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Structural characterization of a water-insoluble (1 → 3)-α-D-glucan isolated from the *Penicillium chrysogenum*

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Abstract

A water-insoluble polysaccharide (WIP) was isolated from *Penicillium* mycelia by extracting with sodium hydroxide. Its structural characteristics were investigated by composition analysis, gel permeation chromatography, 1D and 2D nuclear magnetic resonance (NMR) spectroscopy, and infrared spectroscopy (IR). The results indicated that it was a linear (1 → 3)-α-D-glucan with a molecular weight of 180 kDa.

Keywords: Water-insoluble polysaccharide; *Penicillium chrysogenum*; (1,3)-α-D-glucan; NMR

1. Introduction

Polysaccharides constitute one of the most abundant types of biomacromolecules, which exist widely in all living organisms where they play extensive biological roles Pérez, Kouwijzer, Mazeau, and Engelsen, 1996. In recent years, much interest and studies have been devoted to polysaccharides and their modifications due to their bioactive and medical properties. The (1 → 3)-α-D-glucans, often isolated from the cell wall of fungi, have been less reported compared with other polysaccharides, for example, the (1 → 3)-β-D-glucans family Bao, Liu, Fang, and Li, 2001; Størseth, Hansen, Skjermo, and Krane, 2004. The latter has received considerable attention due to their immunomodulatory properties, such as adjuvant, antimicrobial, antitumor, and radiation protective activities. Some groups have studied the structure and solution properties of a few (1 → 3)-α-D-glucans from different sources, for example, a few short side chains of (1 → 6)-α- or (1 → 4)-α-linked D-glucose residues attached on the O6 of the (1 → 3)-α-D-glucan backbone come from the bacteria of *Streptococcus mutans* Ebisu, Misaki, Kato, and Kotaini, 1974 and *Streptococcus salivarius* Tsumuraya and Misaki, 1979; (1 → 3)-α-D-glucan with a few (1 → 6) branch linkages from *Lentinus edodes* Zhang and Cheung, 2002; linear (1 → 3)-α-D-glucan from *Poria cocos* mycelia Jin et al., 2004. Some bioactive and medicinal properties of (1 → 3)-α-D-glucan derivatives have also been reported. Sulfated linear (1 → 3)-α-D-glucan from the fruit body of *Agrocybe eylindracea* (Fr.) Maire (*Bolbitiaceac*) was obtained, and its antitumor activity against Ehrlich ascites carcinoma was investigated Zhang, Zhang, Zhou, and Zeng, 2000. In addition, a carboxymethylated derivative (1 → 3)-α-D-glucan with antitumor activity against Sarcoma 180 had been synthesized by substitution with chloroacetic acid Kiho, Yoshida, Nagai, Ukai, and Hara, 1989. The relationship between the nature of functionalized groups and physicochemical properties of the chemically modified derivatives of linear (1 → 3)-α-D-glucan, obtained from the spores of *Ganoderma lucidum,* and their immunomodulating activity had been discussed Bao, Duan, Fang, and Fang, 2001. As a by-product of antibiotic industry, a large percentage of *Penicillium* mycelia from industrial fermentation processes are disposed off by incineration, where a small percentage is used as additive to cattle feed and in preparation of

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fertilizers. It is well-known that the main polysaccharides of cell wall of some fungi are chitin and glucans. Furthermore, (1 → 3)-α-D-glucan, usually constituting the cell wall components of fungi and yeasts Jesma and Kreger, 1979, also has been found in few microorganisms. For some *Penicillium* species, such as *Penicillium notatum*, *Penicillium oxalicum*, the cell-wall polysaccharide in the alkali-soluble fraction was partially characterized as an α-glucan with the minor quantities of mannose. Other species such as *Penicillium allahabadense*, *Penicillium erythromellis*, contained mainly galactofuranan Ruperez and Leal, 1987. We have extracted chitosan Tan, Wang, and Shi, 2002 and ergosterol from *Penicillium chrysogenum*, in which a large amount of water-insoluble polysaccharide (WIP, mainly (1 → 3)-α-D-glucans) was found. Although the (1 → 3)-α-D-glucan and its derivatives as mentioned above have bioactive and medicinal importance, surprisingly, the study of the structure of WIP that comes from *P. chrysogenum* has not being given adequate attention in the literature. Since the *P. chrysogenum* mycelia, as a source of WIP, have many economic advantages, research verifying the structure of its WIP is critical. In this paper, we reported the investigation of isolation and structure characteristics of WIP extracted from the cell wall of *P. chrysogenum* by sodium hydroxide.

2. Materials and methods

2.1. Cell-wall preparation and isolation of the water-insoluble fraction

The fresh mycelia of *P. chrysongenum* were provided by North China Pharmacy Co. The deuterium oxide (D_2O), 99.9 atom% D and sodium deuteroxide (NaOD), 40 wt% solution in D_2O, 99 atom% D, produced from Cambridge Isotope Laboratories, Inc. Dextran standards of 100, 150, 180, and 220 kDa were purchased from Pharmacia Co., Ltd. Two hundred grams of fresh mycelia were washed three times with distilled water, followed by freeze-drying to a powder. Twenty grams of powder was extracted with 1 M NaOH for 2 h at 40 °C. After centrifugation at 4000 rpm at 4 °C for 15 min, the supernatant solution was treated by the Sevage method to remove proteins. The pH was then adjusted to 7.0 using hydrochloric acid to precipitate the polysaccharide. In order to remove the water-soluble components, the precipitate was extensively dialyzed against running tap water. The precipitate was then freeze-dried and powdered for the subsequent analysis as WIP.

2.2. Monosaccharide composition and methylation analysis

For the analysis of neutral sugars, the WIP was treated with methanolic 0.6 M HCl over night and hydrolyzed with 3 M trifluoroacetic acid (TFA) for 1 h at 121 °C. The hydrolyzed products were reduced with sodium borohydride and the corresponding alditols were acetylated with pyridine-acetic anhydrite (1:1) for 1 h at 100 °C Laine, Esselman, and Sweeley, 1972. Identification and quantification were carried out by gas-liquid chromatography (GLC) using SP-2480 column (30 m × 0.25 mm, 0.2 μm film thickness) and a temperature program (210–240 °C, 3 min initial hold, 15 °C min^{-1} ramp rate and 7 min final time). The absolute configuration of the sugars was determined as devised by Gerwig, Vliegenthart Gerwig, Kamerling, and Vliegenthart (1978).

Methylation analyses were performed by the usual methodology Ciucanu and Kere, 1984 and hydrolyzed sequentially with 90% formic acid in water at 80 °C for 2 h and 5 M TFA at 100 °C for 5 h. The products were reduced with $NaBD_4$ and then acetylated as above. The corresponding partially methylated alditol acetates were examined by GC–MS using a fused silica SPB-1 column (30 m × 0.25 mm, 0.2 μm film thickness), a temperature program (160–210 °C, 1 min initial hold, 2 °C min^{-1} ramp rate) and a mass detector Q-mass from Perkin-Elmer. Quantification was made according to peak area.

2.3. Gel permeation chromatogramphy

The average molecular weight of the WIP was determined by gel permeation chromatography (Shimadzu LC-6A with refractive index detector) with a Superose-6B column. The WIP powder was dissolved in 0.5 M sodium hydroxide solution, and filtrated with a 0.2 μm filter. This formed a filtrate sample of about 10 mg ml^{-1}. A sample (10 μl) was injected into the column. The separation was operated at room temperature and eluted with 0.5 M NaOH at a flow rate of 0.7 ml min^{-1}. Dextran standards of different molecular weight were used as standards to calibrate the column.

2.4. Structural characterization

The IR spectrum of the WIP was done using a Nicolet FT-IR 360 spectrometer (Nicolet Co. Ltd, USA) Test specimens of polysaccharide film were prepared by the KBr-disk method and 32 scans at a resolution of 4 cm^{-1} were collected. For NMR spectroscopy, a sample of approximately 30 mg WIP was washed three times in D_2O with intermediate lyophilization, and then dissolved in 0.5 M NaOD. 1D NMR and 2D NMR spectra experiments were performed on a Bruker AV 600 NMR spectrometer at 310 K in a 5 mm i.d. NMR tube as relative to external reference TMS at 0 ppm. The water suppression had been used. The frequencies were as follows: 600.13 MHz for the ^1H NMR spectrum and 150.91 MHz for the ^{13}C NMR spectrum. The experiments of DEPT, HSQC, COSY, and TOCSY spectrums (mixing time of 80 ms) were done as usual parameters.

3. Results and discussion

3.1. Isolation and molecular weight of WIP

Dry mycelia were found to contain about 6% by weight of white powder of water-insoluble polysaccharide (WIP). The

WIP was found to be soluble in 0.5 M sodium hydroxide and Me_2SO. Gel permeation chromatography was used to determine the average molecular weight (M_w). A sample (10 mg/ml) of WIP showed a symmetrical peak. The elution time was the same as dextran standard of molecular weight 180 KDa, and, correlation with the calibration curve indicated that its molecular weight (M_w) was about 180 KDa.

3.2. Composition and linkage analysis

Monosaccharide analysis was carried out by GLC. The neutral sugars liberated in the hydrolysis of WIP consisted of glucose with small fractions of mannose and galactose (98.7:0.6:0.7, weight ratio). It indicated that WIP mainly composed of glucose with small quantities of mannose and galactose. Methylation analysis of WIP gave 2, 4, 6-tri-*O*-methyl-D-glucose and a trace of 2, 3, 4, 6-tetra-*O*-methyl-D-glucose (molar percent, 99.9:0.1), as confirmed by GLC and GLC-mass spectrometry of their alditol acetates. The results indicated that the residues in WIP are mainly (1 → 3)-linked.

3.3. Infrared (IR) and NMR spectrum characters

The IR spectrum for WIP (Fig. 1) indicated that it had absorption peaks at 928.90, 846.34, and 820.86 cm^{-1} (characteristic of (1 → 3)-α-D-glucan) Sermour, Julian, Jeanes, and Lamberts, 1980; Zhang, Zhang, and Chen, 1999. It is also important to note that the peak at 820.86 cm^{-1} is only associated with (1 → 3)-linkages Sermour et al., 1980. The ^{13}C NMR spectrum of WIP (Fig. 2) showed 6 narrow signals of approximately equal intensity, among which the only one anomeric carbon signal for the sample was assigned at δ 101.20 ppm, ascribed to the C-1 of glucosyl residues. This indicates that the polysaccharide contains homogeneous repeated units of monosaccharides. In ^{1}H NMR spectrum of WIP (Fig. 3), the α-anomeric ^{1}H region signal was at δ 5.15 ppm ($J_{1,2} = 3.6$ Hz). The H-1 tracks of these resonances in the TOCSY spectrum (not shown) gave the correlations up to proton H-4, typical for pyranose glucose configuration Sletmoen, Maurstad, Sikorski, Paulsen, and Stokke, 2003. The signal at δ 83.66 ppm was assigned to the O-substituted C-3 of glucosyl residues. The appearance of the carbon signals also indicated that it was the (1 → 3)-linked-D-glucopyranosyl with α-anomeric configuration Stroop, Xu, Retzlaff, Abeygunawardana, and Bush, 2002. The inclusion of a pulse angle $\Theta_{y'} = 135^{\circ}$C in the pulse sequence of the experiment inverts only the carbon nuclei bearing two protons, whereas the CH ring carbons appear in the normal upright position Doddrell, Pegg, and Bendall, 1982. By comparing with ^{13}C spectrum and DEPT-135 spectrum (not shown), C6 can be obviously identified at δ 62.03 ppm. There are two different viewpoints for C-4, C-2, and C-5 belongings. The first is that the order from down field to up is C-2, C-5, and C-4 Bao et al., 2001; Woranovicz et al., 1999, the second is C-5, C-2, C-4 Funane et al., 2001; Kiho et al., 1989; Parra et al., 1994; Zhang et al., 1999. According to the correlation of H^1 from TOCSY and COSY spectra (not shown), and C^{13}-H^1 correlation from HSQC(shown as Fig. 4), we have the same order as the second as 71.46, 71.95, and 73.63 ppm, respectively. The ^{1}H and ^{13}C chemical shifts of the WIP sample were fully assigned by using 2D

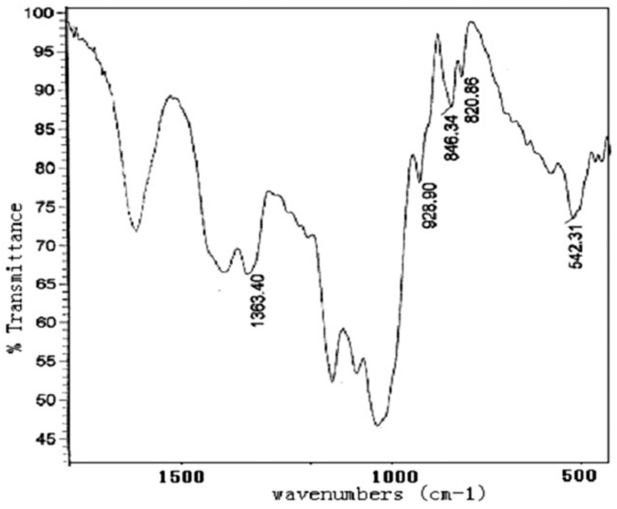

Fig. 1. The infrared (IR) spectra of WIP. The characteristic absorption peaks of (1 → 3)-α-D-glucan were at 928.90, 846.34, and 820.86 cm^{-1}.

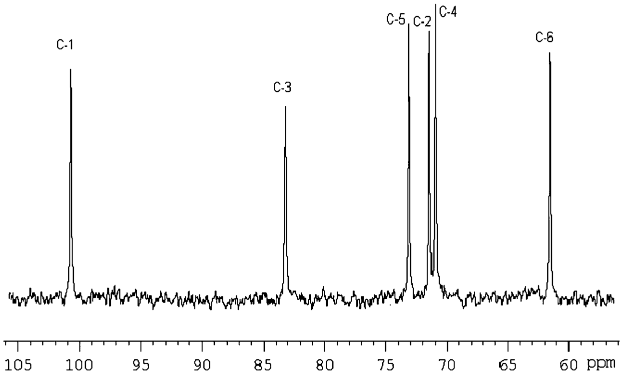

Fig. 2. 150.91 MHz^{13}C NMR spectrum of WIP (310 K, D0.5 M NaOD in D$_2$O). The number of carbon atoms was labeled on each peak to refer the position.

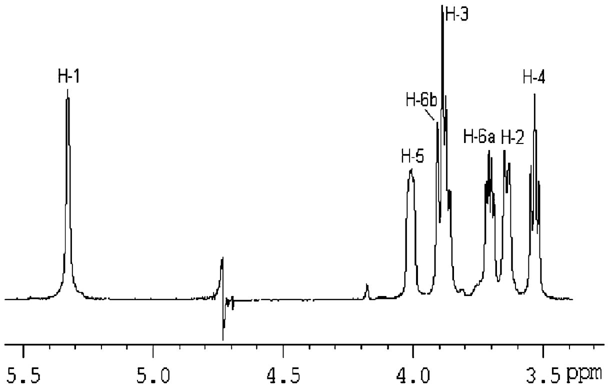

Fig. 3. 600.13 MHz^{1}H NMR spectrum of WIP (310 K, D0.5 M NaOD in D$_2$O). The number of proton attach to the carbon atoms of (1 → 3)-α-D-glucan was labeled on each peak to refer the position.

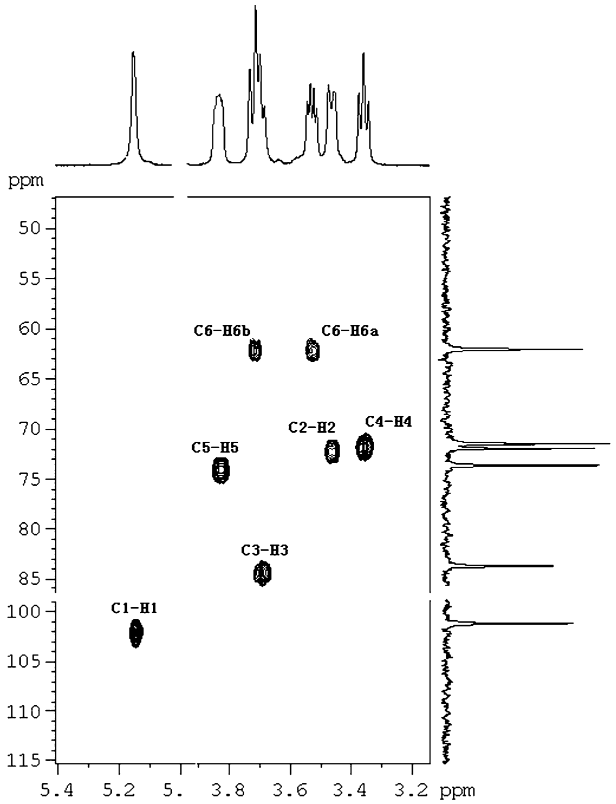

Fig. 4. ^{1}H-^{13}C HSQC spectrum of WIP.

Table 1
The assignments of ^{13}C NMR and ^{1}H spectrum, and corresponding vicinal coupling constants

Assignment	C-1	C-2	C-3	C-4	C-5	C-6	
^{13}C (δ) ppm	101.20	71.95	83.66	71.46	73.63	62.03	
Assignment	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
^{1}H (δ) ppm	5.15	3.46	3.69	3.35	3.83	3.53	3.71
Coupling constant	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6a}$	$J_{5,6b}$	$J_{6a,b}$
(J) Hz	3.6	11.4	9.0	9.6	5.4	–[a]	12.0

[a] The vicinal coupling constant of $J_{5,6b}$ was not shown due to the proton spectrum did not show a quite good resolution for H-5.

homo-(COSY and TOCSY) and hetero-nuclear techniques (HMQC), and presented in Table 1, as well as vicinal coupling constants that able to be calculated.

4. Conclusion

Water-insoluble polysaccharide, the main structural material of *P. chrysogenum* mycelia, was extracted by sodium hydroxide. It was found to be a linear (1 → 3)-α-D-glucan (shown as Fig. 5) with a molecular weight of approximately180 KDa.

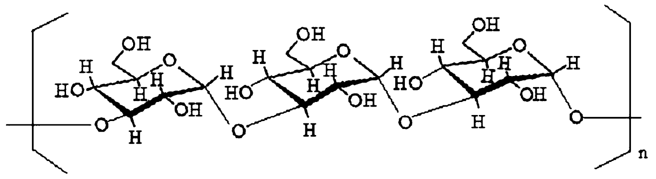

Fig. 5. The structure of linear (1 → 3)-α-D-glucan.

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