td>73.8</td>
<td>76.3</td>
<td>70.3</td>
<td>75.6; 61.5</td>
</tr>
<tr>
<td>αβ- (1→6)-α-D-Glc</td>
<td>5.23</td>
<td>3.55</td>
<td>3.70</td>
<td>3.50</td>
<td>4.00</td>
<td>4.13; 3.90</td>
<td>92.8</td>
<td>72.4</td>
<td>73.7</td>
<td>70.6</td>
<td>71.4; 69.6</td>
</tr>
<tr>
<td>Rβ- (1→6)-β-D-Glc</td>
<td>4.65</td>
<td>3.25</td>
<td>3.49</td>
<td>3.47</td>
<td>3.62</td>
<td>4.18; 3.86</td>
<td>96.7</td>
<td>75.1</td>
<td>76.7</td>
<td>70.6</td>
<td>75.8; 70.8</td>
</tr>
</tbody>
</table>

* 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 αβ-trehalose. It remains unclear why αβ-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(1→ residues with about 10% branching at C-6 by terminal α-D-Glc-(1→6)- or α-D-Glc-(1→6)-α-D-Glc-(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(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$_2$O 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-Galp-(→1-6)-α-L-Fucp-(→1-2)-α-d-Galp-(→1-6)-α-D-Galp-(→1-6)-α-D-Galp-(→1-3)-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 Canodermatia platanum (Usui, Iwasaki, & Mizuno, 1981), Agarius brasiliensis and A. bisporus var. hortensis (Komura et al., 2010), mannosagalactan from Pleurotus pulmonarius (Snyder & et al., 2008), mannofucogalactans from Fomitella fraxinea Imaz. (Cho, Koshino, Yu, & Yoo, 1998), Flammulina velutipes Sing. (Mukimoto & Yamaguchi, 1977), Polyspora pinicola (Fraser, Karacsonyi, & Lindberg, 1967), and fucogalactans from Hericium erinaceus Pers. (Wang, Luo, & Liang, 2004). These structures consist mainly of a backbone of (1→6)-linked α-D-Galp 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.

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References