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AW: idea and design of the experiments, structural experiments, analysis of the results, writing the manuscript, preparing figures ; AC: idea and design of the experiments, structural experiments, analysis of the results, writing the manuscript, revision of the manuscript; IK: idea and design of the experiments, structural experiments, analysis of the results, writing the manuscript, revision of the manuscript; MP: cultivation experiments, preparing figures; MS: cultivation experiments; PP: structural experiments, preparing figures; GJ: genetic analysis; JS: idea and design of the experiments, technical supervision

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SHORT COMMUNICATION

Fruiting bodies of *Hericium erinaceus* (Bull.) Pers. – a new source of water-insoluble (1→3)-α-D-glucan

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Abstract
A water-insoluble polysaccharide (WIP) was isolated from the fruiting bodies of *Hericium erinaceus* HE01 by an alkaline solution with the yield of 5%. Structural and compositional analyses by total acid hydrolysis, methylation analysis, FT-IR, FT-Raman, and ^{1}H NMR spectroscopy as well as other instrumental techniques showed predominantly glucose linked by α-glycosidic bonds and small amounts of mannose, xylose, rhamnose, galactose, and ribose. The methylation analysis showed that (1→3)-linked Glc*p* is the major constituent (70.8%) of the polymer, while the 3,4 substituted D-Glc*p* represents the main branching residue of the glucan. The presence of (1→3)-α-D-glucan in the hyphae of *H. erinaceus* was additionally confirmed by the use of specific fluorophore-labeled antibodies.

Keywords
Hericium erinaceus; (1→3)-α-D-glucan; cell wall; fruiting body; carbohydrate analysis

Introduction

Hericium erinaceus is a famous edible mushroom in China and some other countries in East Asia. Production thereof is relatively easy and many organic materials (sawdust, cereal bran or grains, soybean meal, maize cob rachises, and wastes from sugar cane or agricultural production) were used as a cultivation substrate [1].

The fruiting bodies of *H. erinaceus* have attracted considerable attention because of their antimicrobial and immunomodulatory effects, anti-tumor activities, cytotoxic effect, antioxidant properties, hepatoprotective potential, as well as their promotion of synthesis of the neurogrowth factor [2]. As many other fungi, *H. erinaceus* contains polysaccharides as a major component of their cell wall, and a series of polysaccharides from the mycelium and fruiting body have been purified and their chemical structures studied [3]. Zhang and co-workers [4] described a multibranched (1→3)-α-D-glucan extracted from water-soluble fraction of *H. erinaceus*, but according to our knowledge, there are no reports concerning structural studies on the water-insoluble (1→3)-α-D-glucan.

(1→3)-α-D-Glucan is present in a large number of fungi, but there is very little data concerning its biosynthesis and function. The most of extensive studies were carried out on the biological activity of fungal β-glucan. Water-insoluble (1→3)-α-D-glucan isolated from fungal fruiting bodies usually shows little or no antitumor effect, but their modified products (obtained on the way of, e.g.,: carboxymethylation, hydroxyethylation, sulfation, or aminopropylation) exhibited a strong antitumor activity [5]. Moreover, fungal (1→3)-α-D-glucan has been used as an alternative to the mutan derived from streptococci, which is commonly used for an efficient induction of mutanase, an enzyme able to degrade specific polysaccharides found in dental plaque and hence to prevent dental caries [6]. Due to its insolubility in water, (1→3)-α-D-glucan from *Penicillium chrysogenum* mycelia was a substrate for the preparation of epoxy-activated microsphere as carrier for efficient immobilization of lipase derived from *Candida* sp. [7].

In this article, we reported the detection, isolation, structure, and some properties of the water-insoluble polysaccharide obtained from the fruiting bodies of *H. erinaceus*.

Material and methods

Fungal material and fruiting conditions

The *Hericium erinaceus* (Bull.) Pers. strain was used in the experiment. The voucher specimen (HE01) was lodged in the fungal collection of the Department of Vegetable Crops, Poznań University of Life Sciences, Poland. The strain was classified at the species level by molecular biological analysis of the internal transcribed region (ITS) following the methods described by White and co-workers [8] with further modifications by Wiater and co-workers [9]. Stock culture of the strain was grown at 25°C, on potato dextrose agar (Merck, Darmstadt, Germany). The culture was provided for 14 days. The strain, was permanently stored at 4°C, and it was subcultured every 3 months. The culture was used for the grain spawn (wheat grain) preparing, using a classical method. The preparation was stored at 4°C until it was used for inoculation of the cultivation substrate. The basal substrate composition and cultivation conditions were as described previously [1].

Immunofluorescent labelling of cell wall (1→3)-α-D-glucans

To visualize of the (1→3)-α-D-glucans inside the cell wall of *H. erinaceus*, the specific antibodies labeled with fluorescent dye were used [10]. The presence of (1→3)-α-D-glucan was registered using an excitation wavelength from 470 to 500 nm and emission from 525 to 550 nm (using Olympus BX 51 microscope).

Isolation of (1→3)-α-D-glucans

Fresh fruiting bodies of *H. erinaceus* were lyophilized and milled, and the dried material was used for the isolation of the (1→3)-α-D-glucans according to a method described by Kiho et al. [11].

Carbohydrate analyses

Sugar analyses, including composition analysis, methylation analysis, FTIR, ^{1}H NMR, specific rotation, and viscosity of the alkali-soluble polysaccharides were performed as described previously [9]. The FT-Raman spectrum was recorded on a powdered sample using a Nicolet NXR FT-Raman spectrometer (Madison, USA).

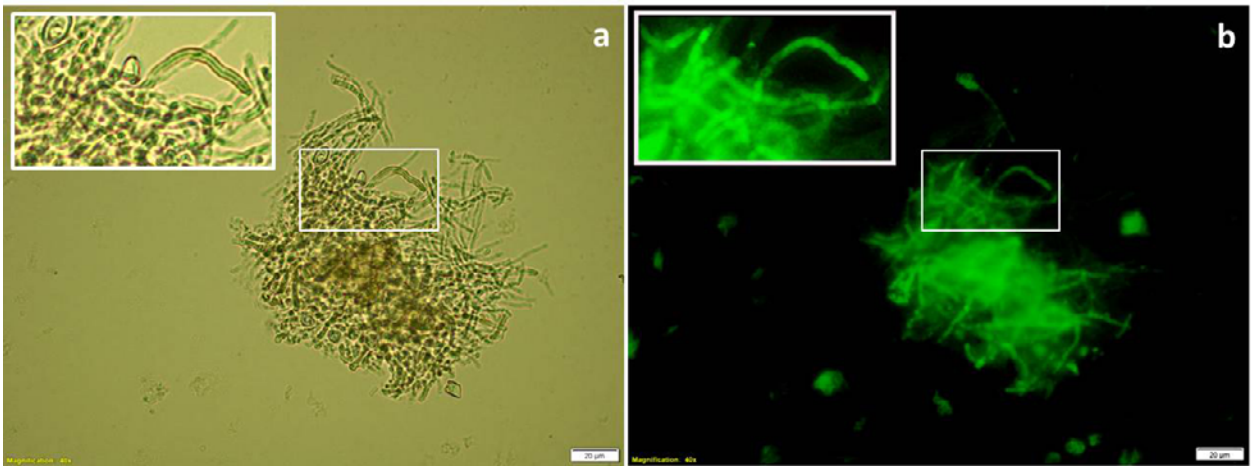

Fig. 1 Visualization of (1→3)-α-D-glucan in the cell wall of *H. erinaceus* HE01 using fluorophore-labeled antibodies (Clone IgM MOPC-104E). **a** Filaments in the light microscopy. **b** The same filaments in fluorescence microscopy. In this experiment, more than 10 preparations were observed, and representative images were chosen for presentation. Scale bar: 20 μm.

Results and discussion

The analyzed strain was identified as *H. erinaceus* based on the sequence of internal transcribed spacer (ITS). Product of length 631 bp was obtained from the PCR amplification, with ITS1–ITS4 primers, and followed by direct sequencing and result was deposited in GenBank under accession number KJ920141. The sequence of this product indicated 100% identity to the *Hericium erinaceus* ITS sequences.

(1→3)-α-D-Glucan is present in a majority of fungi as the main component of the network of glycoproteins and polysaccharides that build the cell wall. In order to detect α-(1→3)-linked D-glucose in the hyphae of *H. erinaceus* HE01, we used specific fluorophore-labeled antibodies (Clone IgM MOPC-104E). As shown in Fig. 1, (1→3)-α-D-glucan was labeled in the hyphae of all the tested species. Moreover, glucan accumulation was seen over the entire length of the hyphae, which confirms the importance of this polymer in the cell wall structure.

The fruiting bodies of *H. erinaceus* HE01 used in this study were cultivated on an artificial substrate (mixture of oak and beech sawdust; Fig. 2). The average yield of matured carpophores from 100 g of the substrate (dry matter) amounted to 42.8 g.

Fig. 2 Cultivated fruiting bodies of *H. erinaceus* HE01.

The water-insoluble polysaccharide (WIP) was obtained from the lyophilized fruiting bodies of *H. erinaceus* HE01 by 1 M NaOH solution extraction with the yield of 5%. The specific optical rotation of the polymers was +45°. The values of viscosity oscillated around 6.7 mPa.

The saccharide analysis showed that the tested polysaccharide consisted mainly of glucose (82.5%). Mannose (7.4%), xylose (7.3%), rhamnose (1.8%), and trace amounts of galactose (0.8%) and ribose (0.2%), were found. All liberated sugars had D absolute configuration [12].

The GC-MS analysis of permethylated alditol acetates revealed that (1→3)-linked Glc*p* is the main component of the glucan backbone chains (70.8%; Tab. 1). The glucan also contained two forms of doubly substituted glucose residues, i.e.: →3,4)-Glc*p*-(1→ and →3,6)-Glc*p*-(1→. The 3,4-substituted D-Glc*p* represented the main branching residue, ca. 26% of the glucan polymer. Moreover, we can suppose that the 3,6-substituted D-Glc*p* formed additional short side chains (1.9%).

Tab. 1 Methylation analysis of WIP from fruiting bodies of *H. erinaceus* HE01.

Methylated sugar	Linkage type	Molar amounts (%)
2,3,4,6-*O*-Me_4-D-glucose	Glc*p*-(1→	1.6
2,4,6-*O*-Me_3-D-glucose	→3)-Glc*p*-(1→	70.8
2,6-*O*-Me_2-D-glucose	→3,4)-Glc*p*-(1→	25.7
2,4-*O*-Me_2-D-glucose	→3,6)-Glc*p*-(1→	1.9

Note: the methylated glucan was hydrolyzed, reduced, and acetylated. For example: 2,3,4,6-*O*-Me_4-D-glucose refers to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

The FT-IR spectrum of the glucan ranging from 400 cm^{-1} to 4000 cm^{-1} are shown in Fig. 3. An intense and broad peak at 3336.72 cm^{-1} for O–H stretching vibrations, a signal at 2926.75 cm^{-1} for C–H stretching vibrations, and a broad peak in the region of 950–1200 cm^{-1} for coupled C–O and C–C stretching and C–OH bending vibrations are characteristic for polysaccharides [13]. The signal at around 1647.96 cm^{-1} could indicate the presence of protein impurities (N–H groups). The glucan preparation had absorption peaks at 846.11 and 822.49 cm^{-1}, characteristic of (1→3)-α-D-glucans. Especially, the peak at 822.49 cm^{-1} is exclusively associated with (1→3)-α-linkages [14]. The lack of absorbance peaks at 890 cm^{-1} suggested that WIP contained only α-linked glucose [15].

The FT-Raman spectrum (Fig. 4) of the WIP had bands at 1465.42, 1268.75, 1127.38, 1076.11, and 1046.32 cm^{-1}, which indicate the presence of polysaccharides [16]. The band at 859.45 cm^{-1} is sensitive to the anomeric structure around the glycosidic bonds and confirmed the α-configuration of the majority of monosugars (glucose) in the WIP of *H. erinaceus* HE01. Moreover, the intense and sharp bands at 940.96 and 547.25 cm^{-1} could be assigned to (1→3)-α-D-glucan [17].

The determination of α-glucosidic linkages in the tested glucan was also based on the ^1H NMR spectrum. In the anomeric region of the spectrum (Fig. 5) the main broad unresolved doublet ($J_{H1,H2} < 3$ Hz) at δ 5.531 ppm was present, which could be assigned to the α-anomeric proton of D-glucose. Any doublets with $J_{H1,H2}$ ~8 Hz was observed, thus indicated the lack of β-anomeric sugars [18].

Taking together the above data, we can conclude that the water-insoluble polysaccharidic material extracted from the fruiting bodies of *H. erinaceus* HE01 contained mainly (1→3)-α-D-glucan.

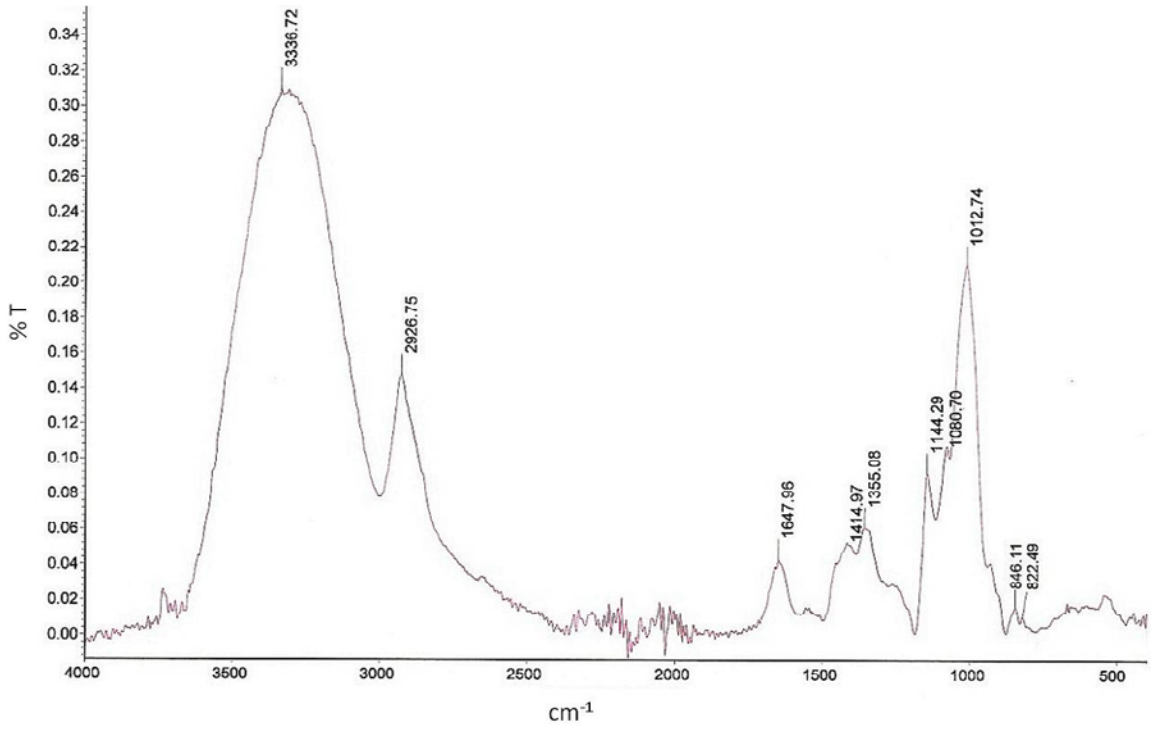

Fig. 3 FT-IR spectrum of WIP from the fruiting bodies of *H. erinaceus* HE01.

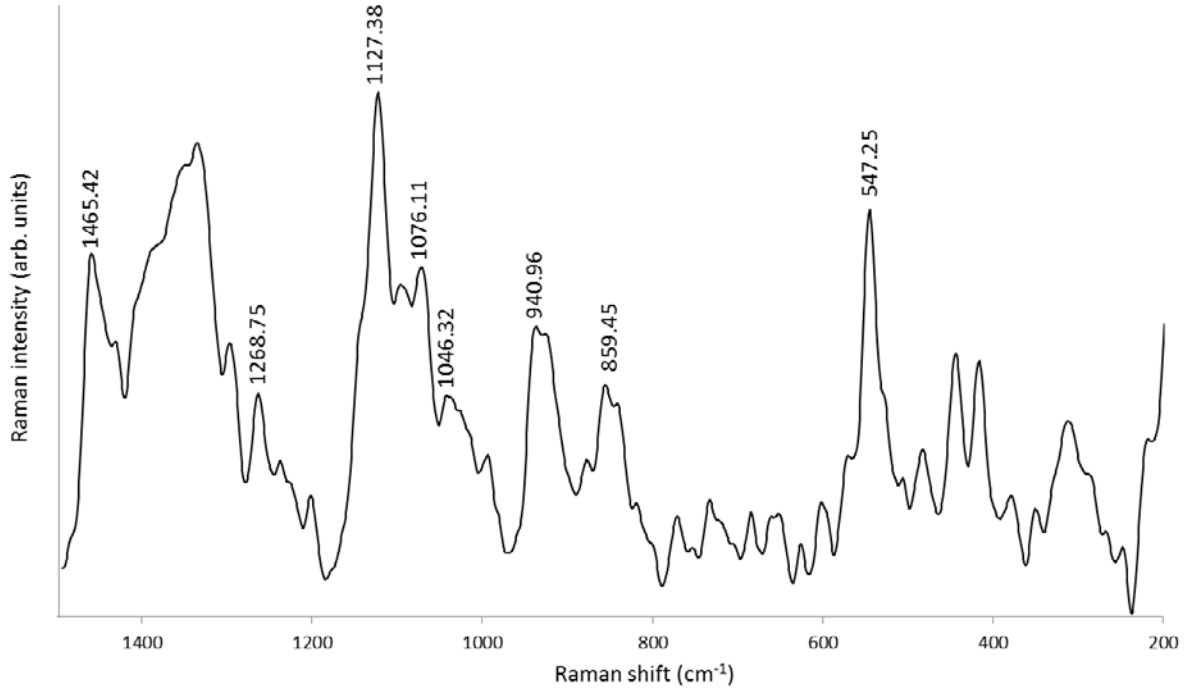

Fig. 4 FT-Raman spectrum of WIP from the fruiting bodies of *H. erinaceus* HE01.

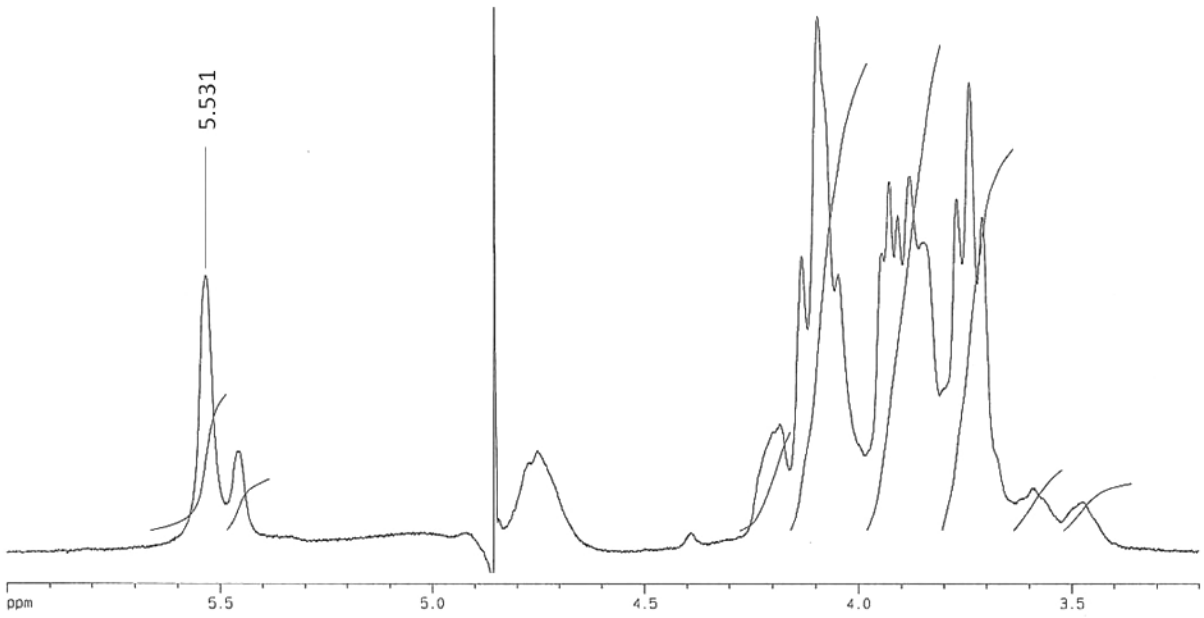

Fig. 5 ^{1}H NMR of WIP from the fruiting bodies of *H. erinaceus* HE01.

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