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Note

The structure of a polysaccharide isolated from *Inonotus levis* P. Karst. mushroom (Heterobasidiomycetes)

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Abstract—*Inonotus levis* biomass was extracted with 5% NaOH containing $NaBH_4$, the insoluble material was discarded and the solution dialyzed. It was further treated with proteinase and the polymeric fraction isolated by gel chromatography. It contained mostly a polysaccharide of the following structure:

$$\beta\text{-GlcA-}(1\rightarrow2)\text{-}\alpha\text{-Gal-}(1\rightarrow6)\text{-}\alpha\text{-Gal3OMe-}(1\rightarrow[\rightarrow6)\text{-}\alpha\text{-Gal-}(1\rightarrow6)\text{-}\alpha\text{-Gal3OMe-}(1\rightarrow]_{5\text{–}10}$$

where a non-reducing terminal glucuronic acid residue was present in about half of the molecules, making thus some of the chains acidic and others neutral. Methyl groups were present at about half of the galactose residues, however, no direct proof of the presence of defined repeating units was obtained due to NMR signals overlap. We believe that these short polymeric chains might be originally attached to a protein *via* serine or threonine residues and were cleaved off due to the alkaline conditions of extraction. Another polymer, co-extracted with this galactan, was a branched phosphorylated mannan with a structure similar to that of the mannan from *Saccharomyces cerevisiae* yeast.

Keywords: *Inonotus levis*; Basidiomycetes; Medicinal mushrooms; Polysaccharides; Phosphorylated mannan; Galactan; NMR spectroscopy

Recently, medicinal mushroom polysaccharides have received special attention due to their wide application. In particular, several groups of polysaccharides from culinary-medicinal mushrooms are attractive because they have potent biological and pharmacological activities including antitumor, immunomodulating, anti-inflammatory, cardiovascular, hypocholesteric, antiviral, antibacterial, antiparasitic, antifungal, antidiabetic, and hepatoprotective activities.[1–9] Various kinds of mushroom polysaccharides, for example, PSK or Krestin from mycelium of *Trametes* (= *Coriolus*) *versicolor* (L.:Fr.) Lloyd, lentinan a water-soluble antitumor polysaccharide from fruiting bodies of *Lentinus edodes* (Berk.) Singer, schizophyllan, or SPG medium product of *Schizophyllum commune* Fr.:Fr., are now commercially available.[1,3]

Some species of the genus *Inonotus*, Hymenochaetaceae, Agarico-mycetideae (e.g., *I. hispidus* (Bull.:Fr.) P. Karst., *I. kanehiare* (Yesuda) Imaz., *I. obliquus* (Pers.:Fr.) Pilat, *I. sciurinus* Imaz., and *I. tabacinus* (Mont.) P. Karst.) are known to have different medical effects.[1,10,11] *I. obliquus* (Chaga) is well known as one of the most popular medicinal species due to its therapeutic effects. Protein-containing polysaccharides from fruit bodies, sclerotia, and mycelia were extracted and purified.[1] The drug 'Befungin' was developed from these species in Russia.

In the course of screening for exopolysaccharide production by submerged cultures of medicinal mushrooms, it was found that another species, *I. levis*, had the capability of producing large amounts of exopolysaccharides. In our preliminary investigation, it was

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found that the polysaccharide produced by the submerged culture of *I. levis* had notable pharmaceutical activities.

Therefore, in the present study data on the structure of polysaccharides isolated from the submerged culture of *I. levis* were examined using NMR spectroscopy, gel- and ion-exchange chromatography.

The 'polysaccharide', obtained by ethanol precipitation of culture liquid, was washed with EtOH to remove lipids and then extracted with 5% NaOH in the presence of $NaBH_4$. The solution was dialyzed and treated with proteinase. The yield of the product was 2.8% (from wet material, so actual yield is ~5 times higher). Monosaccharide analysis of this extract showed the presence of Fuc, Xyl, Gal3OMe, Man, Glc, and Gal in the ratio 0.2:0.4:1.1:3.3:1:2.3. NMR showed that it is mainly a polysaccharide without much protein or lipid contamination. It was further purified by anion-exchange chromatography to give several fractions. All of them contained mixtures of two polysaccharides, which will be further referred to as galactan and mannan, in different ratios. Galactan was a predominant compound and its acidity was due to the presence of a single glucuronic acid (GlcA) residue at the non-reducing end. Some of the galactan had no GlcA and this fraction was not retained on anion-exchange column. The acidity of mannan was due to various degrees of phosphorylation, therefore mannan was spread over a large number of fractions.

In order to obtain galactan free of mannan, the acidic fraction of galactan was dephosphorylated with 48% HF and separated once again on anion-exchange column. Dephosphorylated mannan lost its charge and was eluted in water, whereas the acidity of the galactan remained unchanged and it was retained on the column. Thus, a clean galactan sample nearly free of mannan was obtained.

The molecular mass of the galactan was determined by gel chromatography on Sephacryl S-200HR column as ~5000 Da. NMR data—integration of H-1 signals of terminal monosaccharide relative to the sum of H-1 intensities of in-chain monomers—indicated a lower mass of about 2500 Da. Mannan had broad mass range, starting at as high as ~15,000, lower molecular mass components of it overlapped with galactan.

Monosaccharide analysis of the galactan (GC–MS of alditol acetates) showed the presence of galactose and 3-*O*-methyl galactose (2:1), and small amounts of fucose and glucose (<5%). Mannan fractions contained mannose and 8% each of glucose and xylose.

A set of 2D NMR spectra—COSY, TOCSY, NOESY, HSQC, gHMBC, HSQC-TOCSY was recorded and signals were assigned using the computer program Pronto[12] (Figs. 1 and 2, Table 1). The dominant part of the galactan consisted of a (1→6)-linked linear chain of α-galactopyranose residues, some of them methylated at O-3. The data do not allow to make a conclusion about the presence of the repeating unit, because of the overlap of H-6 signals of both methylated and non-methylated residues. H-1 signals of A and B

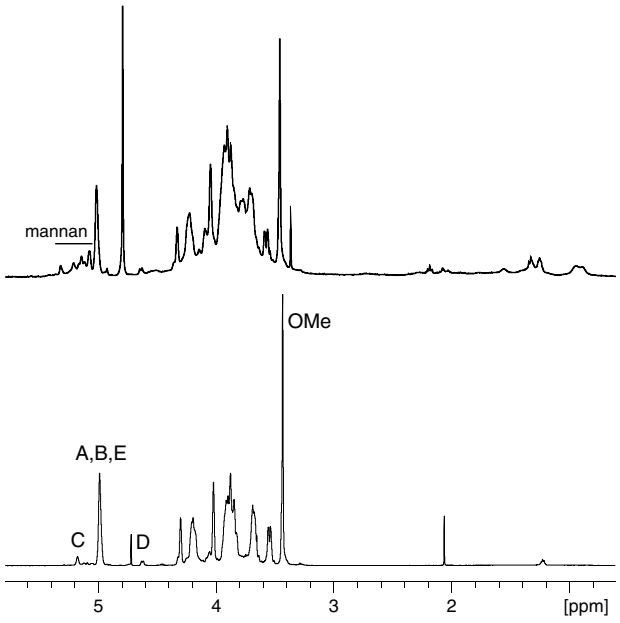

Figure 1. ^{1}H NMR spectra of the *Inonotus levis* polysaccharides. Top, non-purified extract after dialysis; bottom, clean galactan. Letters indicate H-1 signals of the corresponding residues.

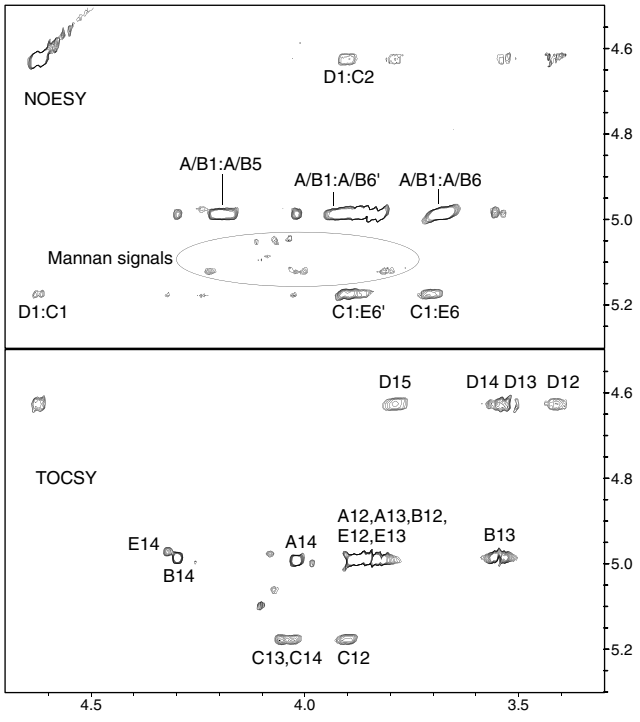

Figure 2. Fragments of TOCSY and NOESY spectra of the galactan from *Inonotus levis*. Intra-ring TOCSY correlations are labeled by residue letter and two unseparated numbers of correlating protons.

Table 1. NMR data for the *Inonotus levis* polysaccharide (δ, ppm)

Unit		1	2	3	4	5	6		OMe
							6a	6b	
A α-Gal	^{1}H	4.98	3.85	3.88	4.02	4.21	3.69	3.91	
	^{13}C	99.8	70.3	71.5	71.4	70.8	68.5		
B α-Gal3OMe	^{1}H	4.98	3.87	3.55	4.30	4.18	3.69	3.91	
	^{13}C	99.8	69.3	80.7	67.1	70.7	68.5		
C α-Gal	^{1}H	5.17	3.90	4.03	4.06	4.00	3.75	3.75	
	^{13}C	100.2	80.4	70.3	71.4	71.4	63.1		
D β-GlcA	^{1}H	4.62	3.41	3.53	3.55	3.78			
	^{13}C	106.0	75.2	77.3	73.6	78.1	176.0		
E α-Gal3OMe	^{1}H	4.96	3.87	3.55	4.32	4.14	3.70		3.43
	^{13}C	99.8	69.3	80.7	67.0	71.4	69.1		58.0

were also nearly exactly overlapped. The methyl signal integral intensity relative to the sum of intensities of H-1 signals of galactose residues was 3:2, which correspond to methylation of half of the galactose residues. This does not agree completely with the results of the monosaccharide analysis, where galactose and 3-*O*-methylgalactose were detected in a 2:1 ratio but may be explained by an underestimated integral intensity of H-1 signals due to signal broadening in the polymeric chain, whereas the methyl signal remained sharp.

Two minor signals for H-1 of the residues D (β-GlcA) and C (α-Gal) were present in the spectra of the acidic fractions of the galactan in a ratio of ~1:15 referred to the sum of H-1 of all in-chain Gal and 3-*O*-Me-Gal residues. H-1 of residue D showed NOE cross-peaks with H-1 and H-2 of Gal C, thus indicating a (1→2) linkage between these residues. H-1 of Gal C correlated with H-6 of the 3-*O*-Me-Gal E residue. All signals of the residue E were slightly shifted from the rest of the 3-*O*-Me-Gal signals, allowing their reliable assignment.

The sequence was confirmed by the observation of HMBC correlations from H-1 of all residues to the transglycosidic carbon atoms.

Absolute D-configuration of the galactose and GlcA was determined by GC of the acetates of 2-butyl-glycosides or esters.[13]

On the basis of experimental data the following structure for the O-specific polysaccharide *I. levis* was proposed:

β-GlcA-(1→2)-α-Gal-(1→6)-α-Gal3OMe-(1→[→6)-α-Gal-(1→6)-α-Gal3OMe-(1→]$_{5\text{-}10}$-ol
D C E A B

The results do not exclude the possibility of random arrangement of Gal and Gal3OMe residues within the chain and of the presence of an excess of non-methylated Gal residues.

NMR data for the mannan and dephosphorylated mannan showed that they have the following general structure:

α-Man-(1→2)-┐
[→6)-α-Man-(1→6)-α-Man-(1→]
α-Man-(1→2)-α-Man-(1→2)-┘

phosphorylated at unknown positions. Chemical shifts, NOE and HMBC were close to that of the corresponding fragments of *S. cerivisiae* mannan.[14] Similar structures are common for yeast mannans.[14,15]

Procedures used for the preparation of polysaccharides from mushrooms usually include many steps of extraction with different salt solutions. Sodium hydroxide extraction is used as a last step, thus it is believed to be the strongest method. We decided that direct application of NaOH extraction will produce representative mixture of most of the polysaccharides found in the biomass, which can be then resolved by chromatographic methods. Only two polysaccharides were thus extracted, a typical fungal mannan and a galactan, not described before as a constituent of mushrooms.

Polysaccharides normally have some aglycon at the reducing end. Alkaline conditions used for the extraction in this work can split the linkage of the O-linked sugar chains of the glycoproteins. Therefore we believe that galactan, as well as mannan chains might be linked to a carrier protein and released from it due to alkaline conditions.

1. Experimental

1.1. Organism and culture condition

I. levis (HAI 796) was kindly provided from the Culture collection of Higher Basidiomyces of the Institute of Evolution (HAI), University of Haifa, Israel. The stock

culture was maintained on malt extract-peptone agar slants, subcultured every half year, and the slants were incubated at 28 °C for 10 days and then stored at 4 °C.

All cultivations were conducted in 250-mL Erlenmeyer flasks filled with 50 mL of defined synthetic medium. Fungal inocula were grown on synthetic medium consisting of the following components (g/L of tap water): glucose, 10.0; $(NH_4)_2SO_4$, 2.0; KH_2PO_4, 1.0; $Na_2HPO_4{\cdot}7H_2O$, 0.5; $MgSO_4{\cdot}7H_2O$, 0.5; yeast extract, 2.0. Phosphate salts were sterilized separately.

The flasks were inoculated with 4-day-old precultures (30–40 mg of biomass dry weight per one flask) and cultivations were carried out on a rotary shaker (160 rpm) at 23–24 °C. All experiments were performed in triplicate. The biomass was obtained by centrifuging samples at 9000 rpm for 15 min, washing the sediment three times with water, and drying to a constant weight. All supernatants were collected: then, the crude polysaccharides were precipitated with the addition of 4 vol of 95% ethanol.

1.2. Isolation of the polysaccharides

The ethanol precipitated product (25 g wet mass) was washed with boiling ethanol (250 mL × 3). The insoluble residue was dried on air and extracted with 5% NaOH–5% $NaBH_4$ (500 mL) overnight at room temperature with magnetic stirring. The insoluble material was removed by centrifugation and the soln dialyzed. After 2 days of dialysis, proteinase K was added (200 mg), and dialysis continued for two more days. The dark brown but transparent soln was treated with charcoal for decolorization, filtered through 10–15 mesh glass filter, and dried to give 700 mg of product. Seventy milligrams of it was separated by anion-exchange chromatography to give a neutral fraction and a broad peak containing carbohydrate components, which was divided into four fractions representing mixtures of galactan and mannan in various proportions.

1.3. NMR spectroscopy

^1H and ^{13}C NMR spectra were recorded using a Varian Inova 500 spectrometer in D_2O solns at 60 °C with acetone standard (2.225 ppm for ^1H and 31.5 ppm for ^{13}C) using standard pulse sequences COSY, TOCSY (mixing time 120 ms), NOESY (mixing time 200 ms), HSQC, gHMBC (optimized for 5 Hz coupling constant), HSQC-TOCSY. Spectra were assigned using the computer program Pronto.[12]

1.4. Monosaccharide identification

The polysaccharide (1 mg) was hydrolyzed (0.5 mL of 3 M TFA, 100 °C, 2 h), evaporated to dryness under a stream of nitrogen, dissolved in water (0.5 mL), reduced with $NaBH_4$ (~5 mg, 30 min), treated with AcOH (0.5 mL), dried. Methanol (1 mL) was added and the mixture was dried twice, the residue acetylated with Ac_2O (0.5 mL, 100 °C, 30 min), dried, analyzed by GLC on DB-17 capillary column (25 m × 0.25 mm) with flame ionization detector (Agilent 6850 instrument) in a temperature gradient 180–240 °C at 2°/min, or on Varian Saturn 2000 instrument equipped with ion-trap mass spectral detector. For absolute configuration determination, a sample of the polysaccharide (1 mg) was treated with (*R*)-2-butanol (0.5 mL) and acetyl chloride (0.05 mL) for 3 h at 100 °C, dried, acetylated as described above, and analyzed by GLC in a temperature gradient 120–240 °C at 2°/min, retention times were compared with those of standard samples prepared from D-galactose and D-glucuronic acid with (*R*)- and (*S*)-2-butanol.[13]

1.5. Gel chromatography

Gel chromatography was carried out on Sephadex G-50 (2.5 × 95 cm), Sepacryl S-200HR (1.6 × 80 cm), and Sephadex G-15 columns (1.6 × 80 cm) in pyridinium-acetate buffer, pH 4.5 (4 mL pyridine and 10 mL AcOH in 1 L water); the eluate was monitored using a refractive index detector.

1.6. Ion-exchange chromatography

Ion-exchange chromatography was performed on Hi-trap Q anion-exchange column containing 5 mL of Q-Sepharose Fast Flow (Amersham Pharmacia Biotech) in a gradient of water—1 M NaCl over 1 h with UV detection at 220 nm; each 3 mL fraction was tested for the presence of carbohydrates by applying a spot (2 μL) onto TLC plate, which was then dried, dipped in 5% H_3PO_4 in MeOH, and developed by heating with a heat gun until brown spots of carbohydrate-containing material became visible. The products were desalted by gel chromatography on Sephadex G-15 column.

1.7. Dephosphorylation

The polysaccharide sample (15 mg) was dissolved in 48% HF (0.2 mL), kept for 24 h at 5 °C, diluted with water (20 mL), and dialyzed against water. The products were then separated by anion-exchange chromatography to give unretained dephosphorylated mannan and acidic galactan polysaccharide.

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