Structural investigation of cell wall polysaccharides extracted from wild Finnish mushroom *Craterellus tubaeformis* (Funnel Chanterelle)

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Abstract

Craterellus tubaeformis (Funnel Chanterelle) is among the most abundant wild mushrooms in Finland. Three polysaccharide fractions were sequentially extracted from the fruiting bodies of *C. tubaeformis*, using hot water, 2% and 25% KOH solutions, respectively, and purified. The monomer composition, molecular weight, and chemical structure were determined using chromatographic and spectroscopic methods. Thermogravimetric analysis was performed as well. The hot water extract consisted mainly of high-molecular weight →2,6)-α-Man-(1→ and →6)-α-Gal-(1→ chains, covalently bound to proteins. The alkali extracts consisted of acidic →6)-β-Glc-(1→, with branches of short →3)-β-Glc-(1→ chains or single β-Glc residues. The use of alkali influenced the glycosidic linkages, molecular mass and thermal stability of the polysaccharide fractions. The use of KOH 2% increased the amount of low molecular weight polysaccharides, resulting in bimodal molecular weight distributions, with little impact on the thermal stability. Conversely, extraction with KOH 25% provided low molecular weight polysaccharides with substantially reduced thermal stability.

1. Introduction

Mushrooms have been considered worldwide as medicinal resources and have traditionally been used for the prevention and treatment of multiple medical conditions. The cell wall components, in particular the β-glucans, have been identified as major responsible for the biological activities of mushroom extracts. These carbohydrate polymers typically consist of glucose units linked by β-(1→3) and β-(1→6) glycosidic bonds (Synytsya et al., 2009). It is generally accepted that the mushroom cell wall is composed of three type of layers: an outer layer of heterosaccharides and proteins extensively modified with carbohydrates, a middle layer representing the β-glucan network and an inner layer of chitin covalently bound to β-glucans. Variations in layer composition are depending upon species, growth conditions and stage of maturity (Chen & Cheung, 2014). Chitin, linear →4)-β-GlcNAc-(1→, is a minor component of the cell wall. Glucans, conversely, are the major structural polysaccharides (Synytsya et al., 2009). Both linear and branched β-glucans have been reported. Glucans serve as the structural constituent to which other cell wall components are covalently attached or bound via hydrogen bonds. The branched glucans can then be cross-linked together, to chitin and/or to glycoproteins. These proteins are N- or O-glycosylated to various degrees, depending on the species, with different oligosaccharides, usually composed of mannose and galactose (Chen & Cheung, 2014).

Polysaccharides have been extracted and characterized from medicinal mushrooms, such as *Ganoderma lucidum* (Lingzhi) or *Lentinus edodes* (Shitake) (Synytsya & Novák, 2013), but also from mushrooms commonly consumed as food, such as *Pleurotus* spp. (Smiderle et al., 2008) and *Agaricus* spp. (Ruthes et al., 2013). An extensive body of research suggests that mushroom β-glucans modulate non-specific reactions of the immune system. In addition, mushroom polysaccharides have been reported to lower the levels of cholesterol and blood sugar, as well as to scavenge free radicals (Giavasis, 2014) and to act as prebiotics in the human gut (Zhao & Cheung, 2011). There is increasing attention towards these molecules in the nutraceutical market, due to their potential for application as dietary supplements and ingredients for food and feed. Additionally, in China and Japan, mushroom polysaccharides are used as adjuvants in cancer therapy (Leung et al., 2006).

The biological properties of mushroom cell wall polysaccharides largely depend on their physicochemical properties, such as molecular weight, degree of branching, presence of proteins, type of glycosidic linkage, and monomer composition. The extraction and purification processes have been shown to impact the physicochemical properties and biological activities (Giavasis, 2014). The choice of extraction

methods determines the layers of the mushroom cell wall to be extracted but also influences the molecular structures of the extracted polymers (Chen & Cheung, 2014).

Craterellus tubaeformis (Funnel Chanterelle) is a basidiomycete belonging to the family Cantharellaceae. It is a common edible mushroom of the Finnish forests, picked during late autumn until early winter (also called "Winter Mushroom"). The mushroom is generally available in large amounts even during poor mushroom years. It is considered a good source of vitamin D2 (Teichmann et al., 2007), with an high content of amino acids contributing to umami and bitter tastes (Manninen et al., 2018). To our knowledge, the only study reported on the bioactive water-soluble compounds from *C. tubaeformis* has been performed by O'Callaghan and coworkers (O'Callaghan et al., 2015), which showed mild anti-inflammatory activity from the raw extract obtained with hot water from this mushroom.

The high forest yield and large availability of this mushroom make it a good raw material for β-glucan exploitation. For such purpose, investigation of the polysaccharides composing the cell wall of *C. tubaeformis* is necessary. The extraction of the macromolecules situated in the lower layers of the cell wall requires the disruption of its strong covalent and non-covalent polymer network, which is usually accomplished with alkali solutions. The stepwise use of alkali allows the fractionation of the cell wall components (Chen & Cheung, 2014). In this study, we have extracted the polysaccharides from *C. tubaeformis* with a stepwise method using three different extraction media of increasing alkalinity. The surface layer of the cell wall was extracted with hot water. A mild alkali solution was used to extract the middle layers of the cell wall, while a strong alkali solution was used to deplete the mushroom cell wall of extractable polysaccharides. We investigated the monomer composition, molecular weight, and structure of the polysaccharides of *C. tubaeformis* and the impact of the extraction methods on their physical and chemical properties. Since industrial treatment of dietary fiber fractions commonly involves high temperatures, the thermal stability of the produced polysaccharide fractions was analyzed with thermogravimetric analysis.

2. Materials and methods

2.1. Fungal material

Fresh fruiting bodies of *C. tubaeformis* were purchased from Sienestä Oy (Kontiomäki, Kainuu, Finland). Fresh mushrooms were dried in an oven for 8 h at 60 °C, to obtain 100 g of dried sample of *C. tubaeformis*.

101 2.2. Polysaccharide extraction and purification

102 Dried mushrooms were ground into a powder form in a mortar (particle size < 3 mm). Prior to
103 polysaccharide extraction, fats and phenolic compounds were removed from the mushroom samples by
104 extracting three times with 500 mL of ethanol (95% v/v purity), each extraction time lasting for 6 hours. The solid
105 residue was subsequently extracted three times with 500 mL of deionized water by solvent reflux (100 ˚C) for 6
106 hours during each extraction. Next, the extraction mixture was filtered, and the supernatants of the three
107 extractions were combined. After filtration, the residue of the hot water extraction was extracted with 500 mL
108 of 2% KOH solution. The extraction was carried out three times at 80 °C, for 3 hours each time (Smiderle et al.,
109 2008). After filtration, the residue was subsequently extracted three times with 500 mL of 25% aqueous
110 potassium hydroxide by refluxing for 3 hours during each extraction (Smiderle et al., 2006). The supernatants
111 were combined after filtration. After extraction, the supernatants of the alkali extractions were neutralized with
112 acetic acid.

113 After evaporation, proteins were removed from the concentrated extracts using the Sevag method and
114 collecting the supernatant after centrifugation at 1225 *x g* for 10 minutes. All the extracts were dialyzed against
115 running tap water for 24 hours using a cellulose membrane (cut-off 12-14 kDa). After dialysis, the
116 polysaccharides were precipitated by addition of cold ethanol (3:1 v/v) and overnight storage at 4 °C. The
117 polysaccharides were collected by centrifugation (13000 *x g* for 20 minutes at 4 °C). Water soluble and insoluble
118 polymers were separated using the freeze-thawing process (Gorin & Iacomini, 1984) three times. Soluble and
119 insoluble polymers were finally separated by centrifugation (13000 *x g* for 20 minutes at 4 °C), with insoluble
120 polysaccharides being precipitated after centrifugation. The collected supernatants, which contained the soluble
121 polysaccharides were freeze-dried and were coded as Crat HW, Crat 2%, and Crat 25%, respectively.

122 The soluble polysaccharides from the hot water and alkali extractions were purified using anion-
123 exchange chromatography. An aliquot of the fractions was dissolved in a minimum amount of deionized water
124 and then loaded on a column (30 cm, 2.2 cm i.d.) packed with DEAE-cellulose, previously equilibrated with
125 deionized water. The column was eluted with potassium chloride (0.05 M) solution and fractions, 5 mL each,
126 were collected. To follow the elution of polysaccharides, the sugar content of each fraction was monitored with
127 the phenol-sulfuric acid method (Section 2.3). The fractions were collected based on the sugar content and
128 profile, dialyzed (12-14 kDa as cut-off molecular weight) against deionized water for 24 hours, changing the

129 water every 4 hours, and freeze-dried. The fractions were coded as Crat HW1, Crat 2%1, and Crat 25%1,
130 respectively, and were further investigated.

131 2.3. Sugar and protein contents and methylation analysis

132 Sugar content of the polysaccharide fractions was measured in triplicate with the phenol-sulfuric acid
133 method adapted for microplate (Masuko et al., 2005). Protein content of semi-purified and purified
134 polysaccharide fractions was measured in triplicate with a modified Lowry method (Markwell et al., 1978).
135 Methylation of Crat 2%1 and Crat 25%1 was performed singularly according to literature (Ciucanu & Kerek,
136 1984). The permethylated polysaccharides were hydrolyzed with HCl 2 M in MeOH at 100 °C for 3 hours,
137 silylated and analyzed with GC-MS (Laine et al., 2002).

138 2.4. Average molecular weight analysis

139 The average molecular weight and molecular weight distribution of polysaccharides in the fractions Crat
140 HW1, Crat 2%1, and Crat 25%1 were determined by high performance size exclusion chromatography (HPSEC),
141 using a Waters 2690 system equipped with a TSK-GMPW column (30 cm x 7.5 mm i.d.) and coupled with a Waters
142 2487 UV detector and a Shimadzu 20A refractive index detector. An aqueous solution of sodium nitrate (0.1 M)
143 was used as mobile phase. A calibration curve was obtained with a series of standard pullulans with molecular
144 weights ranging from 6.20 x 10^3 to 8.05 x 10^5 Da (Pullulan Kit, Polymer Standards Service, Germany).
145 Polysaccharide samples and pullulan standards were dissolved in the mobile phase at a concentration of 1
146 mg/mL and 50 µL of sample or pullulan standard were injected singularly. The system temperature was kept at
147 40 ˚C and the flow rate at 0.5 mL/min. Instrumental error was measured with an injection in triplicate.

148 2.5. Polysaccharide hydrolysis and monomer composition analysis

149 For determination of the monosaccharide composition, the obtained polysaccharides were hydrolyzed
150 in triplicates with 2 M TFA at 100 ˚C for 6 hours, in test tubes with Teflon screw cap. Hydrolysates were filtered
151 through a 0.45 µm regenerated cellulose membrane and pipetted into autosampler vials. *Myo*-inositol solution
152 was added as standard in each vial and the solutions were evaporated to dryness with nitrogen flow and heating.
153 The vial content was silylated by adding 500 µL of TriSil (Thermo Scientific, Bellefonte, PA, USA), shaking for 7
154 minutes, and heating at 70 ˚C for 1 h. After silylation, 1 µL was injected in a Shimadzu GC-2010 Plus gas

chromatograph equipped with a flame ionization detector and a SPB-1 column. After injection (split mode 1:15), the initial column temperature was held at 150 °C for 2 min, then increased to 210 °C with a rate of 4 °C/min and to 275 with a rate of 40 °C/min and kept at 275 °C for 5 minutes. The injector and FID temperatures were 210 °C and 290 °C, respectively. Helium was used as carrier gas. Sugar standards (glucose, mannose, galactose, xylose, rhamnose, arabinose, glucuronic acid, galacturonic acid, glucosamine and fucose) were silylated and analyzed in the same way.

2.6. FT-IR spectroscopy

Fourier transform infrared spectroscopy was performed with a Bruker Vertex 70 spectrometer equipped with a single bounce (angle of incidence 45˚) attenuated total reflection accessory (VideoMVP, Harrick), employing a diamond hemisphere and a sampling surface of 0.5 mm^2. Spectra were recorded in duplicate directly on the freeze-dried polysaccharides, sampling the region 5000-450 cm^{-1} with a resolution of 2 cm^{-1}. Samples were subjected to 128 scans.

2.7. TGA-MS

Thermal analyses of the semi-purified mushroom polysaccharides were carried out with a thermogravimeter (STA 449 C, Netzsch Instruments, Germany), coupled to a mass spectrometer (QMS 403 C Aëolos, Pfeiffer Vacuum Technology, Germany) for the detection of the evolving gases. Approximately 9 mg of freeze-dried samples were loaded in open aluminum oxide crucibles and heated from 25 ˚C to 600 ˚C, first with a heating rate of 10 ˚C/min until 190 ˚C, then at a lower rate of 2 ˚C/min until the end of the analysis. Starch and bovine serum albumin were used as reference compounds for pure polysaccharide and pure protein, respectively, and were analyzed in the same way. Inert atmosphere was granted by a nitrogen flow of 50 mL/min. A helium flow of 40 mL/min was used as protective gas. The gas transfer line (1.5 m long) was kept at 240 ˚C for preventing condensation of the evolved gases. TGA instrument was controlled with Netzsch Proteus software and the mass spectrometer was controlled with Inficon Quadstar 32-bit software. TGA data, in addition to its first and second derivative (DTGA and DDTGA, respectively), were exported as function of time.

2.8. Nuclear Magnetic Resonance spectroscopy

The samples were prepared by dissolving 10 mg of polysaccharide fraction in 600 µL D_2O (99.9% D). A small drop of acetone was added as internal reference (δ_{1H} = 2.23 ppm, δ_{13C} = 29.6 ppm). All ^{1}H and 2D NMR spectra were recorded on a Bruker AVANCE III spectrometer operating at 600.20 MHz (^{1}H) and 150.92 MHz (^{13}C) equipped with a Prodigy TCI inverted CryoProbe optimized for proton detection. ^{13}C NMR spectra were recorded on a Bruker AVANCE III spectrometer operating at 500.20 MHz (^{1}H) and 125.78 MHz (^{13}C) equipped with a Prodigy BBO CryoProbe. Characterization was carried out using a standard set of experiments: ^{1}H, ^{13}C, double-quantum filtered correlated spectroscopy (DQF-COSY), total correlation spectroscopy (TOCSY, 80 ms mixing time), nuclear overhauser effect spectroscopy (NOESY, 300 ms mixing time), heteronuclear single quantum coherence (HSQC, multiplicity edited, CH/CH_3 positive and CH_2 negative) and heteronuclear multiple bond correlation (HMBC, with a three-fold low-pass J-filter to suppress one-bond correlations). All NMR spectra were recorded at 308 K.

3. ***Results and discussion***

3.1. Polysaccharide extraction and purification

The protocol for the sequential extraction of polysaccharides from dried *C. tubaeformis* is summarized in **Supplementary Figure A.** After removal of lipids and part of the phenolic compounds with ethanol, the mushroom material was subsequently extracted with hot water, KOH 2% and KOH 25%. The three raw fractions produced underwent the same purification process, resulting in three semi-purified polysaccharide fractions. The yields (w/w) of semi-purified extracts from the hot water, KOH 2% and KOH 25% extractions were 1.12%, 4.25% and 3.04%, respectively. The hot water extraction yield is in agreement with reported yields from *Pleurotus tuber-regium* (1.6%) (Chen & Cheung, 2014) and from *Cantharellus cibarius* (1.9%) (Nyman et al., 2016). The yield granted by mild alkali was approximately the same obtained in the reference work (4%) (Smiderle et al., 2008) and higher than the reported yield from *Flammulina velutipes* (1%) (Smiderle et al., 2006) but lower than the one reported from *C. cibarius* (6%) (Nyman et al., 2016). The yield of the polysaccharide extraction with 25% KOH was higher than the yield, in the reference work (0.9%) (Smiderle et al., 2006).

The sugar contents of these three extracts, measured with the phenol-sulfuric acid method (w/w), were 32.4%, 64.4% and 67.5%, respectively. The protein content (w/w) of the semipurified polysaccharide fractions was 39.7%, 13.4%, and 2.7%, for Crat HW, Crat 2%, and Crat 25%, respectively. For the purified polysaccharide

fractions, it was 38.6% for the hot water fraction and below 2% for both alkali fractions, while their sugar content was 73.4%, 87.9%, and above 95%, respectively (**Table 1**). The higher amount of protein in the hot water extract is coherent with the reported amount of the same extract from *P. tuber-regium* (Chen & Cheung, 2014), while, differently from our result, the hot water extract from *C. cibarius* had a protein content of 6% (Nyman et al., 2016). After purification, the protein content of alkali fractions was below 2%, in agreement with literature. The presence of protein in the mild alkali fraction is in agreement with the presence of a layer of glycoproteins between the outer layer and the β-glucan layer, as indicated by the mushroom cell wall model.

The use of KOH 2% solution as extraction media after the hot water extraction almost quadrupled the final yield of the semi-purified polysaccharides and doubled the sugar content compared to the hot water extraction. The results suggest that sequential extraction with alkali solution of increasing concentrations could be a feasible starting point for efficient exploitation of mushroom polysaccharides.

3.2. High-performance size-exclusion chromatography

The HPSEC-RID chromatograms of Crat HW1, Crat 2%1 and Crat 25%1 fractions are reported in **Figure 1**. All purified fractions consisted of two polysaccharide populations with distinct bimodal molecular weight distributions of high and low M_w. Average molecular weight values are reported in **Table 1**. Relevant peaks below the penetration limit of the column were not observed. Different from Crat HW1 and Crat 25%1, the mild alkali fraction was composed of two polymer populations with close peak areas (area ratio 1.49, **Table 1**).

The use of alkali as extraction media resulted in polymer populations with molecular weights lower by one order of magnitude, compared to the population of high M_w. This may have been caused by depolymerization of polysaccharides of higher molecular weights. The small population of polysaccharides of lower molecular weight in Crat HW1 may, on the other hand, originate from the starting material. The proximity of molecular weight of the two small populations of Crat HW1 and Crat 2%1 could be an indication of homogeneity of degradation products.

3.3. Monomer composition of polysaccharides

The monomer compositions of the Crat HW1, Crat 2%1 and Crat 25%1 fractions, expressed as relative molar percentages, are reported in **Table 1**. Crat HW1 consisted mainly of mannose (34.4%), while Crat 2%1 contained glucose as the major component (69.5%). This amount indicates Crat 2%1 consisted mainly of glucans. The predominance of glucose in Crat 2%1 indicates that this hexose is the main monomer of both the polymer populations of this fraction. Glucose was the main component of Crat 25%1 as well (46.7% of the total sugars), followed by mannose (24.8%). The monomer composition of Crat HW1 indicates that hot water is an inefficient medium for extracting glucans from *C. tubaeformis*, and harsher conditions are needed for disrupting the cell wall in order to improve the extractability of these polymers. The high amounts of mannose, galactose and fucose in Crat HW1 indicate that hot water extracts mainly proteoglycans and heterosaccharides of the outer layer of the cell wall, as confirmed by infrared spectroscopy (**Figure 2** and Section 3.4).

Polysaccharides with high contents of mannose and galactose have been isolated from the hot water extract of multiple mushrooms (Ruthes, Smiderle, & Iacomini, 2016). These results are in agreement with the generally accepted mushroom cell wall model (Chen & Cheung, 2014) and with our study on the disruption of the cell wall of *A. bisporus* (Trygg et al., 2018, submitted and under revision). However, the increased presence of mannose and galactose in Crat 25%1 suggests the presence of heterosaccharides in the lowest layer of the cell wall of *C. tubaeformis*. Polymers with high content of galactose and mannose have been already isolated from fungi with alkali, for example from *F. velutipes* (Smiderle et al., 2006) and *Cordyceps militaris* (Smiderle et al, 2013).

Table 1. Sugar and protein contents, monomer composition and average molecular weight of Crat HW1, Crat 2%1 and Crat 25%1.

Fraction	Sugar content[a]	Protein content[a]	Monosaccharide composition (mol% of total)[a]							Average Molecular weight (x 10^5 Da)[b]		
	(w/w %)		Xylose	Galactose	Glucose	Mannose	Arabinose	Fucose	Rhamnose	Population 1	Population 2	Area ratio[c]
Crat HW1	73.4 (2.0)	31.6 (1.6)	22.0 (0.3)	17.8 (0.6)	11.9 (0.5)	32.6 (0.6)	0.8 (0.0)	14.0 (0.3)	0.8 (0.0)	3.96 (0.12)	0.17 (0.01)	5.26
Crat 2%1	87.9 (5.5)	<2	5.7 (0.0)	4.1 (0.2)	68.5 (0.5)	17.8 (0.1)	2.5 (0.1)	1.3 (0.1)	0.1 (0.0)	5.08 (0.15)	0.17 (0.01)	1.49
Crat 25%1	>95%	<2	7.6 (0.1)	12.2 (1.2)	45.6 (0.5)	24.2 (0.3)	1.9 (0.0)	8.3 (0.5)	0.2 (0.0)	5.42 (0.16)	0.09 (0.00)	0.09

[a]Values are given as mean (n=3), with s.d. reported in brackets. [b]Instrument error in brackets. [c]Ratio of peak areas between population 1 and population 2 peaks, respectively.

3.4. FT-IR spectroscopy

The FT-IR spectra of the three polysaccharide fractions Crat HW1, Crat 2%1 and Crat 25%1 are shown in **Figure 2**. The figure shows the vibration bands in the area 3600-935 cm^{-1} and, in the inserted expansion, the area of the recorded spectra between 1000 and 700 cm^{-1}. The broad band around 3320 cm^{-1} was assigned to the O-H stretching, due to both polysaccharide hydroxyl groups and sample moisture. The absorption bands between 2920 and 2850 cm^{-1} were, in turn, assigned to C-H (pyranoid ring and CH_2) bond stretching. The bands in the area 1160 - 935 cm^{-1} represent a typical pattern of polysaccharides backbones, the strong and overlapping bands corresponding to the stretching of the C-O and C-O-C (glycosidic) bonds. The IR bands between 1650 and 1590 cm^{-1} originated from C=O stretching of protein amides. In the case of Crat 2%1, this vibration band overlapped with the asymmetric stretching vibration of carboxylates. The assignment is confirmed by the band at 1411 cm^{-1}, produced by the symmetric stretching of the carboxylate anion. The spectrum of Crat HW1 showed a small band at 1743 cm^{-1}, assigned to the stretching of the ester group. The bands in the area 1470-1160 cm^{-1} of the spectrum were due to C-H bending, CO-H bending, O-H bending and C-C stretching (Mohaček-Grošev et al., 2001). The presence of the ester band, coupled with the C-H vibration, suggests the presence of lipids in the Crat HW1 fraction. Additionally, the carboxylate anion vibrations suggest the presence of uronic acid units in Crat 2%1 and Crat 25%1 fractions.

The low absorbance bands in the region 950-800 cm^{-1} resulted from the C-H bending of the anomeric proton (Synytsya & Novak, 2014). In particular, vibrations around 890 cm^{-1} were produced by β-linkages and those between 830 and 800 cm^{-1} by α-glycosidic linkages (Novák et al., 2012). Bands around 920 cm^{-1} were attributed to α-glycosidic linkages as well.
The α-linkage vibration at 800 cm^{-1} (**Figure 2**, expansion) was relatively more intense in the spectra of the polysaccharides extracted with hot water, and was assigned to the α-mannan chain (Mohaček-Grošev et al., 2001). The anomeric C-H vibration around 865 cm^{-1} was assigned to an α-linkage (Chylińska et al., 2016) and more specifically to α-galactan (Kacuráková et al., 2000; Synytsya et al., 2009). The outer layer of the mushroom cell wall is rich in proteoglycans with mannose and galactose as major carbohydrate monomers. This information, together with the results from the monomer composition of Crat HW1, confirms that hot water

mainly extracted the polysaccharides from the outer layer of the cell wall, i.e., the proteoglycans. The vibration at 892 cm^{-1} had the highest intensity in the anomeric region of the spectrum of Crat 2%1, and it was assigned to the β-linkage, suggesting that the β-glucans were more abundant in this fraction. The vibrations at 833 cm^{-1} and 916 cm^{-1} were assigned to α-glycosydic linkage (Mohaček-Grošev et al., 2001; Chylińska et al., 2016).

3.5. Thermogravimetric analysis

Thermogravimetric analysis was carried out to investigate the effect of the mushroom cell wall disruption with alkaline solution on the thermal stability of the extracted polysaccharides. The analysis was performed using a low temperature gradient until complete degradation (600 ˚C) of the polymers, in order to thoroughly investigate the effect of alkali on the polysaccharides. The thermogravimetric profiles and the DTGA curves of the extracted polysaccharides are illustrated in **Figure 3**. The polysaccharides decomposed in a relatively narrow temperature range, starting at about 195 °C, after the loss of moisture and volatile compounds at approximately 100 °C. The degradation range was very similar for all polysaccharides studied, ending at approximately 350 °C, while minor degradations occurred until 441 °C. Crat HW and Crat 2% fractions had comparable maximum rates of decomposition, -1.72%/min and -2.02%/min, respectively, at very close temperatures (292 ˚C and 298 ˚C, respectively). The Crat HW decomposition process exhibited a sharp DTGA peak, while Crat 2% polysaccharides DTGA peak showed a shoulder of -0.74%/min at 260 ˚C. The main mass loss of Crat HW and Crat 2% polysaccharides took place in the same temperature range, 226 - 348 ˚C (43 - 104 min), while Crat 25% polysaccharides exhibited a wider range (196 - 348 ˚C, 25 - 103 min) and the lowest mass loss rate. The polysaccharides extracted with KOH 25% showed, on the other hand, a faster degradation process, starting with higher mass loss rate at lower temperatures (i.e., the DTGA shoulder of -0.55 %/min at around 210 °C), and with earlier mass loss peak of -1.07%/min at 256 °C. This temperature was close to the Crat 2% DTGA shoulder.

The differential thermal analysis of *C. tubaeformis* polysaccharides (**Supplementary Figure E**) indicated, during the thermal degradation, the presence of two broad endothermal events, with temperature ranges of 25-196 ˚C and 196-295 ˚C, respectively. The first one could be attributed to water desorption, the second one to the first step of the degradation of polysaccharides. After 295 ˚C, in all the profiles, the curve has an inflection

and an increase, suggesting the presence of an exothermal event, which could be assigned to the main thermal degradation.

The thermal profile of Crat HW is in agreement with previous thermal stability studies of fungal polysaccharides. A mannan isolated from *Agaricus brasiliensis* with hot water had maximum degradation rate at 301 ˚C, with a DTGA profile similar to Crat HW. A branched β-glucan, isolated from the fruiting bodies of the same fungus with the same method, showed a similar DTGA profile, with a maximum degradation rate at 314 ˚C (Cardozo et al., 2013). The presence of thermal degradation earlier than 290 ˚C could be due to the presence in the fractions of polymers with lower molecular weight, since glycosidic linkage of terminal units breaks at lower temperatures (Dumitriu, 2005; Collard & Blin, 2014). Crat 25% DTGA showed also, after the loss of volatiles, a consistent peak in the DTGA in the beginning of the slow temperature program (-0.26%/min, 197 °C), which could be attributed to the degradation of carbohydrate monomers and oligomers or oligopeptides. The great reduction in thermal stability of Crat 25% could be as well explained by the increased amounts of terminal units, due to the reduction in molecular weight consequent to alkali hydrolysis of the polysaccharide chain. A decrease in thermal stability of polysaccharide fractions obtained in a stepwise process using alkali solutions of increasing concentration was noticed during the extraction of hemicelluloses (Sun et al., 2013). The authors correlated the decrease in thermal stability to the decrease in molecular weight of the polysaccharides.

3.6. Nuclear Magnetic Resonance Spectroscopy

3.6.1. Crat HW1

The obtained 1D ^{1}H and 2D COSY, TOCSY, HSQC, HMBC, HSQC-TOCSY and NOESY NMR spectra allowed the identification of the main polysaccharide chains.

The anomeric proton producing the signal at 4.95 ppm correlated, in the HSQC spectrum (**Figure 4**, left), with the carbon resonating at 98.4 ppm. In the COSY spectrum, the anomeric proton interacted with a proton signal at 3.85 ppm (H1-H2 interaction), and this proton produced an HSQC interaction with a carbon at 72.0 ppm, whose chemical shift was ascribed to C2 of glucose. The HMBC correlation between the anomeric signal at 4.95 ppm and the C6 signal at 66.8 ppm was assigned to a →6)-α-Glc-(1→ chain. The HSQC interaction between carbon signal at 66.8 ppm and proton at 4.17 ppm (H6') further proved the α-(1→6) linkage.

The doublet at 4.98 ppm had a corresponding C1 signal resonating at 98.8 ppm. The HMBC correlations of this proton with the carbon at 70.9 ppm and at 67.0 ppm were interpreted as H1-C2 and H1-C6 interactions, respectively. These correlations were produced by the terminal non-reducing unit of the →6)-α-Glc-(1→ chain (Mondal et al., 2004). This assignment was confirmed by the NOESY spectrum, which showed H1-H6 and H1-H6' interactions and a strong H1-H3 interaction at 4.98/3.74 ppm (confirmed by HSQC interaction 3.74/73.2) in the terminal unit.

The proton signal at 5.32 ppm (**Figure 4**, left) gave an HMBC interaction with a C2 carbon 78.8 ppm and an HSQC interaction with the C1 at 100.6 ppm. Such interactions and the chemical shift of the proton signal were in agreement with a →2)-α-Man-(1→ unit. The NOE spectrum showed a strong correlation between the proton at 5.32 ppm and the signal at 4.12 ppm. This proton was confirmed to be O-substituted H2 by the HSQC spectrum. The HSQC-TOCSY showed an intra-residue interaction between the H1 and the O-substituted C2, further confirming the 5.32 ppm signal assignment (Galinari et al., 2017).

The H1 at 5.13 ppm had an HSQC correlation with a C1 at 102.7 ppm and an HMBC interaction with a carbon at 78.8 ppm. The COSY interaction with an O-substituted H2 indicated that the 5.13 ppm signal was produced by the monomer of a →2)-α-Man-(1→ chain (Ustyuzhanina et al., 2018).

The most intense H1 signal of the ^{1}H spectrum α-anomer region was at 5.04 ppm. It interacted with a C1 signal resonating at 98.0 ppm and it produced HMBC interactions with an O-substituted C6 (69.3 ppm). The presence of two HSQC-TOCSY (not shown) H1-C6 signals under the 5.04 signal and the separation of the HMBC peak (**Figure 4**, left) suggested that the 5.04 ppm signal was an overlapping of two α-(1→6) H1 signals. Both →6)-α-Gal-(1→ and →6)-α-Man-(1→ were proposed as responsible for such signal pattern (Smiderle et al., 2013).

The proton peak at 5.16 ppm had an HMBC and HSQC-TOCSY correlations with an O-substituted C6 carbon (70.3 ppm). This anomeric proton had a COSY interaction with O-substituted H2 (4.12 ppm), which, on the other hand, produced a NOESY correlation with the H1 of the →2)-Man-(1→ unit at 5.32 ppm. These correlations and the agreement of chemical shifts with the literature indicated that the proton resonating at 5.16 ppm belonged to a →2,6)-α-Man-(1→ unit (Smiderle et al., 2013).

The proton signal at 5.09 ppm was due to the overlapping of two different anomeric signals, at 5.095 ppm and 5.091 ppm. The proton signal at 5.095 ppm (corresponding to a C1 at 97.9 ppm) had a NOESY correlation at 5.095/4.03 ppm, which could be assigned to a H1-H6' inter-residue interaction (HSQC correlation 4.03/70.2 ppm). The COSY spectrum showed a 5.095/3.88 ppm peak, the latter being the chemical shift of an unsubstituted H2. Such H1 was assigned to a →6)-α-Man-(1→ residue. The proton at 5.091 ppm, with a corresponding C1 at 102.4 ppm, showed a 5.091/4.19 NOESY correlation, which was assigned to a H1-H2 inter-residue interaction. The COSY spectrum showed a weak 4.19/5.23 ppm correlation, with the latter signal assigned to H1 of →2)-α-Gal-(1→ (confirmed by weak HSQC correlation 5.23/101.3). The H1 at 5.091 ppm was then assigned to a α-L-Fuc-(1→ branching unit of →2)-α-Gal-(1→ (Fan et al., 2006; Ruthes et al., 2013; Galinari et al., 2017). The α-L-Fuc-(1→ unit was further confirmed by the deoxyhexopyranose C6 1.33/18.0 HSQC correlation.

3.6.2. Crat 2%1

The polysaccharides of Crat2%1 were investigated using 1D ^{1}H, 2D COSY, TOCSY, HMBC, HSQC, and HSQC-TOCSY NMR spectroscopy. The HSQC spectrum (**Figure 4**, right) showed four broad correlations: 4.55/104.0; 4.52/104.2; 4.73/104.2; 4.77/103.9. They were assigned to →3,6)-β-Glc-(1→, →6)-β-Glc-(1→, β-Glc-(1→ , and →3)-β-Glc-(1→ units, respectively. The signal assignment was provided by HSQC interactions at 3.75/86.1 ppm, 4.22/70.3 ppm and 3.86/70.3, which were assigned to O-substituted H3/C3, H6'/C6 and H6''/C6, respectively. The typical splitting of the proton of unsubstituted position 6 was visible as well, with the signal pair at 3.92/62.0 ppm and 3.74/62.0 ppm. The HMBC spectrum showed a H1-C6 correlation broad signal at 4.52/70.3 ppm and H6'-C1 at 4.22/104.2 ppm and H6''-C1 at 3.86/104.2 ppm. The HMBC spectrum showed as well a 4.55/86.1 correlation, which indicated an intra-residue interaction between the H1 and the O-substituted C3, proving the →3,6)-β-Glc-(1→ unit. This was further confirmed by the HSQC-TOCSY spectrum, which showed an intra-unit correlation between the H1 at 4.55 pm and an O-substituted C3 together with a O-substituted C6. Moreover, in this spectrum, the intra-unit correlation between H1 at 4.77 ppm and O-substituted C3 was visible as well, confirming the assignment of this H1 to a →3)-β-Glc-(1→ unit. The assignments were in agreement with the HSQC-TOCSY spectrum, which showed H1 correlations with O-substituted C3 only for 4.55 ppm and 4.77 ppm signals.

The results of the glycosidic linkage analysis, which focused on the β-glucan structure, were in agreement with the signal integration of the ^{1}H spectrum: the ratio between →6)-Glc-(1→ and →3)-Glc-(1→ was 1:1.09, and the ratio between →6)-Glc-1)→ and →3,6)-Glc-1)→ was 1:0.63. The ratio between →6)-Glc-(1→ and terminal glucose unit was 1:0.06. Due to the dominance of glucose and of →6)-Glc-(1→ and →3)-Glc-(1→ units in the polysaccharides of Crat 2%1, it can be assumed that both polymer populations would contain the glycosidic units identified with NMR spectroscopy. It can be speculated that low molecular weight population likely has different ratios between the two types of β-Glc linkage units, compared to the population of high molecular weight. In particular, a lower ratio between →6)-Glc-(1→ and →3,6)-Glc-1)→ would be expected, due to the a shorter backbone length consequent to alkali hydrolysis, compared with the population of high molecular weight.

3.6.3. Crat 25%1

The NMR spectra of Crat 25%1 had patterns similar to Crat 2%1 spectra. The linkage analysis indicated however an increase of the ratio between →6)-Glc-1)→ and →3)-Glc-1)→ units (1:0.73), which was also seen in the integration of the ^{1}H NMR signals. In contrast, the ratios between →6)-Glc-1)→ and terminal glucose and →6)-Glc-1)→ and →3,6)-Glc-1)→ decreased to 1:0.22 and 1:0.9, respectively. These differences can be ascribed to the depolymerizing effect of the extraction method.

The two proton peaks at 5.44 ppm and 5.89 ppm marked the clear difference between Crat 2%1 and Crat 25%1. The proton at 5.44 ppm gave an HSQC correlation with a carbon signal at 100.8 ppm, ascribable to a glycosidic C1 (**Supplementary Figure F**). Basing on its chemical shift and the coupling constant (4.93 Hz), it was assigned to a β-isomer. The proton at 5.89 ppm gave a HSQC correlation with a carbon at 107.4 ppm and HMBC correlations with carbons at 144.7 ppm and 169.8 ppm. These signals were ascribed to a double bond and a carboxyl group, respectively. The anomeric proton had a HMBC correlation with the carbon at 144.7 ppm, similar to the proton at 5.86 ppm.
The proton signal at 5.89 ppm was assigned to the β-position of an α,β-unsaturated uronic acid (Adorjan et al., 2006; Jongkees & Withers, 2011). The COSY spectrum of Crat 25%1 showed short range correlations at 5.89/4.26 ppm and 5.44/3.94 ppm, which allowed the assignment of 3.94 ppm to H2, 4.26 to H3 and 5.89 to H4. The HSQC spectrum allowed the assignments of C2 and C3 positions to 70.4 ppm and 66.7 ppm, respectively. The HSQC-

TOCSY spectrum further confirmed the assignments. The H1 of the uronic acid gave an HMBC signal with a carbon at 84.0 ppm, indicating that the uronic acid formed a β-(1→3) linkage. This was further confirmed by the HMBC correlation of the C1 position with a proton at 3.88 ppm, which, on the other hand, produced an HSQC interaction signal with a carbon at 84.0 ppm (O-substituted C3). The proton at 3.88 ppm gave an HSQC-TOCSY interaction with the carbon signal at 103.9 ppm and a TOCSY correlation with the H1 signal at 4.77. Such correlations were interpreted as a glycosidic linkage between uronic acid and →3)-β-Glc-(1→ side chain of the β-glucan.

The ^{1}H signal ratio between the uronic acid and the →6)-β-Glc-(1→ was 1:12, indicating a seldom presence of the former unit in the polysaccharide chain.

3.7. Structures of the polysaccharides from *C. tubaeformis*

Table 2. NMR assignments of polysaccharides isolated from *C. tubaeformis*

H1/C1 assignment	^{13}C δ (ppm)	^{1}H δ (ppm)	NOESY signal	HMBC signal
→6)-β-Glc-(1→	104.2	4.52	4.52/4.22	4.52/70.3
→3,6)-β-Glc-(1→	104.0	4.55	4.55/4.22	4.55/70.3
β-Glc-(1→	104.2	4.73	4.73/3.75	4.73/86.1
→3)-β-Glc-(1→	103.9	4.77	4.77/3.75	4.77/86.1
→6)-α-Glc-(1→	98.4	4.95	4.95/3.85	4.95/66.8
α-Glc-(1→	98.8	4.98	4.98/3.85	4.98/67.0
→6)-α-Man-(1→ →6)-α-Gal-(1→	98.0	5.04	5.04/3.89	5.04/69.3
α-L-fucose-(1→	103.8	5.091	5.091/4.19	5.091/78.9
→6)-α-Man-(1→	102.3	5.095	5.095/4.03	5.095/70.2
→2)-α-Man-(1→	102.7	5.13	5.13/4.14	5.13/78.8
→2,6)-α-Man-(1→	102.6	5.16	5.16/4.03	5.16/70.3
→2)-α-Gal-(1→	101.3	5.23	[a]	5.23/70.0[b]
→2)-α-Man-(1→	100.6	5.32	5.32/4.12	5.32/78.8
β-4,5-en-UroA-(1→	100.8	5.44	5.44/3.89	5.44/84.0

[a]The assignment was based on COSY (Section 3.6.1). [b]Weak signal.

A summary of the identified anomeric signals of the different polysaccharides extracted from *C. tubaeformis* is reported in **Table 2**. Our results suggest that these polysaccharides represent the extractable part of the cell wall network of *C. tubaeformis*. The polymers extracted with hot water were mainly α-heterosaccharides, as cross-confirmed by the monomer composition and IR spectra. The elucidation of the

anomeric protons of Crat HW1 indicated that such polymers were mainly →6)-α-Man-(1→ and →6)-α-Gal-(1→. The α-mannan was branched in C2 position, possibly to another →6)-α-Man-(1→ chain, while the α-galactan was branched in C2 position with single L-fucose units. These two polymers have been already reported as cell wall components extractable with hot water, for example the fucogalactan from *Agaricus bisporus* (Ruthes et al., 2013) and *Coprinus comatus* (Fan et al., 2006), and the mannan from *C. cibarius* (Nyman et al., 2016). →6)-α-Man-(1→ chains have been extracted from yeasts (Galinari et al., 2017) and other Ascomycetes (Henry et al., 2016) as well. The monomer composition analysis indicated a relevant amount of xylose among the monomers of Crat HW1. By contrast, NMR spectra lacked in clear xylose signals. However, a →3)-α-Man-(1→ chain with →3)-β-Xyl-(1→ units has been extracted from *F. velutipes* using KOH 25%, mentioned in Section 3.3 (Smiderle et al., 2006).

NMR spectroscopy confirmed that the main component of the alkali fractions was a β-glucan. The ^{1}H and ^{13}C chemical shifts of its units are reported in **Supplementary Table A**. A glucan with →3)-β-Glc-(1→ backbone and →6)-β-Glc-(1→ branches have been isolated from *Pleurotus pulmonarius* (Smiderle et al., 2008) with KOH 2%. Such β-glucan has been isolated also from *F. velutipes,* using KOH 2% as well. Our results indicated however that Crat 2%1 and Crat 25%1 consisted of a →6)-β-Glc-(1→ backbone with branches in C-3 position. Such branches consisted of single β-Glc-(1→ units or short →3)-β-Glc-(1→ chains. In the case of Crat 25%1, a β-4,5-enuronic acid sparsely constituted the terminal unit of the side chain. A polymer similar to Crat 2%1 has been extracted from *C. cibarius* (Nyman et al., 2016). The differences in the ratios between →3)-β-Glc-(1→ and →6)-β-Glc-(1→ units of Crat 2%1 and Crat 25%1 were ascribed to the harsher extraction conditions of Crat 25%1, which reduced the branch length and broke down the backbone. The presence of β-4,5-enuronic acid can be ascribed to the alkali β-elimination reaction of a β-uronic acid unit. Crat 2%1 differed from the β-glucans extracted from *C. cibarius* and from the non-mushroom Basidiomycete *Malassezia restricta* (Stalhberger et al., 2014) in the degree of branching and by the presence of an uronic acid residue: Nyman proposed a backbone/branching points ratio of 8:2 in the repeating unit, while Stalhberger reported a variable frequency of branching every 2-10 units. Our results suggest an average frequency of branching every 2 units. The short length of the side chain (1-2 residues) is the common feature of these polysaccharides.

4. Conclusion

The cell wall components of the mushroom *C. tubaeformis* were extracted with a stepwise method of increasing alkalinity, purified and investigated. The polysaccharides extracted with hot water from *C. tubaeformis* were mainly branched →6)-α-Man-(1→ and →6)-α-Gal-(1→ chains, covalently bound to proteins. This indicates that for utilization of *C. tubaeformis* as a source of β-glucan, hot water is an inefficient extraction medium. Such polysaccharide was, on the other hand, the main component of the extracts produced by disruption of the cell wall. The β-glucan extracted with KOH 2% was an acidic →6)-β-Glc-(1→ chain. Structural investigation suggested that this polymer was highly branched with β-Glc-(1→3)-β-Glc-(1→ dimers or single β-Glc-(1→ residues. The same polysaccharide was extracted with KOH 25%, with a reduction of branch and backbone lengths. The extraction with KOH 2% resulted in a bimodal molecular weight distribution, while extraction with KOH 25% resulted in polysaccharides of low molecular weights. The TGA study indicated that the use of KOH 2% as extraction medium for β-glucan has little effect on the thermal stability of the extracted polymers, whereas KOH 25% remarkably decreased the thermal stability of the polysaccharides.

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Declaration of interest

The authors declare no conflict of interest related to the publication of this article.

Appendix A. Supplementary material

Supplementary Figure A: Polysaccharide extraction scheme; Supplementary Figure B: HPSEC-RID-UV chromatograms; Supplementary Figure C: TGA-DTGA profile of BSA; Supplementary Figure D: TGA-DTGA profile of starch; Supplementary Figure E: Differential thermal analysis plots; Supplementary Figure F: HMBC-

HSQC spectra of Crat 25%1 (β-4,5-en-uronic acid signals); Supplementary Table A: ^{13}C and ^{1}H chemical shifts for *C. tubaeformis* β-glucan.

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Figure captions

Figure 1. HPSEC-RID chromatograms of Crat HW1, Crat 2%1 and Crat 25%1.

Figure 2. FT-IR spectra of Crat HW1, Crat 2%1 and Crat 25%1, with an expansion of the anomeric region (900-700 cm^{-1}).

Figure 3. TGA (dashed line) and DTGA (solid line) profiles of Crat HW, Crat 2% and Crat 25%. Temperature program is represented by the dotted line.

Figure 4. HMBC (blue) and HSQC (red) NMR spectra of Crat HW1 (anomeric region, left) and Crat 2%1 (right). NMR spectra were recorded in D_2O at 308 K. See **Table 2** and **Supplementary Table A** for signal assignments.