Carbohydrate Polymers 91 (2013) 603–608

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

# Chemical characterization of a water insoluble (1 → 3)-α-D-glucan from an alkaline extract of *Aspergillus wentii*

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ARTICLE INFO

*Article history:*
Received 20 June 2012
Received in revised form 2 August 2012
Accepted 17 August 2012
Available online 24 August 2012

*Keywords:*
(1 → 3)-α-D-Glucan
Water-insoluble polysaccharide
*Aspergillus wentii*
NMR
FT-IR

ABSTRACT

The chemical structure of a water insoluble α-glucan isolated from the cell wall of *Aspergillus wentii* was described on the basis of total acid hydrolysis, methylation analysis, and 1D and 2D NMR studies (TOCSY, DQF-COSY, NOESY and HSQC) as well as other instrumental techniques. It was established that the analyzed preparation contained a linear polymer composed almost exclusively of (1 → 3)-linked α-D-glucose, with a molecular mass of about 850 kDa. The polymer was divided into subunits separated by a short spacers of (1 → 4)-linked α-D-glucoses. Each subunit contained about 200 glucose residues.


## 1. Introduction

Molds of the genus *Aspergillus* are distributed worldwide. They can be found in many different environments from the Arctic region to the tropics. Probably, they are the most frequently encountered fungi in human surroundings (Gómez-Miranda et al., 2004). Fungal biomass (including *Aspergillus* biomass) arising as an industrial by-product can be utilized in various ways. Mycelia can be used in the production of food and feed as protein and lipid supplements. The biomass is a source of flavors, antibiotics, vitamins and other metabolites, as well as enzymes and cell wall polysaccharides (Maziero, Cavazzoni, & Bononi, 1999). Generally, the cell wall of most fungi is composed of different types of glucans, chitin, and glycoproteins. (1 → 3)-α-D-Glucan is found in a large number of fungi, but little is known about its function and biosynthesis. In *Aspergillus* species, (1 → 3)-α-D-glucan polymers represent 9–32% of total cell-wall carbohydrates (Bobbit, Nordin, Roux, Revol, & Marchessault, 1977; Borgia & Dodge, 1992; Leal, Guerrero, Gómez-Miranda, Prieto, & Bernabe, 1992). It has been suggested that in the case of *Aspergillus nidulans* this polysaccharide serves as the main reserve material accumulated in the cell wall during vegetative growth (Zonneveld, 1973). In vivo, (1 → 3)-α-D-glucan has been detected in the biofilm matrix in pulmonary aspergilloma (caused by *A. fumigatus*), where hyphae remained strongly aggregated, but not during invasive aspergillosis, where hyphae were disseminated in the lung. This observation suggests that (1 → 3)-α-D-glucans could be involved in biofilm cohesion (Fontaine et al., 2010).

It is generally accepted that some (1 → 3)-β-D-glucans (e.g., lentinan, schizophyllan, and grifolan) obtained from fungi can be used as immunomodulators in the treatment of cancers (Bao, Duan, Fang, & Fang, 2001). Compared with the intensive study of β-glucan, relatively little work has been done on the biological activity of (1 → 3)-α-D-glucan. The few reports available deal with the structure and immunomodulatory properties of (1 → 3)-α-D-glucans from different sources (Huang, Zhang, Cheung, & Tan, 2006; Unursaikhan, Xu, Zeng, & Zhang, 2006; Zhang, Zhang, Zhou, Chen, & Zeng, 2000). It has been found that water-insoluble (1 → 3)-α-D-glucans isolated from mycelia or fruiting bodies usually have little or no antitumor effect, while their modified (e.g., carboxymethylated, sulfated, aminopropylated, or hydroxyethylated) products show a potent antitumor activity (Gan, Ma, Jiang, Xu, & Zeng, 2011; Kiho, Yoshida, Nagai, & Ukai, 1989; Wiater et al., 2011).

In our previous studies, it has been shown that a cell wall preparation from the mycelium of *Aspergillus wentii* effectively induced the production of (1 → 3)-α-glucanase (mutanase) in cultures of *Trichoderma harzianum* and *Paenibacillus curdlanolyticus*. Moreover, this cell wall preparation fully substituted streptococcal mutan

*Abbreviations:* AW-WIG, *Aspergillus wentii* water-insoluble glucan.

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0144-8617/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2012.08.060

((1 → 3),(1 → 6)-α-D-glucan) when used in the culture medium as the sole carbon source (Pleszczyńska, Wiater, & Szczodrak, 2010; Wiater, Szczodrak, Pleszczyńska, & Próchniak, 2005). In contrast to the streptococcal biopolymer, this new and powerful stimulant of inductive mutanase synthesis is inexpensive, easily available, and safe for humans. Moreover, the cell wall-induced mutanase, compared to other mutanases, shows a relatively high hydrolytic potential and good compatibility with other glucanases in its action on insoluble mutans of streptococcal origin. Consequently, the (1 → 3)-α-glucanase induced by the mycelium of *A. wentii* could be applied as an active ingredient in toothpaste or mouthwash to prevent the accumulation of glucose biopolymers. Thus, the inductive properties of *A. wentii* preparation suggested the presence of (1 → 3)-α-D-glucose-containing polymer in the cell wall of this microorganism. In this paper we elucidate the structural features and properties of the water-insoluble glucan isolated from *A. wentii* cell wall.

## 2. Materials and methods

### 2.1. Microorganism and culture conditions

*A. wentii* CIM 449 was obtained from the Collection of Industrial Microorganisms (Warsaw, Poland). The microorganism was maintained on potato dextrose agar slants at 4 °C. The composition of the medium and the growth conditions were as described by Hasegawa, Nordin, and Kirkwood (1969).

### 2.2. Isolation of the water-insoluble polysaccharide

Fresh mycelia of *A. wentii* were lyophilized and milled. The dried material was used for the isolation of the alkali-soluble α-glucan according to a method described by Kiho et al. (1994). Briefly, the dried material (100 g) was successively extracted with methanol, NaCl (9 g/l), hot water, $Na_2CO_3$ solution (50 g/l), and, finally, 1 M NaOH containing 0.2 g $NaBH_4$/l for 24 h at room temperature. The alkali extract was neutralized with 1 M HCl. The precipitated polysaccharide fraction was washed several times with water, collected by centrifugation, and lyophilized.

### 2.3. Immunofluorescent labeling of the A. wentii cell wall (1 → 3)-α-D-glucan

To localize the (1 → 3)-α-D-glucan within the cell wall of *A. wentii*, fluorescently labeled antibodies were used (Fujikawa et al., 2009). Fresh mycelium of *A. wentii* on Lab-Tek II Chamber slides (Nunc, Rochester, USA) was fixed with a 3% (v/v) formaldehyde solution in distilled water at 65 °C for 30 min. The fixed fungal cells were washed three times in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4$, 1.5 mM $KH_2PO_4$, pH 7.4) before being infiltrated with 1% (v/v) Tween 20 in PBS (PBS-T). To detect the presence of the (1 → 3)-D-α-glucan, a 150 μl solution of mouse IgM MOPC-104E (0.1 mg/ml in PBS) (Sigma) as a primary antibody and 150 μl Alexa Fluor 488 goat anti-mouse IgM (μ-chain specific) (0.1 mg/ml in PBS) (Sigma) as a secondary antibody were used. Samples were incubated with the primary antibodies overnight at 4 °C in a wet chamber. Incubation with the secondary antibodies was performed for 2 h in the dark at 37 °C. Before observation under a fluorescent microscope (Olympus BX 51), the antibody-labeled cells were rinsed three times with PBS. The (1 → 3)-α-D-glucan was observed at an excitation wavelength of 470/500 nm and an emission wavelength of 525/550 nm.

### 2.4. Sugar composition and methylation analysis

For sugar analysis, the polysaccharide was hydrolyzed with 2 M trifluoroacetic acid (TFA) (100 °C, 4 h). The sugars were converted into alditol acetates (Sawardeker, Sloneker, & Jeanes, 1965).

Methylation was performed according to the method of Hakomori (1964), and the methylated polysaccharides were purified on a Sep-Pak C18 cartridge (York, Darvill, McNeil, Stevenson, & Albersheim, 1986). The resulting material was hydrolyzed in 2 MTFA (100 °C, 4 h) and reduced with $NaBD_4$. The partially methylated alditols were converted into acetate derivatives.

The absolute configuration of the liberated monosaccharides was established by an analysis of acetylated *R*-(−)2-butylglycosides, according to Gerwig, Kamerling, and Vliegenthart (1978). All sugar derivatives were analyzed by GC–MS.

### 2.5. Gel permeation chromatography

The average molecular mass of the glucan was determined by gel permeation chromatography with a Sepharose CL-6B column (0.7 cm × 90 cm). The glucan (2 mg) was dissolved in a 1 M sodium hydroxide solution (0.5 ml). The separation was done at room temperature and eluted with 1 M NaOH at a flow rate of 0.3 ml $min^{-1}$. Carbohydrates in the eluate were determined by the phenol–$H_2SO_4$ assay (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Dextrans of known molecular masses were used as standards to calibrate the column.

### 2.6. β-Elimination

Alkaline degradation of polysaccharide was carried out for seven days at room temperature in a saturated calcium hydroxide solution according to a procedure described by Grün et al. (2005). Amylose, nigerose, and laminarine (all from Sigma) were used as reference compounds.

### 2.7. Smith degradation

For Smith degradation (Smith & Montgomery, 1956), the polysaccharide was suspended in 0.015 M sodium periodate at a concentration of ~1 mg/ml. The mixture was placed in the dark at 4 °C and continuously mixed for 48 h. The reaction was stopped by adding ethylene glycol. The oxidized polysaccharide was reduced with an excess of $NaBH_4$ for 18 h at room temperature. Then, excess borohydride was removed by the addition of glacial acetic acid. The product was dialyzed against distilled water and hydrolyzed in 10% acetic acid at 100 °C for 1 h.

### 2.8. Miscellaneous methods

GC–MS was carried out on a Hewlett-Packard gas chromatograph (HP 5890A) connected to a mass selective detector (MSD HP 5971). The chromatograph was equipped with a capillary column HP-5MS (30 m × 0.25 mm). The carrier gas was helium with a flow rate of 0.7 ml $min^{-1}$. The temperature program for alditol acetates and partially methylated alditol acetates was 150 °C for 5 min, then raised to 310 °C at 5 °C $min^{-1}$, and kept for 10 min.

For NMR analysis, a glucan sample was dissolved in $Me_2SO$-$d_6$. $^1H$ 1D and 2D NMR spectra were recorded at 500 MHz on a Varian Unity plus 500 instrument at 80 °C using standard Varian software. The following two-dimensional NMR experiments were performed: DQF-COSY, TOCSY, NOESY, and geHSQC. The $^1H$ and $^{13}C$ resonances were measured relative to the methyl group signal of $Me_2SO$-$d_6$ ($\delta_H$ 2.50/$\delta_C$ 39.50).

An infrared absorption spectrum (FT-IR) between 400 and 4000 $cm^{-1}$ was recorded using a Perkin Elmer FT-IR

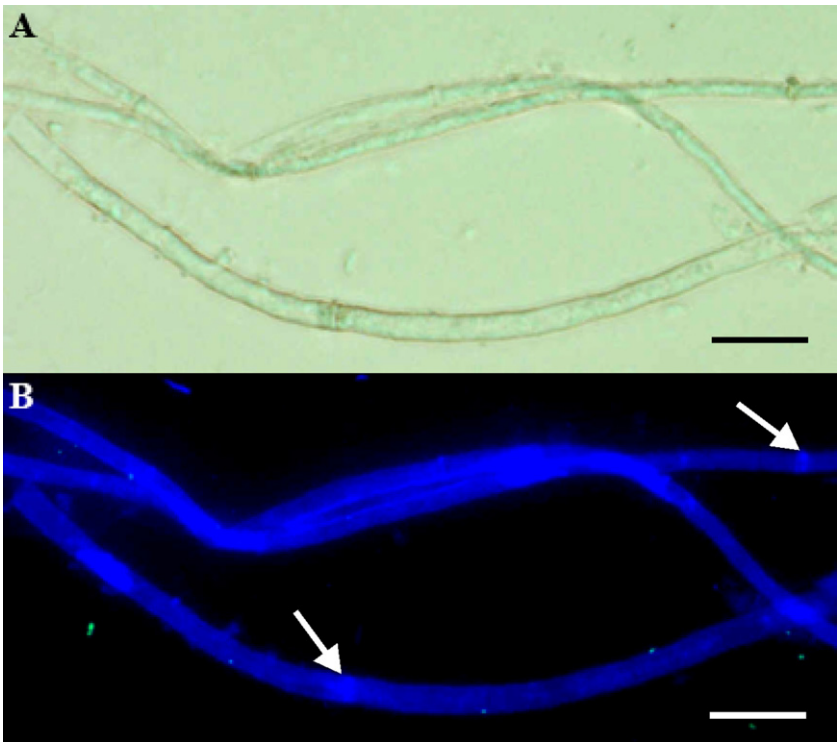


**Fig. 1.** Localization of (1 → 3)-D-α-glucan in hyphae of *A. wentii* by means of fluorophore-labeled antibodies. (A) Filaments in the light microscopy and (B) fluorescent image of the same filaments. Arrows indicate the accumulation of glucan in the hyphal septum. Twenty samples were observed and typical images are shown. Scale bar = 20 μm.

spectrophotometer (Model 1725X, Perkin Elmer, Norwalk, CT, USA) with the specimen prepared as a KBr disk.

Optical rotations were determined in 1 M NaOH water solution with a Perkin-Elmer 343 polarimeter.

## 3. Results and discussion

In our previous studies we have shown that the mycelium of *A. wentii* strongly stimulates the production of microbial (fungal and bacterial) (1 → 3)-α-D-glucanases (Pleszczyńska et al., 2010; Wiater et al., 2005). These results could suggest the presence of a (1 → 3)-α-D-glucan in the cell wall of *A. wentii*. This type of glucan has been isolated from mycelia of several members of the genus *Aspergillus*, e.g., *Aspergillus niger*, *A. nidulans*, *Aspergillus flavipes*, and *Aspergillus ochraceus* (Bobbit et al., 1977; Borgia & Dodge, 1992; Leal et al., 1992), but, to the best of our knowledge, not from *A. wentii*. In order to test the presence of a (1 → 3)-α-D-glucan in the cell wall of *A. wentii*, specific fluorophore-labeled antibodies were used. As shown in Fig. 1, (1 → 3)-α-D-glucan was clearly visible in hyphae. Moreover, a significant accumulation of this glucan was observed in hyphal septa.

The yield of the water-insoluble fraction (AW-WIG) was 6.5% of dry fungal mass. Monosaccharide analysis showed that this preparation consisted mainly of glucose with traces of galactose. The absolute configuration of glucose was shown to be D. Methylation analysis of the glucan gave 2,4,6-tri-*O*-methyl-D-glucose accompanied by a small amount of 2,3,6-tri-*O*-methyl-D-glucose and traces of fully methylated (terminal) glucose alditol acetate derivatives (Table 1).

AW-WIG was dissolved in 1 M sodium hydroxide, and the average molecular mass was estimated by gel permeation chromatography on a Sepharose CL-6B column (0.7 cm × 90 cm). The material was eluted close to a void volume of the column giving a molecular mass ($M_w$) of about 850 kDa.

The FT-IR spectrum of AW-WIG ranged from 400 $cm^{-1}$ to 4 000 $cm^{-1}$, as shown in Fig. 2. An intense and broad absorption peak at 3402 $cm^{-1}$ for O—H stretching vibrations, a peak at 2927 $cm^{-1}$ for C—H stretching vibrations, and a broad absorption band in the region of 950–1200 $cm^{-1}$ for coupled C—O and C—C stretching and C—OH bending vibrations are characteristic of polysaccharides (Liu et al., 2008). The peaks at around 2926 $cm^{-1}$ and 2360 $cm^{-1}$ indicate the presence of C—H bonds. The signals at 2342 $cm^{-1}$ and 1647 $cm^{-1}$ might be indicative of protein impurities (N—H absorbing groups) (Yang et al., 2012). The AW-WIG preparation had absorption peaks at 930, 849, and 822 $cm^{-1}$. All of them are characteristic of α-(1 → 3)-D-glucans. Especially, the peak at 822 $cm^{-1}$ is exclusively associated with α-(1 → 3)-linkages (Seymour, Julian, Jeanes, & Lamberts, 1980). No absorbance band was observed at 890 $cm^{-1}$, suggesting that AW-WIG contained glucose linked exclusively by α type glycosidic bonds (Zhang, Zhang, & Cheng, 1999).

The 1D and 2D $^1H$ NMR (DQF-COSY, TOCSY, NOESY) and 2D $^1H$–$^{13}C$ NMR (HSQC) experiments were performed to independently verify the data from chemical analyses and infrared spectroscopy as well as to determine the anomeric configuration of glucose residues. The NMR data are summarized and listed in Table 2. The only spin system identified was characteristic of α-D-glucose. Therefore, the HSQC spectrum (Fig. 3.) contained only seven signals attributed to directly bonded carbon and proton

**Table 1**
Results of methylation analysis of *A. wentii* water-insoluble glucan.

| | Methylated sugar | Mol (%) | Linkage type |
|---|---|---|---|
| 1 | 2,3,4,6-*O*-$Me_4$-D-glucose | 0.1 | α-D-Glcp-(1→ |
| 2 | 2,4,6-*O*-$Me_3$-D-glucose | 99.4 | →3)-α-D-Glcp-(1→ |
| 3 | 2,3,6-*O*-$Me_3$-D-glucose | 0.5 | →4)-α-D-Glcp-(1→ |

The glucan preparation was methylated before being hydrolyzed, reduced and acetylated. Therefore methylated sugars mentioned refer to permethylated alditol acetates identified by GC–MS (e.g. 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).

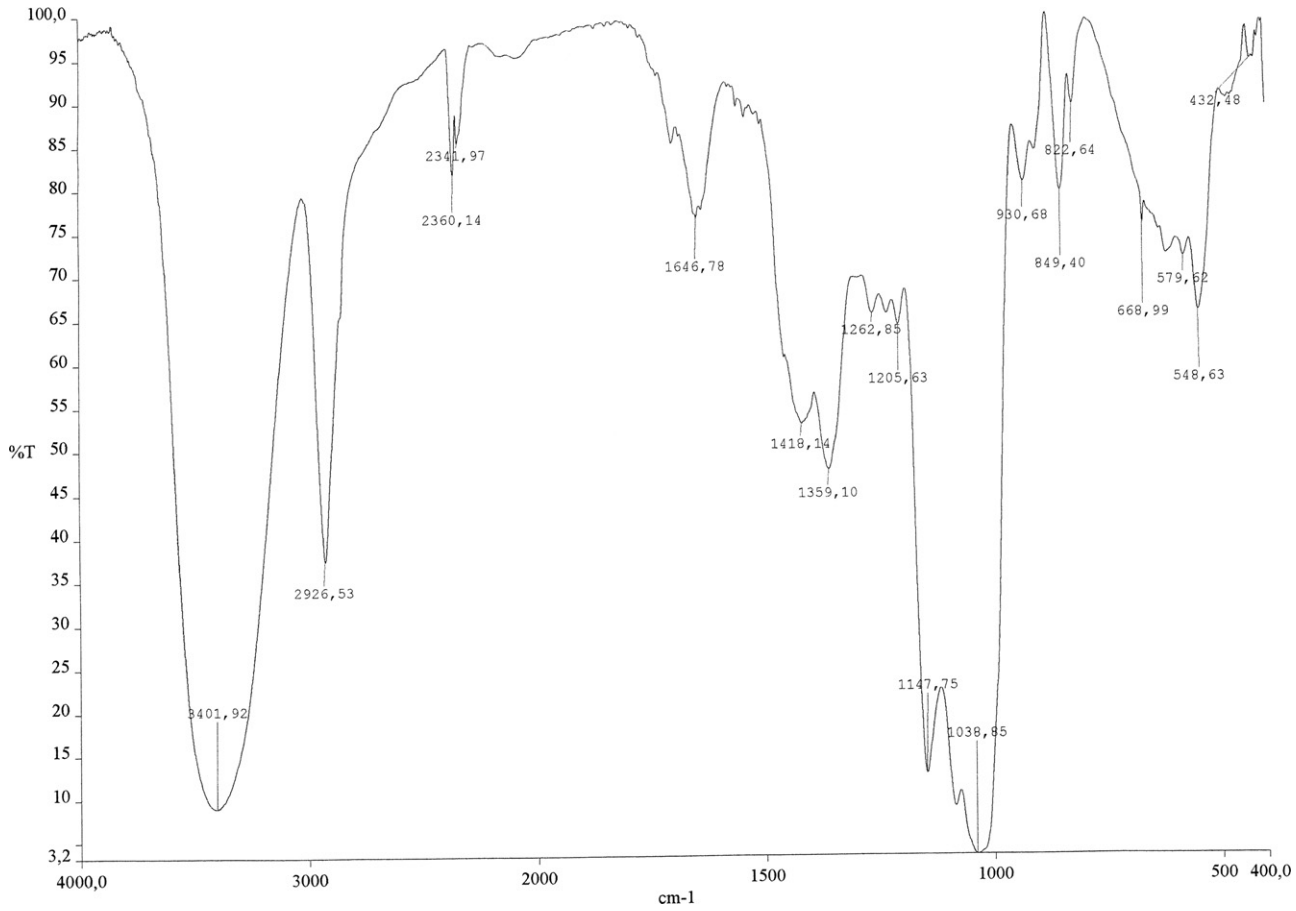


**Fig. 2.** Infrared (FT-IR) spectrum of AW-WIG. For details see the text.

nuclei correlations. Because the AW-WIG preparation was dissolved in $Me_2SO$-$d_6$ prior to the NMR analysis, the $^1H$ NMR spectra contained hydroxyl proton signals. Three resonances (4.173, 4.463, and 4.865 ppm) could be assigned to protons from hydroxyl groups. They did not generate cross-peaks in the HSQC spectrum (Fig. 3 and the $^1H$ NMR spectrum within it). These signals were assigned to OH-6, OH-2, and OH-4 of the glucose ring, respectively. The absence of an OH-3 hydroxyl signal in the $^1H$ NMR spectrum together with a downfield shift of C-3 (82.55 ppm, Table 2) indicated that the glucose residues were (1 → 3)-linked. The low-field anomeric proton signal at 5.092 ppm and the anomeric carbon signal at 99.00 ppm as well as the small value of the $^3J_{H1,H2}$ coupling constant (ca. 3.5 Hz) are typical of the α-anomer of glucose. The high optical rotation, $[\alpha]_D^{25}$ +216, additionally confirmed an α-glucan structure.

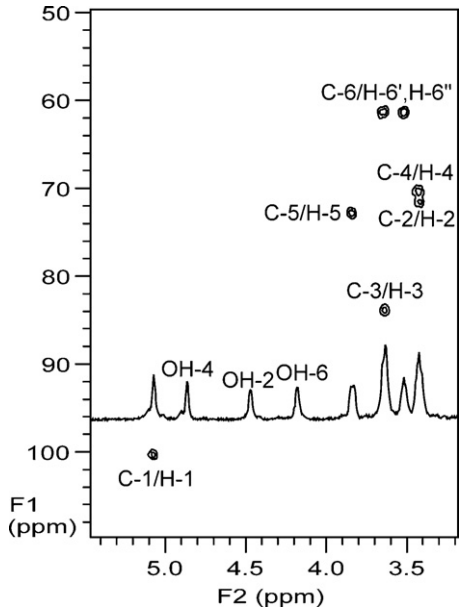


**Fig. 3.** Part of the $^1H$–$^{13}C$ HSQC spectrum of AW-WIG.

Altogether, the NMR data presented here afford the conclusion that AW-WIG is a (1 → 3)-linked α-D-glucan type. Signals corresponding to (1 → 4) linked-glucose (found during the methylation analysis) and the terminal glucose were not detected by NMR spectroscopy (i.e., their amounts were below the 0.5% – a detection limit of the method).

To determine the type of linkages between the glucose residues at the reducing end of the glucan, the preparation was subjected to prolonged (seven-day) alkaline β-elimination. This procedure successively cleaves glycosidic bonds starting from the reducing end of a polysaccharide and releases *iso*-saccharinic acids (two isomers) from 4-substituted glucose or *meta*-saccharinic acids (also two isomers) from 3-substituted glucose. Reaction products, found in supernatant, after neutralization of solution (calcium hydroxide), evaporation of water and derivatization with trimethylsilyl reagent were analyzed by GC–MS (Grün et al., 2005). During alkaline β-elimination, only *meta*-saccharinic acid isomers were released from the AW-WIG polysaccharide. This means that the reducing end of the macromolecule is terminated by (1 → 3)-linked α-D-glucose.

To determine whether the (1 → 3)-α-D-glucan contained internal (1 → 4)-linked residues, the molecular mass of the

**Table 2**
Assignments of $^{1}H$ and $^{13}C$ NMR spectra and corresponding coupling constants of *A. wentii* α-glucan.

| H | H-1 | H-2 | H-3 | H-4 | H-5 | H-6′ | H-6″ | OH-2 | OH-3 | OH-4 | OH-6 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| δ (ppm) | 5.084 | 3.428 | 3.650 | 3.439 | 3.853 | 3.650 | 3.528 | 4.463 | nd | 4.865 | 4.173 |
| C | | C-1 | | C-2 | | C-3 | | C-4 | | C-5 | C-6 |
| δ (ppm) | | 99.00 | | 70.239 | | 82.55 | | 69.05 | | 71.45 | 59.93 |

nd, not detected.

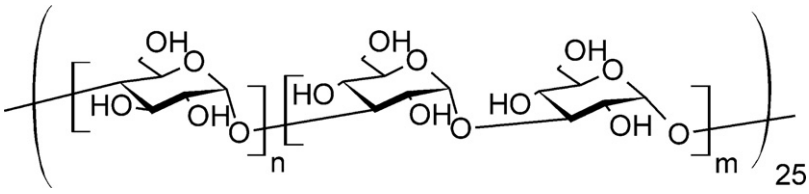


**Fig. 4.** Predicted structure of the linear subunit of (1 → 3)-D-α-glucan of *A. wentii* (AW-WIG preparation). The number of (1 → 4)-linked glucoses was estimated at 1–2 (*n*), whereas the number of (1 → 3)-linked glucoses was estimated at 200 (2*m*).

Smith-degraded polymer was measured. Smith degradation dramatically decreased the average molecular mass of the polymer, indicating that (1 → 4)-linked residues are spread along the intact polymer. The calculated average mass of the product of the oxidation procedure (∼35 kDa) points to the presence of ca. 25 subunits built of about 200 (1 → 3)-linked glucose residues separated by a short sequence of (1 → 4)-linked α-D-glucoses.

## 4. Conclusions

The water-insoluble polysaccharide (AW-WIG) which is the main structural component of *A. wentii* mycelia was extracted with 1 M NaOH aqueous solution. It was found to be a linear α-D-glucan (as shown in Fig. 4) with a molecular mass of approximately 850 kDa. The polymer consisted of ca. 25 subunits built of about 200 residues of (1 → 3)-linked α-D-glucoses separated by a short spacer of (1 → 4)-linked α-D-glucoses. Because (1 → 3)-α-D-glucans usually possess antitumor activity, future studies of the *A. wentii* glucan should concentrate on this issue.

## Acknowledgement

This work was financially supported by BW/BS/BiNoZ/UMCS Research Programs.

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