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NMR characterization of the polysaccharidic fraction from *Lentinula edodes* grown on olive mill waste waters

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Abstract—A high-field NMR study of the polysaccharidic fraction extracted from *Lentinula edodes* mycelium grown on olive mill waste waters is reported. Diffusion-ordered NMR spectroscopy (DOSY) was applied to the polysaccharidic fraction. The results showed the presence of two polysaccharides of different sizes, whose structures were revealed using one- and two-dimensional NMR techniques. These two polysaccharides were identified as xylan and lentinan.

Keywords: *Lentinula edodes*; Olive mill waste waters; Lentinan; Xylan; NMR; DOSY

1. Introduction

Basidiomycetes constitute a natural source of biologically active metabolites. Many basidiomycetes have been classified by the National Cancer Institute of the United States as antitumor agents exhibiting an immunomodulatory activity.[1] The therapeutic activity is mainly related to polysaccharides or protein-bound polysaccharides, such as glucans, heterogalactans, and glucanproteins, which are present either in the mycelium or in the fruit body.[2–6] Among these polysaccharides are β-D-glucans, which are of particular interest because of their pharmacological properties. Most of the β-D-glucans exhibiting a biological activity have been extracted from *Grifola frondosa*, *Ganoderma lucidum*, *Trametes versicolor*, *Schizophyllum commune*, *Lentinula edodes*, and *Flammulina velutipes*.[7]

β-D-glucans are composed of a β-(1→3)-linked-D-glucopyranose backbone to which β-(1→6)-D-glucopyranosyl residues are randomly branched. Their activity has been shown to depend on their structure and conformation.[8–10] More specifically, lentinan is a β-(1→3)-D-glucan that has been extracted from *L. edodes*, a mushroom widely cultivated in oriental countries. To the backbone of lentinan, two β-(1→6)-D-glucopyranosyl residues are branched every five β-D-glucopyranosyl residues.[9] This specific structure is reported to be responsible for the antitumor, antibacterial, antiviral, anticoagulatory as well as the wound-healing activities of lentinan; in particular, lentinan has a strong antitumor activity against sarcoma 180 in mice, with a complete regression of the tumor after 10 doses of 1 mg/kg.[11]

It has been shown that lipids, such as oleic and palmitic acids, stimulate the growth of *L. edodes* mycelium.[12] Because olive mill waste waters (OMWW) contain lipids, they appear as a suitable source of nutrients for the growth of *L. edodes* mycelium. In addition, in a strategy of bioremediation, the production of mycelial biomass from agricultural wastes appears highly attractive.

In this paper, the study of the polysaccharidic fraction extracted from *L. edodes* mycelium grown on OMWW is reported. Because the activity of a polysaccharide can be

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affected by its structure and by the degree of branching, a careful structural analysis of the polysaccharidic fraction was carried out, using gas chromatography and NMR spectroscopy, including conventional 2D ^{1}H–^{1}H COSY, TOCSY, and ^{1}H–^{13}C HSQC experiments as well as ^{1}H-detected diffusion-ordered NMR spectroscopy (DOSY) experiments.

2. Results and discussion

L. edodes is commonly cultivated on lignocellulosic substrates; because lipids stimulate the mycelium growth, they are usually added to the growth medium. OMWW (olive mill waste waters) contain, on average, 1–1.5% of lipids, mainly palmitic and oleic acids, and are therefore a suitable growing medium for *L. edodes*. The complete chemical characterization of OMWW is reported in Table 1.[13]

In our case, it was observed that the growth of *L. edodes* on OMWW led to a 2-fold increase in mycelial biomass with respect to the growth on the control medium consisting of malt extract and peptone (Fig. 1). From each mycelial biomass, a polysaccharidic fraction was extracted. It must be pointed out that, from the same amount of mycelial biomass, grown either on OMWW or on the control medium, the same amount of polysaccharidic fraction (0.80–0.85% dry weight) was extracted. Subsequently, both polysaccharidic fractions were analyzed by gas chromatography (GC) and NMR spectroscopy, and the results were the same; therefore, only the analysis of the fraction extracted from the mycelium grown on OMWW is reported here.

The GC analysis, performed on the hydrolyzed sample (see Experimental) allowed the monosaccharidic composition to be obtained (Table 2): glucose and xylose were present in large amount (>99% area), whereas ribose, arabinose, and mannose, were present only in trace (<1% area). The xylose/glucose molar ratio was 1:7.

Table 1. Chemical characterization of olive mill waste waters

pH	4.7–5.5
Water	90.4–96.5%
Dry matter	3.5–9.6%
Organic matter	2.6–8.0%
Lipids	0.5–2.3%
Proteins	0.17–0.4%
Carbohydrates	0.5–2.6%
Organic acids	Traces
Polyalcohols	0.9–1.4%
Pectines, gums, tannines	0.23–0.50%
Glucosydes	Traces
Polyphenols	0.3–0.8%
Ashes	0.2–0.5%
P_2O_5	0.03–0.07%
SO_3, SiO_2, FeO, MgO	traces – 0.03%
CaO	0.01–0.03%
K_2O	0.11–0.24%
Na_2O	0.01–0.03%
Suspended solids	0.7–1.1%
Dry matter	3.5–9.6%

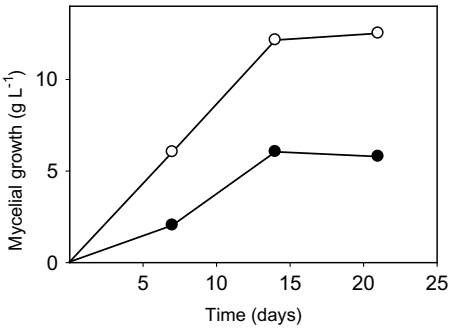


Figure 1. Growth of *L. edodes* mycelium on olive mill waste waters (empty circles) and on the control medium (filled circles).

Table 2. Gas chromatographic retention times and areas of the monosaccharides identified in the polysaccharidic fraction

Peak	Residue	Retention time (min)	Area
1	Ribose	21.534 ± 0.015	12343 ± 34
2	Arabinose	22.192 ± 0.006	9449 ± 38
3	Xylose	24.362 ± 0.040	140700 ± 47
4	Mannose	27.275 ± 0.009	6284 ± 9
5	Glucose	29.375 ± 0.007	1289560 ± 59
6	Inositol[a]	30.422 ± 0.005	1045238 ± 906

[a] Inositol was used as an internal standard.

The gel filtration chromatography showed a broad peak with a molecular weight ranging from 200 to 350 KDa; the fraction corresponding to this broad peak was analyzed by NMR.

The ^{1}H spectrum of the polysaccharidic fraction in 0.5 M NaOD aqueous (D_2O) solution is reported in Figure 2 as horizontal projection. All signals were rather broad suggesting the presence of high molecular weight compounds.

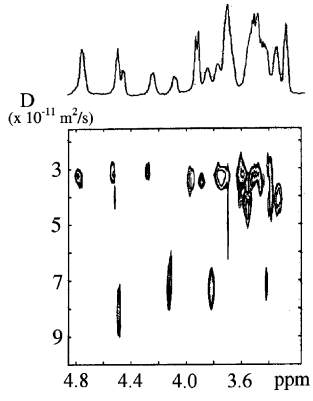


Figure 2. ^{1}H-detected DOSY spectrum of the polysaccharidic fraction in 0.5 M NaOD aqueous (D_2O) solution at 300 K. The 600.13 MHz ^{1}H spectrum of the sample is also reported.

In order to check whether the sample was a single compound or a mixture, a diffusion-ordered NMR experiment was performed. The DOSY experiment is one way of displaying pulsed field gradient NMR data,[14] and has been previously used for many applications.[15–21] This experiment yields a pseudo 2D NMR spectrum with chemical shifts in one dimension (horizontal axis) and diffusion coefficients in the other one (vertical axis). Therefore, DOSY spectroscopy allows one to distinguish compounds according to differences in their size.

In Figure 2, a ^{1}H-detected DOSY of the polysaccharidic fraction is reported. All ^{1}H signals were classified according to their self-diffusion coefficient. In particular two groups of signals characterized by a distinct self-diffusion coefficient were observed. Therefore, two compounds of different sizes were present. The structural elucidation of these two compounds, hereafter referred to as compounds X and A, is discussed separately.

2.1. Structural elucidation of compound X

Compound X exhibited the major diffusion coefficient and hence the minor molecular size. The structure was revealed using 1D and 2D NMR experiments. ^{1}H–^{1}H COSY (data not shown) and ^{1}H–^{1}H TOCSY experiments (Fig. 3) showed that all the ^{1}H resonances due to compound X belonged to the same spin system; in fact, proton H-1x at 4.49 ppm was correlated to other five protons at 3.33, 3.55, 3.82, 3.40, and 4.15 ppm, respectively. The corresponding ^{13}C assignment was obtained by a ^{1}H–^{13}C HSQC experiment (Table 3).

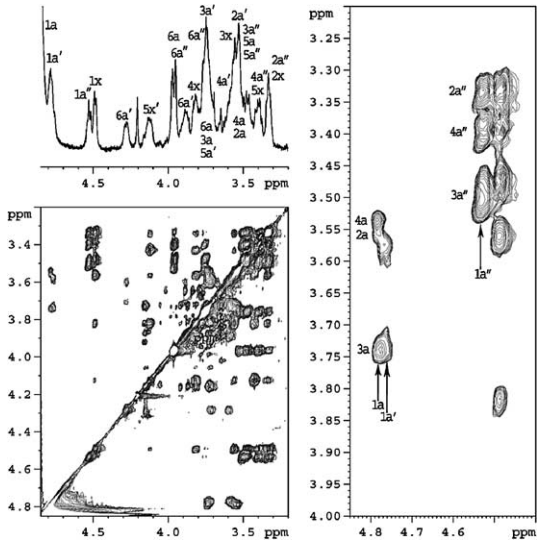


Figure 3. ^{1}H–^{1}H TOCSY map of the polysaccharidic fraction in 0.5 M NaOD aqueous (D_2O) solution at 300 K. The ^{1}H spectrum of the sample with the corresponding assignment is also reported. Labels x and a refer to compounds X and A, respectively. Cross-peaks between anomeric protons and correlated protons are evidenced in the expansion of the anomeric region.

Table 3. ^{1}H and ^{13}C assignments of compound X in 0.5 M NaOD aqueous (D_2O) solution at 300 K

Proton	$\delta 1_H$ (ppm)	Carbon	$\delta 13_C$ (ppm)
H-1x	4.49	C-1x	104.4
H-2x	3.33	C-2x	74.3
H-3x	3.55	C-3x	76.3
H-4x	3.82	C-4x	78.5
H-5x, H-5x′	3.40, 4.15	C-5x	65.6

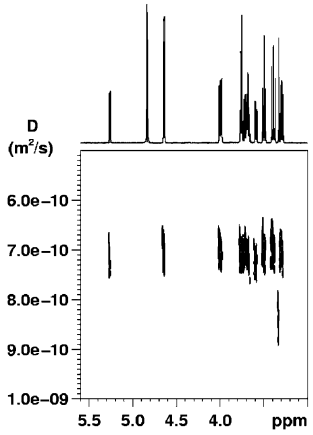


Figure 4. ^{1}H-detected DOSY spectrum of a xylose sample in 0.5 M NaOD aqueous (D_2O) solution at 300 K. The 600.13 MHz ^{1}H spectrum of the xylose sample is also reported.

These results suggested the presence of β-xylose units. In order to determine whether the compound was a monosaccharide or a polysaccharide, a DOSY experiment was performed on a xylose sample (Fig. 4). The comparison between the diffusion coefficients of compound X (7×10^{-11} m/s^2, Fig. 2) and xylose (7×10^{-10} m/s^2, Fig. 4) indicated that compound X had a much larger molecular size than xylose; therefore, compound X was generically reported as xylan.[22] Finally, the low-field chemical shift of the C-4x carbon at 78.5 ppm indicated that the monomeric units were linked in position 4.

2.2. Structural elucidation of compound A

With respect to compound X, compound A had a minor diffusion coefficient and hence a major molecular size. The ^{1}H resonances (Fig. 3) were assigned by means of 2D experiments. Three different spin systems of different intensity, labeled as a, a′, and a″, were identified by ^{1}H–^{1}H COSY and ^{1}H–^{1}H TOCSY experiments. The ^{13}C assignment corresponding to these spin systems was obtained by means of a ^{1}H–^{13}C HSQC experiment. The ^{1}H and ^{13}C chemical shift values of these three spin systems suggested the presence of glucose residues (Fig. 3). The ^{1}H and ^{13}C assignments of these residues are reported in Table 4. The chemical shift values of the

Table 4. ^{1}H and ^{13}C assignments of compound A in 0.5 M NaOD aqueous (D_2O) solution at 300 K

Proton	$\delta 1_H$ (ppm)	Carbon	$\delta 13_C$ (ppm)
H-1a	4.78	C-1a	105.3
H-2a	3.55	C- 2a	75.6
H-3a	3.73	C-3a	88.2
H-4a	3.53	C-4a	70.5
H-5a	3.50	C-5a	77.0
H-6a, H-6a	3.74, 3.96	C-6a	63.1
H-1′a′	4.77	C-1′a′	105.5
H-2′a′	3.55	C-2′a′	76.4
H-3′a′	3.72	C-3′a′	88.6
H-4′a′	3.59	C-4′a′	70.4
H-5′a′	3.70	C-5′a′	77.1
H-6′a′, H-6′a′	3.88, 4.26	C-6′a′	71.0
H-1″a″	4.53	C-1″a″	105.1
H-2″a″	3.33	C-2″a″	75.5
H-3″a″	3.48	C-3″a″	78.3
H-4″a″	3.40	C-4″a″	72.3
H-5″a″	3.50	C-5″a″	78.3
H6″a″, H6″a″	3.74, 3.96	C-6″a″	63.1

anomeric protons H-1a, H-1a′ and H-1a″ at 4.78, 4.77, and 4.53 ppm, respectively, indicated that the anomeric protons were in a β-configuration. The chemical shift values of C-3a and C-3a′ at 88.2 and 88.6 ppm, respectively, indicated the presence of glucosyl residues linked in position 3.[22] Hence, compound A consisted of a backbone made of β-(1 → 3)-D-glucopyranosyl residues (a and a′ spin systems).

In addition, the chemical shift value of the C-6a′ methylene group at 71.0 ppm was typical of a branch in position O-6;[22] therefore, the glucosidic residues a′ and a″ were linked in position O-6. All these observations were consistent with the presence of β-(1 → 3)-D-glucopyranosyl residues containing branch points on the β-(1 → 6)-D-glucopyranosyl residues (Scheme 1).

The integral of the anomeric ^{1}H resonances of the a and a′ residues of the backbone compared with the integral of the anomeric ^{1}H resonances of the a″ residues allowed the content of branching to be measured: the sample had a 40% of branched units, that is, it had two branches every five D-glucopyranosyl residues. Therefore, in agreement with the literature,[22] this polysaccharide was identified as lentinan. Besides, the integral performed on the anomeric ^{1}H resonances due to xylan and lentinan agreed with the xylose/glucose ratio of 1:7 determined by GC.

3. Experimental

3.1. Organism

L. edodes (SMR 0090), stored at the International Bank of Edible Saprophytic Mushrooms, was cultured on agar slopes of synthetic medium containing 3% malt extract.

3.2. Preparation of inoculum

Mycelial pellets were obtained by growing mycelium in shake cultures in 100 mL Erlenmeyer flasks containing 50 mL of synthetic liquid medium (0.5% peptone and 3% malt extract) at 25 °C, 125 rpm for 10 days. Afterwards pellets were homogenized aseptically in an omni mixer homogenizer for 3 s and inoculated into flasks for mycelial growth.

3.3. Mycelial growth

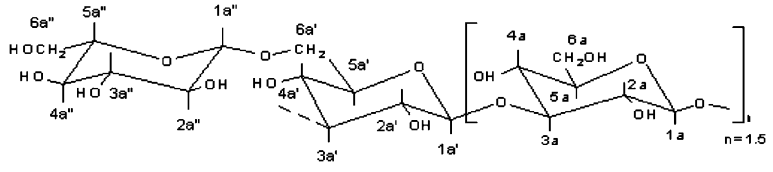
50 mL of mycelial suspension (equivalent to 1.5–1.6 g of dry weight) were inoculated in 2500 mL flasks containing 1000 mL of:

(a) Control medium = 3% malt extract and 0.5% peptone;
(b) Olive mill waste waters (OMWW) (dry weight = 4.85% and organic matter = 89.0% dry weight); the pH was adjusted at 5.8.

The flasks were incubated for 21 days at 25 °C, H = 70% and stirred at 100 rpm. Mycelial growth was assayed by weight after 7, 14, and 21 days from inoculation.

3.4. Extraction of the polysaccharidic fraction[23]

21-days old mycelial biomass obtained from both control and OMWW was filtered through gauze, washed with water, and freeze-dried. Mycelium polysaccharides were extracted with boiling water (15 mg/mL at 100 °C for 15–18 h) under stirring. The suspension was centrifuged at 5000 g for 20 min and the surnatant was precipitated twice with ethanol (1/1 v/v) overnight at 4 °C under stirring. The precipitate was re-dissolved in boil-



Scheme 1. Structure of (1 → 3)-β-D-glucan-containing glucopyranosyl residues branched in position 6.

ing water and then precipitated with 0.2 M CTA-OH (cetyltrimethylammonium hydroxide) at pH 12, overnight at 4 °C. The precipitate was separated by centrifugation (5 min at 9000 g), washed with ethanol, and centrifuged again; 20% acetic acid was then added to the precipitate (5 min at 0 °C under stirring). After centrifugation for 5 min at 9000 g, 50% acetic acid was added to the precipitate (3 min at 0 °C). The suspension was centrifuged and the obtained precipitate was solubilized in a 1.5 M NaOH solution. The soluble fraction was washed twice with ethanol, once with ethyl ether and once with MeOH. Finally, the obtained polysaccharidic fraction was dialyzed, freeze-dried, and used for the chemical characterization.

3.5. Gas chromatography

A portion of the polysaccharidic fraction was derivatized to alditol acetates as follows: 5 mg of sample were hydrolyzed with 2 mL of 2 N trifluoroacetic acid at 100 °C for 16 h and then dried with N_2 at 50 °C. One milliliter of 10 mM inositol (internal standard), 0.1 mL of 1 M NH_3 and 1 mL of $NaBH_4$ (2% in DMSO) were added and heated at 40 °C for 90 min. Then 0.1 mL of acetic acid, 0.2 mL of 1-methylimidazole and 2 mL of Ac_2O were added and left for 10 min at room temperature. After addition of 4 mL of water, the solution was cooled and 1 mL of CH_2Cl_2 was added. The CH_2Cl_2 phase was separated and analyzed using a GC Hewlett–Packard 5890A equipped with a flame ionization detector. A capillary column, SP-2330 FS (Supelco) (30 m × 0.25 mm × 0.20 μm film thickness), was used with He as carrier gas at 110 kPa. Injector and detector temperatures were 250 and 280 °C, respectively; an initial column temperature of 150 °C was held for 2 min and then increased to 250 °C, at a rate of 4 °C/min, for 10 min. The split ratio was 1:20. The analyses were performed in triplicate and the identity of each sugar peak in the chromatograms was determined by comparison with the retention times observed for standard monosaccharidic solutions (Sigma products).

3.6. Gel filtration chromatography

Gel filtration chromatography was performed on Sepharose CL-4B (fine grade Pharmacia) with a 0.7 × 60 cm column and flow rate 26 mL h^{-1}. Samples of about 6 mg/mL were applied and eluted with 0.01 M Tris(hydroxymethyl)aminomethane buffer pH 7.2 containing 1 M NaOH. Fractions of 1 mL were collected and their absorbance was measured at 280 nm. A calibration curve was obtained by measuring the elution volumes of reference substances, namely Blue Dextran, Aldolase, Catalase, and Ferritin.

3.7. NMR spectroscopy

The polysaccharidic fraction (≈2 mg) was solubilized in 0.5 M NaOD aqueous solution (D_2O) under stirring at room temperature (300 K). ^1H and ^{13}C spectra were recorded at 300 K on a Bruker AVANCE AQS600 spectrometer operating at 600.13 and 150.9 MHz, respectively, with a Bruker z-gradient probe head. All one- (1D) and two-dimensional (2D)[24] spectra were recorded using a soft presaturation of the HOD residual signal. Chemical shifts were reported with respect to a trace of 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS) used as an internal standard. The ^1H and ^{13}C assignments were obtained using ^1H–^1H COSY (Correlation spectroscopy), ^1H–^1H TOCSY (total correlation spectroscopy) and ^1H–^{13}C HSQC (heteronuclear single quantum coherence) experiments[24] with gradient selection of the coherence. All 2D experiments were acquired using a time domain of 512 data points in the F1 and 1024 data points in the F2 dimension, the recycle delay was 1.2 s. The ^1H–^1H TOCSY experiment was acquired with a spin-lock duration of 80 ms. The ^1H–^{13}C HSQC experiment was performed using a $^1J_{C-H}$ coupling constant of 150 Hz. The number of scans was optimized to achieve a good signal-to-noise ratio. For all 2D experiments a matrix of 512 × 512 data points was used; the ^1H–^1H COSY spectrum was processed in the magnitude mode whereas all other 2D experiments were processed in the phase sensitive mode.

DOSY experiments[25] were performed with a pulsed field gradient unit capable of producing magnetic field gradients in the *z*-direction with a strength of 55.4 G/cm. The stimulated echo pulse sequence using bipolar gradients with a longitudinal eddy current delay was used. The strength of the gradient pulses, of 2.3 ms duration, was incremented in 16 experiments, with a diffusion time of 100 ms and a longitudinal eddy currents delay of 5 ms. After Fourier transformation, phase, and baseline corrections, the diffusion dimension was processed using the Bruker XWINNMR software package (version 2.5).

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