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Structural characterization of a polysaccharide and a β-glucan isolated from the edible mushroom *Flammulina velutipes*

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Abstract

Two polysaccharides were isolated from the basidiomycete *Flammulina velutipes*, via successive hot extraction with water, 2% and 25% aq. KOH, and then submitted to freeze-drying. The precipitate formed by repeated freeze-thawing from the 2% aq. KOH extraction PK2 was analyzed by determination of its monosaccharide composition, as well as by methylation analyses using GC–MS, mono- (^{13}C, ^{1}H NMR) and bidimensional (^{1}H (obs.), ^{13}C HMQC) spectroscopy, and controlled Smith degradations. It was established to be a branched β-glucan, with a main chain of (1 → 3)-linked-Glc*p* residues, substituted at O-6 by single-unit β-Glc*p* side chains. The precipitate formed by repeated freeze-thawing from the 25% KOH extraction PK25 contained Xyl, Man, and Glc and was heterogeneous by HSPEC and extraction with DMSO gave a soluble xylomannan (XM). It was homogeneous with a molar mass 30.8×10^4 g/mol ($dn/dc = 0.186$). Using the above chemical analyses, it was a xylomannan with Man and Xyl in a 3:2 molar ratio. Its main chain consisted of (1 → 3)-linked α-Man*p* units, mainly substituted at O-4 by β-Xyl*p* units or with some β-Xyl*p*-(1 → 3)-β-Xyl*p* groups.

Keywords: *Flammulina velutipes*; Basidiomycete; Branched β-glucan; Xylomannan

1. Introduction

Edible mushrooms have, since ancient times, been consumed with the goals of maintaining health and promotion of longevity (Manzi and Pizzoferrato, 2000). As well as for their medicinal or nutritional properties, they were especially appreciated for their texture and flavor. Nowadays, following nutritional investigations, they are well known as a very rich food supplement, due to their favorable protein, carbohydrate, and dietary fiber contents (Manzi and Pizzoferrato, 2000; Leung et al., 1997; Mallavadhani et al., 2006; Manzi et al., 2004).

The biological importance of these basidiomycetes arises from their chemical components, especially various biologically active polysaccharides. Among these, the β-glucans from different organisms have been the most studied. Edible fungi from the genera *Ganoderma*, *Agaricus* and *Lentinus* have been widely investigated (Wasser, 2002; Kües and Liu, 2000), and have also demonstrated some interesting biological properties, such as immunomodulatory and antitumor activity (Guterrez et al., 2004; Zheng et al., 2005; Peng et al., 2005).

Also studied has been another genus of edible mushrooms, that of *Flammulina*, the main one being *Flammulina velutipes* (Curt. ex Fr.) Sing. This is popularly known by its Japanese name "enokitake" which, however, was first cultivated in China during the 8th century. Its consumption is now worldwide and ranks in fourth place in the production and consumption of edible mushrooms (Leifa et al., 2001).

Studies on *F. velutipes* polysaccharides have demonstrated strong immunomodulatory and antitumoral activities for its glucans and heteropolysaccharides (Yoshioka et al., 1973; Ikekawa et al., 1982; Otagiri et al., 1983). Ikekawa et al. (1982) reported a β-(1 → 3)-glucan and two heteropolysaccharides to have antitumoral activity. This was

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followed by a report by Otagiri et al. (1983), who found an intensification of antitumor-immunity by a protein-bound polysaccharide containing glucose, galactose, mannose, xylose, and arabinose.

Apart from these studies, which focused on biological properties, little is known about the detailed structure of these polysaccharides. A study carried out by Mukumoto and Yamaguchi (1997) revealed the structure of a mannofucogalactan from the fruiting bodies of *F. velutipes*. This polysaccharide was obtained by cold water extraction that was precipitated at a concentration 50% of acetone in water and it consisted of a main chain of (1 → 6)-linked-α-Gal*p* units, every third of which are substituted by 3-*O*-α-D-Man*p*-L-Fuc*p* or L-Fuc*p* residues.

Since there is a lack of information on the detailed structure of the polysaccharides that are present in *F. velutipes*, we now have fractionated extracts of its fruiting bodies and characterized a previously known branched (1 → 3), (1 → 6)-linked β-glucan (Ikekawa et al., 1982) and a poorly characterized xylomannan.

2. Results and discussion

A dry sample of *F. velutipes* was submitted to successive aq. and 2% aq. KOH, and 25% aq. KOH extraction at 100 °C. The fractions obtained from 2% and 25% aq. KOH, named K2 and K25, respectively, were submitted to several freeze-thawing procedures until no more precipitates were formed (Fig. 1).

After centrifugation of the fractions, soluble SK2 (1% yield) and SK25 (0.9% yield) and insoluble PK2 (3.7% yield) and PK25 (2.6% yield) subfractions were isolated (Fig. 1).

PK2 contained mainly glucose (Table 1) consistent with a predominant glucan. ^{13}C NMR and ^{1}H (obs.), ^{13}C HMQC spectra (Fig. 2a and b, respectively) (Table 3) had signals corresponding to all carbons from the polysaccharide: C-1/H-1 at δ 103.1/4.53 corresponding to 3-*O*-substituted units (A) (Fig. 2c, while those at δ 103.1/4.23 are from 3,6-di-*O*-substituted units (B). The β-configuration was shown by H-1 signals at high field and C-1 signals at low field. The resonances at δ 86.7 and 86.3 arise from substitutions at O-3 in units A, while those at δ 86.0 and 76.7 are from similar substitutions in units B and free O-3 from non-reducing end units of β-Glc*p* (C), respectively. Signals at δ 76.4; 76.2 and 74.9 arise from C-5 of units A, A

Table 1
Monosaccharide composition of the fractions obtained from *F. velutipes*

Fractions	Monosaccharides (%)[a]		
	Xyl	Man	Glc
PK2	–	–	100
PK25	18	32	50
XM	40	60	–
SM1	9	91	–
SM2	3	97	–

[a] Alditol acetates obtained on successive hydrolysis, $NaBH_4$ reduction, and acetylation, analyzed by GC–MS.

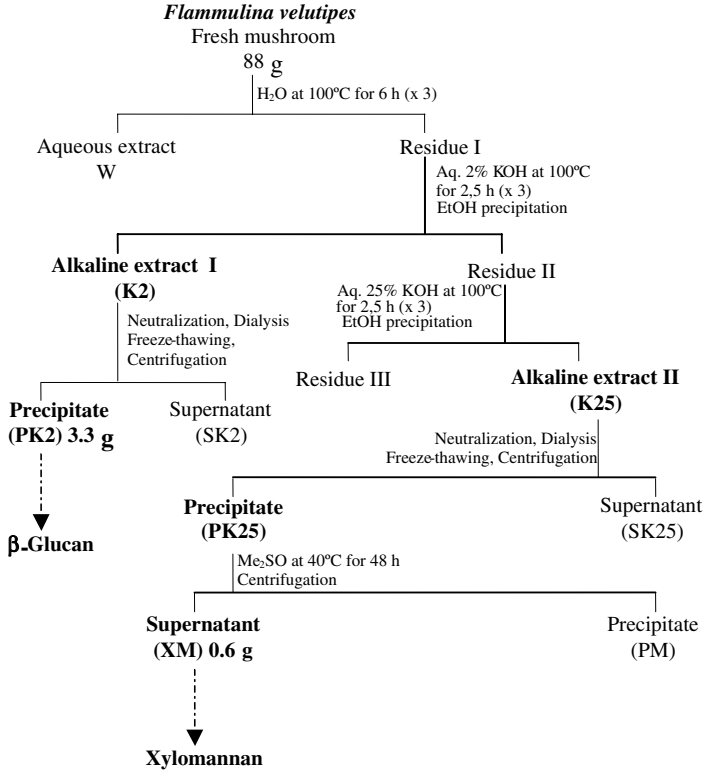

Fig. 1. Extraction and purification of glucan (PK2) and xylomannan (XM).

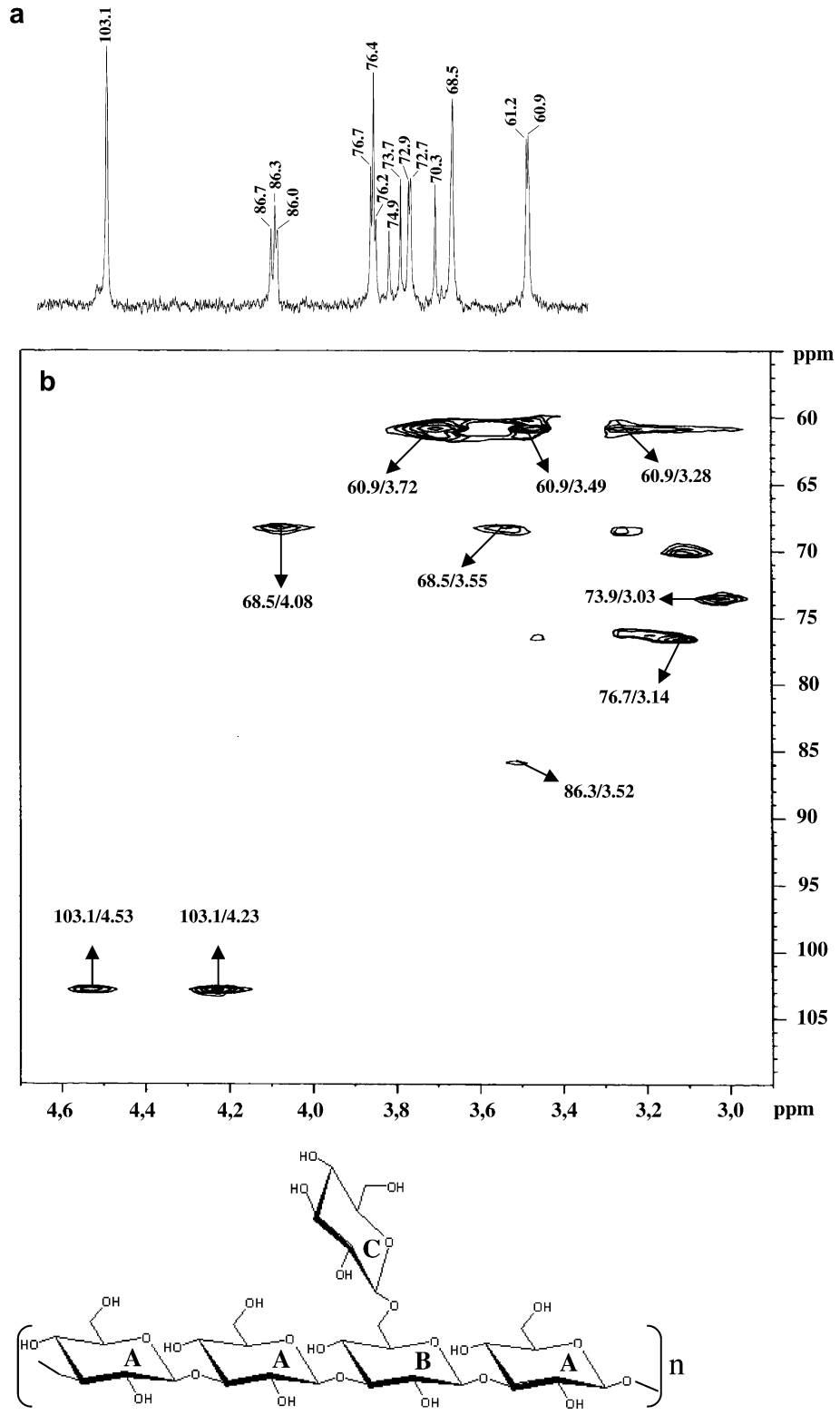

Fig. 2. ^{13}C NMR (a) and HMQC (b) spectra, and chemical structure (c) of glucan (PK2), in DMSO-d_6 at 70 °C, chemical shifts are expressed in ppm.

and/or C and B, respectively. *O*-Substituted and non-substituted $-CH_2$ resonances are shown at δ 68.5 and 61.2; 60.9, respectively (Yoshioka et al., 1985).

These data agree with those of a methylation analysis, which showed mainly the alditol acetates of 2,3,4,6-Me_4Glc (20%), 2,4,6-Me_3Glc (58%), and 2,4-Me_2Glc (19%), showing the presence of a branched (1 → 3), (1 → 6)-linked β-glucan (Table 2). A controlled Smith degradation (Abdel-Akher et al., 1952; Hay et al., 1965) was carried out on the glucan and the final product of high molecular weight was analyzed by ^{13}C NMR spectroscopy. It proved to be a linear (1 → 3)-linked β-glucan with six typical signals at δ 102.9; 86.1; 76.3;

Table 2
Partially *O*-methylalditol acetates formed on methylation analysis of polysaccharides isolated from *F. velutipes*

Partially *O*-methylated alditol acetates	Fractions (%)				Linkage type[b]
	PK2[a]	XM[a]	SM1[a]	SM2[a]	
2,3,4,6-Me_4-Glc	20	–	–	–	Glc*p*-(1→
2,4,6-Me_3-Glc	58	–	–	–	3→)-Glc*p*-(1→
2,3,6-Me_3-Glc	2	–	–	–	4→)-Glc*p*-(1→
2,3,4-Me_3-Glc	2	–	–	–	6→)-Glc*p*-(1→
2,4-Me_2-Glc	19	–	–	–	3,6→)-Glc*p*-(1→
2,3,4-Me_3Xyl	–	31	8	–	Xyl*p*-(1→
2,4-Me_2Xyl	–	6	1	–	3→)-Xyl*p*-(1→
2,3-Me_2Xyl	–	1	–	–	4→)-Xyl*p*-(1→
3,4-Me_2Xyl	–	1	–	–	2→)-Xyl*p*-(1→
2-MeXyl	–	1	–	–	3,4→)-Xyl*p*-(1→
2,3,4,6-Me_4-Man	–	Tr.[c]	1	3	Man*p*-(1→
2,4,6-Me_3-Man	–	28	82	97	3→)-Man*p*-(1→
2,6-Me_2-Man	–	32	8	–	3,4→)-Man*p*-(1→

[a] Analyzed fractions and percentage of peak area relative to total peak area.
[b] Based on derived *O*-methylalditol acetates.
[c] Trace.

72.8; 68.4 and 60.9, arising from C-1, C-3, C-5, C-2, C-4 and C-6, respectively (Fig. 3) (Carbonero et al., 2001). These results show such a main chain, partially substituted at O-6 by single-unit β-Glc*p* side chains, typical of certain basidiomycetous fungi, and with varying degrees of substitution. These were first described as an exocellular product of *Sclerotium glucanicum* (Johnson et al., 1963).

PK25 consisted of glucose (50%), mannose (32%) and xylose (18%). This fraction was submitted to a further purification step with Me_2SO at 40 °C for 48 h. A soluble (XM; 0.7% yield) and an insoluble (PM; 1% yield) fraction were obtained (Fig. 1). XM was free of a glucose contaminant, containing Man (60%) and Xyl (40%) and was homogeneous with a molar mass of 30.8×10^4 g/mol ($dn/dc = 0.186$).

Methylation analysis (Table 2) of XM indicated a complex, highly substituted xylomannan. Apart from a high content of non-reducing end units of Xyl*p* (2,3,4-Me_3-Xyl, 31%), 3-*O*-substituted (2,4,6-Me_3Man, 28%) and 3,4-di-*O*-substituted Man*p* (2,6-Me_2-Man, 32%), many other minor derivatives were detected. These corresponded to 3-*O*-(6%), 4-*O*-(1%), 2-*O*-(1%), and 3,4-di-*O*-substituted Xyl*p* units (1%).

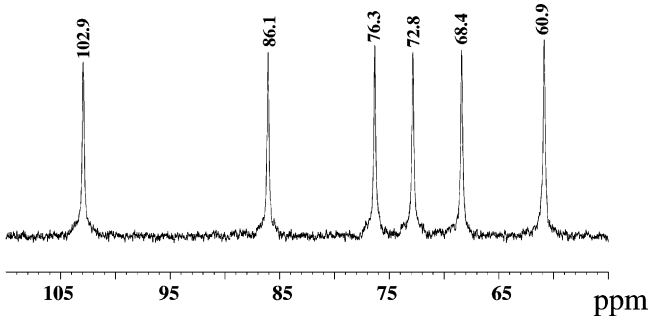

Fig. 3. ^{13}C NMR spectrum of Smith degraded glucan (PK2), in DMSO-d_6 at 70 °C, chemical shifts are expressed in ppm.

^{13}C NMR and HMQC spectra (Fig. 4a and b, respectively) (Table 3) also showed a complex structure for XM. The anomeric region contained signals (C-1/H-1) at δ 103.9/4.88, corresponding to β-Xyl*p* units (Vinogradov et al., 2004): low field C-1 resonances are typical of β-Xyl*p* units and others at δ 101.7/5.59; 101.5/5.61; 99.7/5.72 arising from C-1/H-1 of α-Man*p* units. Such low field H-1 signals are typical of α-configurations in the pyranosyl series (Hall and Johnson, 1969). Other resonances can also be assigned, such as those at δ 76.7, 72.4, 69.8, and 65.6 for C-3, C-2, C-4 and C-5 of non-reducing end units of Xyl*p* (Vinogradov et al., 2004), and those at δ 62.5; 61.3 and 60.4 for non-substituted O-6 of α-Man*p* units. The area integrals of H-1 signals of α-Man*p* units (δ 5.71 and 5.59), showed a Man to Xyl ratio of 2.40:1.76, data in agreement with that of the monosaccharide composition (Fig. 5).

For determination of the main chain structure of XM, it was submitted to two successive, controlled Smith degradations. Respective polymeric fractions were obtained, SM1 with a Xyl to Man ratio of 9:91, and SM2 with a ratio of 3:97, showing a considerable and then almost complete removal of Xyl*p* side chains. This agreed with the methylation analysis of SM1 and SM2 (Table 2), which also showed that after two successive, controlled Smith degradations, the main chain of (1 → 3)-linked Man*p* units had been completely exposed, and after one, there was still single-unit side chains (8%) of Xyl*p*.

The ^{13}C NMR spectrum of SM1 (Fig. 6a) contained six predominant signals arising from (1 → 3)-linked α-Man*p* units of the main chain, with minor ones from β-Xyl*p* units.

That of SM2 (Fig. 6b) only had six resonances at δ 101.4; 78.4; 73.5; 69.4; 66.1 and 61.1, which correspond to C-1, C-3, C-5, C-2, C-4 and C-6 of α-Man*p* units, respectively (Vinogradov et al., 2004).

The above analysis data show that the xylomannan is composed of a main chain of (1 → 3)-linked α-Man*p* units, partially substituted at O-4 with single unit side chains of β-Xyl*p*, with a small proportion of β-Xyl*p*-(1 → 3)-β-Xyl*p* groups.

Xylomannans with similar main chains were obtained from the mushrooms *Armillaria mellea* (Bouveng et al., 1967) and *Polyporus tumulosus* (Angyal et al., 1974). *A. mellea* presented a partly substituted main chain with branching points at O-4 by Xyl*p*-(1 → 4)-side chains (Bouveng et al., 1967), while *P. tumulosus* contained substitutions at O-4 by single units or different linkages of Xyl*p* side chains (Angyal et al., 1974).

Beside these heteropolymers, other fruit body fucoxylomannans of basidiomycetes, were also isolated. These structures were observed in *Ganoderma lucidum*, which had a main chain formed by (1 → 4)-linked Man*p* units (configuration not known at the time) (Miyazaki and Nishijima, 1982), *Polyporus pinicola* (Axelsson et al., 1969) and *Fomes annosus* (Axelsson et al., 1971), which contained (1 → 3)-linked β-Man*p* main chains (configuration of the linkage determined by optical rotation).

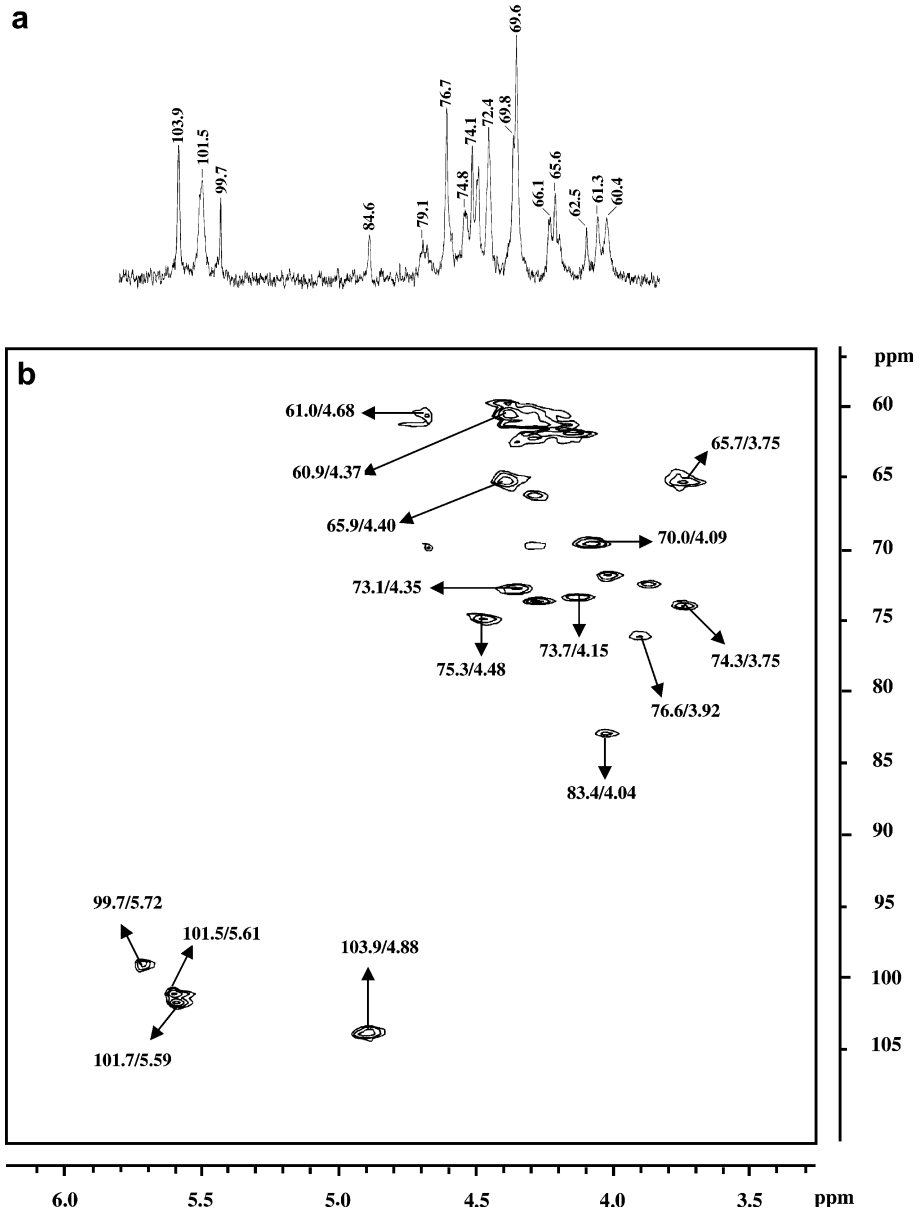

Fig. 4. ^{13}C NMR (a) and HMQC (b) spectra of xylomannan (XM), in DMSO-d_6 at 70 °C, chemical shifts are expressed in ppm.

3. Concluding remarks

In conclusion, we characterized an uncommon xylomannan, and refined the chemical characterization of a β-glucan isolated from *F. velutipes*, which was previously mentioned but never fully characterized. Since many polysaccharides isolated from several basidiomycetes are related to specific biological properties, further studies should be carried out to determine in greater detail the biological role of these polymers.

4. Experimental

General experimental procedures: All solutions were evaporated at <40 °C under reduced pressure. Centrifugation was carried out at 9000 rpm for 15 min, at 25 °C. Alditol acetate mixtures were analyzed by GC–MS using a Varian model 3300 gas chromatograph linked to a Finnigan Ion-Trap, model 810-R12 mass spectrometer, using a DB-225 capillary column (30 m × 0.25 mm i.d.) programmed from 50 to 220 °C at 40 °C/min, then held at that temperature. Partially *O*-methylated alditol acetate mixtures were similarly analyzed, but with a program from 50 to 215 °C at 40 °C/min. The homogeneity and molar mass of the XM fraction were determined by high-performance size-exclusion chromatography (HPSEC-MALLS), using a Waters 510 HPLC pump at 0.6 ml/min, with four gel permeation columns in series with exclusion sizes of 10^6–5×10^3 Da, using a refractive index (RI) detector. Poly(ethylene oxide) of MW = 11,600 was used as standard to calibrate the columns. The eluent was 0.1 mol/l aq.

Table 3
Assignments of signals in the NMR spectroscopic analyses of glucan and xylomannan from *F. velutipes*

Signal (C/H)[a]	Assignment
Glucan	
103.1/4.53	C-1/H-1 of 3-*O*-substituted glucopyranosyl units (unit A)
103.1/4.23	C-1/H-1 of 3,6-di-*O*-substituted glucopyranosyl units (unit B)
86.7 and 86.3	C-3 of 3-*O*-substituted glucopyranosyl units (unit A)
86.0	C-3 of 3-*O*-substituted glucopyranosyl units (unit B)
76.7	C-3 of non-substituted non-reducing end glucopyranosyl units
76.4	C-5 of 6-*O*-substituted glucopyranosyl units (unit A)
76.2	C-5 of 6-*O*-substituted glucopyranosyl units (unit A or C)
74.9	C-5 of 6-*O*-substituted glucopyranosyl units (unit B)
68.5	C-6 of 6-*O*-substituted glucopyranosyl units
61.2 and 60.9	C-6 of non-substituted glucopyranosyl units
Xylomannan	
103.9/4.88	C-1/H-1 of β-xylopyranosyl units
101.7/5.59, 101.5/5.61 and 99.7/5.72	C-1/H-1 of α-mannopyranosyl units
76.7	C-3 of non-reducing end xylopyranosyl units
72.4	C-2 of non-reducing end xylopyranosyl units
69.8	C-4 of non-reducing end xylopyranosyl units
65.6	C-5 of non-reducing end xylopyranosyl units
62.5, 61.3 and 60.4	C-6 of non-substituted mannopyranosyl units

[a] The chemical shifts are expressed as ppm, δ.

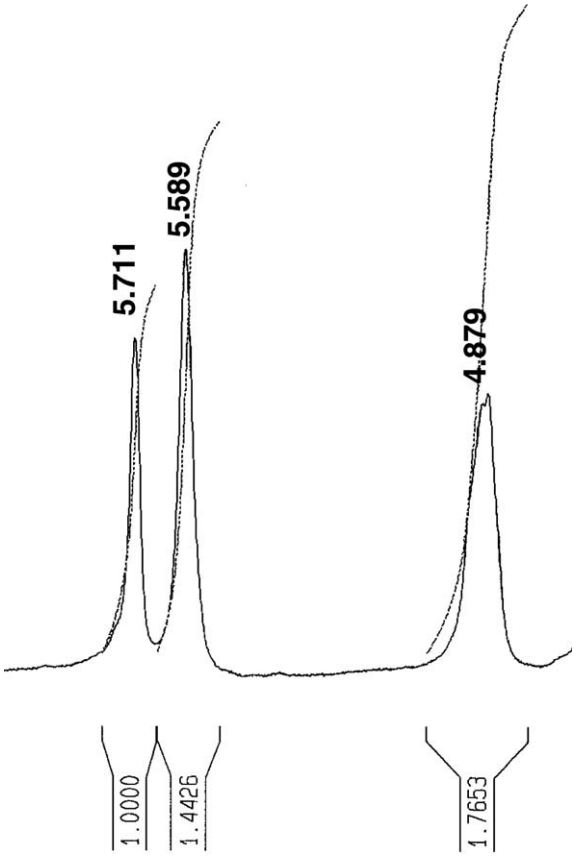

Fig. 5. Anomeric region of ^{1}H NMR spectrum of xylomannan (XM), in DMSO-d_6 at 70 °C, chemical shifts are expressed in ppm.

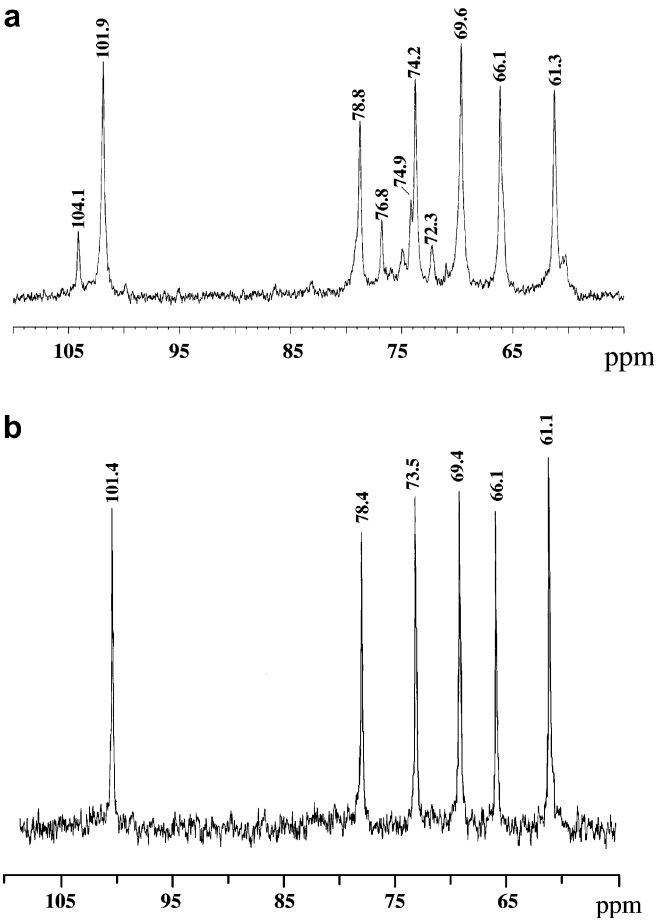

Fig. 6. ^{13}C NMR spectra of successively Smith degraded xylomannan SM1 (a) and SM2 (b), in DMSO-d_6 at 70 °C, chemical shifts are expressed in ppm.

$NaNO_2$ with 200 ppm aq. NaN_3. XM fraction was dissolved in the eluent medium, filtered using a membrane (0.22 μm), and injected (250 μl loop) at a 2 mg/ml concentration. The specific refractive index increment (d*n*/d*c*) was also determined.

Fungal material: F. velutipes is recognized by a sticky, orange-brown cap of 1–3 cm broad and dark, finely pubescent stipe of 1.5–7 cm tall and 0.2–0.7 cm thick. The basidiomycete was purchased at the Public, Municipal Market of Curitiba, State of Parana (PR), Brazil, and was identified by the Prof. Dr. Fábio Rosado from the Centro Universitário de Maringá (CESUMAR), Maringá-PR.

Polysaccharide extraction and purification: The dried fungus (88 g) was milled and submitted to aqueous (×3 at 100 °C, 1000 ml) and alkaline extractions.

The residue from aqueous extraction residue was submitted to further alkaline extraction at 100 °C, using 2% (×3, 1000 ml) and then 25% (×3, 1000 ml) aq. KOH. The combined aqueous extracts were evaporated to a small volume and polysaccharide precipitated by addition to excess EtOH (3:1). A similar procedure was used with the alkaline extracts, except that they were neutralized with AcOH prior to precipitation. All products were dialyzed against

tap water for 48 h, concentrated under reduced pressure and freeze-dried. The extracts were then dissolved in H_2O and the solutions submitted to freezing followed by mild thawing at 4 °C. Soluble fractions, following centrifugation, were obtained from hot aqueous (SW), 2% aq. KOH (SK2), and 25% KOH (SK25) extracts. Corresponding insoluble material was obtained and called PW, PK2, and PK25 (Fig. 1).

Monosaccharide composition: Each fraction (2 mg) was hydrolyzed with 2 M TFA at 100 °C for 8 h, followed by evaporation to dryness. The residue was successively reduced with excess of $NaBH_4$ and acetylated with Ac_2O–pyridine (1:1, v/v; 2 ml) at room temperature for 12 h (Wolfrom and Thompson, 1963a,b). The resulting alditol acetates were analyzed by GC–MS as indicated above and identified by their typical retention times and electron impact profiles (Jansson et al., 1976).

Methylation analysis: Per-*O*-methylation of the isolated polysaccharides (10 mg each) was carried out using powdered NaOH in DMSO–MeI (Ciucanu and Kerek, 1984). The products were treated with 50% v/v aq. H_2SO_4 (0.5 ml v/v, 1 h, 0 °C), followed by a dilution until it reached 5.5% (addition of 4.0 ml of distilled H_2O). The solution was kept at 100 °C for 12–18 h, and was neutralized with $BaCO_3$ (Saeman et al., 1954), filtered, and the filtrate evaporated to dryness. The residues were converted into partially *O*-methylated alditol acetates as described above, and analyzed by GC–MS (as described above).

NMR analyses: ^{13}C and ^{1}H NMR and HMQC spectroscopic determinations were carried out using a 400 MHz Bruker model DRX Avance spectrometer incorporating Fourier transform. Samples were dissolved in D_2O or DMSO-d_6 and examined at 50 or 70 °C. Chemical shifts are expressed in ppm (δ) relative to the resonance of acetone at δ 30.20 (^{13}C) and 2.22 (^{1}H) for experiments in D_2O, and of DMSO-d_6 at δ 39.70 (^{13}C) and 2.40 (^{1}H) for those examined in this solvent.

Controlled Smith degradation: Fractions PK2 and XM (150 mg each) were submitted to oxidation with 0.05 M aq. $NaIO_4$ (20 ml) for 72 h at 25 °C in the dark (Abdel-Akher et al., 1952; Hay et al., 1965). Samples were then dialyzed against tap H_2O for 48 h and treated with $NaBH_4$ (pH 9–10) for 20 h. The solutions were dialyzed again, and freeze-dried. The products were then submitted to partial acid hydrolysis (TFA, pH 2.0, 30 min, 100 °C), and dialyzed against tap H_2O using membranes with a size exclusion of 2 kDa and retained material was freeze-dried.

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