

Antrodia camphorata polysaccharides exhibit anti-hepatitis B virus effects

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Abstract

Polysaccharides were extracted from fruiting bodies and cultured mycelia from five *Antrodia camphorata* strains. Polysaccharide profiles of the five strains, as determined by high-performance anion-exchange chromatography, showed varying yields and composition of neutral sugars. *A. camphorata* fruiting bodies also had different polysaccharide patterns compared to the cultured mycelium. Analysis of 26-day-old mycelia showed that the neutral sugars galactose, glucose, mannose, and galactosamine were predominant. All mycelia polysaccharide preparations exhibited anti-hepatitis B virus activity. Polysaccharides from strain B86 at a concentration of 50 $\mu\text{g ml}^{-1}$ showed the highest level of anti-hepatitis B surface antigen effect, which was higher than α -interferon at a dosage of 1000 U ml^{-1} . Only strains B86 and 35398 had substantial anti-hepatitis B e antigen activities. None of the polysaccharides exhibited cytotoxic effects. © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Antrodia camphorata (Chinese name, niu-chang-chih) is a medicinal fungus of the family Basidiomycetes that has been used as a folkdrug in Taiwan for the treatment of tumorigenic diseases. Chemical compounds found in *A. camphorata* include sesquiterpene lactone, steroids and triterpenoids [1–6]. The triterpenoids have anti-cholinergic and anti-serotonergic activities [6].

Polysaccharides are common structural and storage polymers in living organisms, representing more than 75% of the dry weight of plants [7]. Compositional analysis of glycoconjugates is important in structural studies of these compounds. Polysaccharides are potentially useful, biologically active ingredients for pharmaceutical uses due to a

variety of biological activities, such as mitogenic activity, activation of alternative-pathway complement and plasma-clotting activity [8]. Tumor-inhibition activity has been documented in numerous mushroom polysaccharide fractions such as a glucan–protein complex from *Ganoderma tsugae* [9], lentinan (a 1→3-linked β -D-glucan) from *Lentinus edodes* [10], and schizophyllan from *Schizophyllum commune* [11]. We report here on the physicochemical properties of polysaccharides in *A. camphorata*. Quantification and profile analysis of the carbohydrates were carried out by anion-exchange chromatography with pulsed amperometric detection [12]. The effects of these polysaccharides on the hepatitis B virus (HBV) were also examined.

2. Materials and methods

2.1. *A. camphorata* strains

A. camphorata isolates, accessions 35396 and 35398, were obtained from the Culture Collection and Research Center (CCRC, Taiwan) [13]. Taiwan strains, B85 from

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Taitung, B86 from Hsinchu, and B71 from Alishan, were gifts from fungi specialist Dr. T.T. Chang. Fresh samples of fruiting bodies were collected from Ilan, in northern Taiwan.

2.2. Liquid culture of *A. camphorata*

A. camphorata was maintained on potato dextrose agar (PDA) slants and transferred to fresh medium at 3-week intervals. For each pasteurized Petri dish, 25 ml of PDA medium (39 g l⁻¹) was used. *A. camphorata* was inoculated at the center of Petri dishes which were then incubated at 28°C for 19 days. The fine mycelia of *A. camphorata* on the media surface were cut into pieces (approximately 1 × 1 cm) before transferring to 250-ml culture flasks containing 25 ml of potato dextrose broth (48 g l⁻¹) with 20 g l⁻¹ glucose and pH 5.6. Polysaccharides were isolated from 26-day-old cultures. Following incubation, mycelia were rapidly washed with 1 l of 250 mM NaCl while aspirating to remove contaminating extracellular polysaccharides. Samples were then lyophilized and stored at 4°C. Growth media for solid and liquid cultures were purchased from Sigma Co. (St. Louis, MO, USA). LC grade organic solvents were purchased from E. Merck Co.

2.3. Isolation of polysaccharides

The lyophilized mycelia of *A. camphorata* were extracted with 80°C water in a 1:100 (w/w) ratio for 6 h. The extracts were cooled and 4 volumes of 95% ethanol were added, then allowed to precipitate overnight at 4°C. The precipitated polysaccharides were collected by centrifugation and lyophilized, resulting in a crude brownish polysaccharide sample.

2.4. High-performance anion-exchange chromatography (HPAEC) analysis of polysaccharides

Crude polysaccharides (200 µg) were separated by HPAEC (Dionex BioLC) equipped with a gradient pump, a pulsed amperometric detector (PAD-II) using a gold working electrode, and an anion-exchange column (Carbopac PA-100, 4.6 × 250 mm). Samples were applied using an autosampler (AS3500, SpectraSYSTEM®) via a microinjection valve with a 200-µl sample loop. Polysaccharide analysis was carried out in a linear gradient of 100–700 mM NaOAc in 90 mM NaOH for 90 min at ambient temperature. Data were collected and integrated on a PRIME DAK system (HPLC Technology, Ltd., UK).

2.5. Hydrolysis of polysaccharides

A series of experiments were conducted to determine the optimum acid concentration, i.e. a concentration sufficient

to release the maximum amount of compounds but with minimal degradation and/or polycondensation. Comparable yields with 4 and 6 N HCl at 80°C were obtained. Hydrolysis was conducted on 1 mg of the crude polysaccharide sample with 6 N HCl in a heating block at 80°C for 6–8 h. The samples were cooled and the acid evaporated. The hydrolyzed polysaccharides were resuspended in Milli-Q water and filtered through a Millipore-GX nylon membrane prior to analysis.

2.6. General analytical methods

Total carbohydrate content was measured by the phenol-sulfuric acid method [14]. Total protein content was measured by the Bradford method [15]. Specific optical rotation was determined by an automated polarimeter (Jasco P1010) at 589 nm.

2.7. Compositional determination of the polysaccharides

The monosaccharide fractions of the polysaccharide hydrolysates were separated by HPAEC (Dionex BioLC) as mentioned above, with an anion-exchange column (Carbopac PA-10, 4.6 × 250 mm). The analysis of monosaccharides was carried out at an isocratic NaOH concentration of 18 mM at ambient temperature.

2.8. Anti-HBV test

Anti-viral analyses were performed as previously described [16]. Briefly, the HBV-producing cell line MS-G2 was plated onto 24-well flat-bottomed tissue culture plates at a density of 3 × 10⁵ cells ml⁻¹ well. After an overnight incubation to ensure that all cells were properly attached, the cells were challenged with the tested polysaccharides or with Interferon alfa-2a (Roche, Co., Mannheim, Germany) for comparison. Test samples were dissolved in autoclaved Milli-Q water at various concentrations (i.e. 10, 25, and 50 µg ml⁻¹). An additional set was treated with an equal volume of autoclaved Milli-Q water as a control. Subsequently, the cultures were collected at 3-day intervals and assayed for anti-viral activity. Anti-viral activities were assessed by analyses of anti-hepatitis B surface antigen (HBsAg) and anti-hepatitis B e antigen (HBeAg) values using an ELISA (enzyme-linked immunosorbent assay) kit (EverNew, Co., Taipei, Taiwan). The results were determined at 492 nm by a Power Wave × ELISA reader (Bio-Tek Instruments, Inc., USA). The inhibition percentage was calculated as %inhibition = (OD_{control} - OD_{sample}) / OD_{control} and compared with the positive control group (α-interferon). The results from three replications were expressed as the mean ± standard error of the mean. Cell damage was tested using an AST (aspartate transaminase) kit (Fuji). AST values higher than 25 I.U. l⁻¹ served as an indication of cell damage.

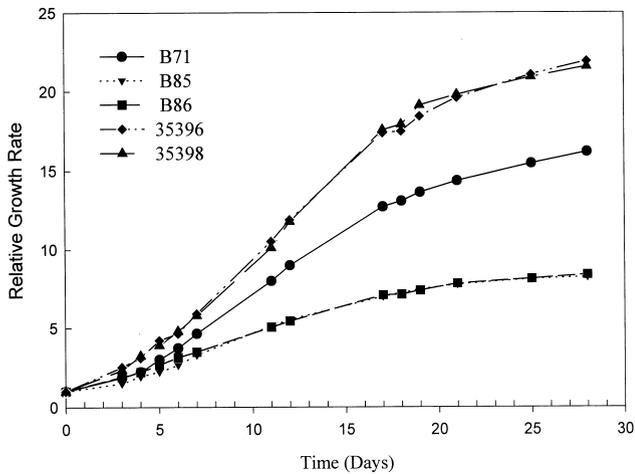


Fig. 1. Growth properties of five strains of *A. camphorata*.

3. Results and discussion

3.1. Growth-curve determination

The growth rate of *A. camphorata* mycelia was determined as the relative mass change (dry weight) over time (Fig. 1). Comparisons were made between five Taiwan strains. Strains 35396 and 35398 grew faster than the others. Generally, exponential phase was reached before 18 days of incubation.

3.2. Polysaccharide profiles of *A. camphorata*

Comparisons were made between polysaccharides isolated from the fruiting bodies of *A. camphorata* and cultured mycelium (Fig. 2). Polysaccharides were resolved in a linear gradient of 100–700 mM NaOAc in 90 mM NaOH for 90 min. The chromatograph was subdivided into three parts (I, II, III). Compounds located in part I were estimated to be small molecules. There were abundant polysaccharide species present in this range in strains B85, B86, and 35398. The intermediate-sized population of polysaccharides from the fruiting body (part II) contained numerous species. In the high molecular mass range (part III), several species of polysaccharides were present in the fruiting bodies but with low molarity. In cultured mycelia, two or three polysaccharide species were present in this range with greater amounts observed in strains B71 and 35398.

3.3. Physicochemical properties of polysaccharide of *A. camphorata*

Compositional analysis of the sugar moieties, and their

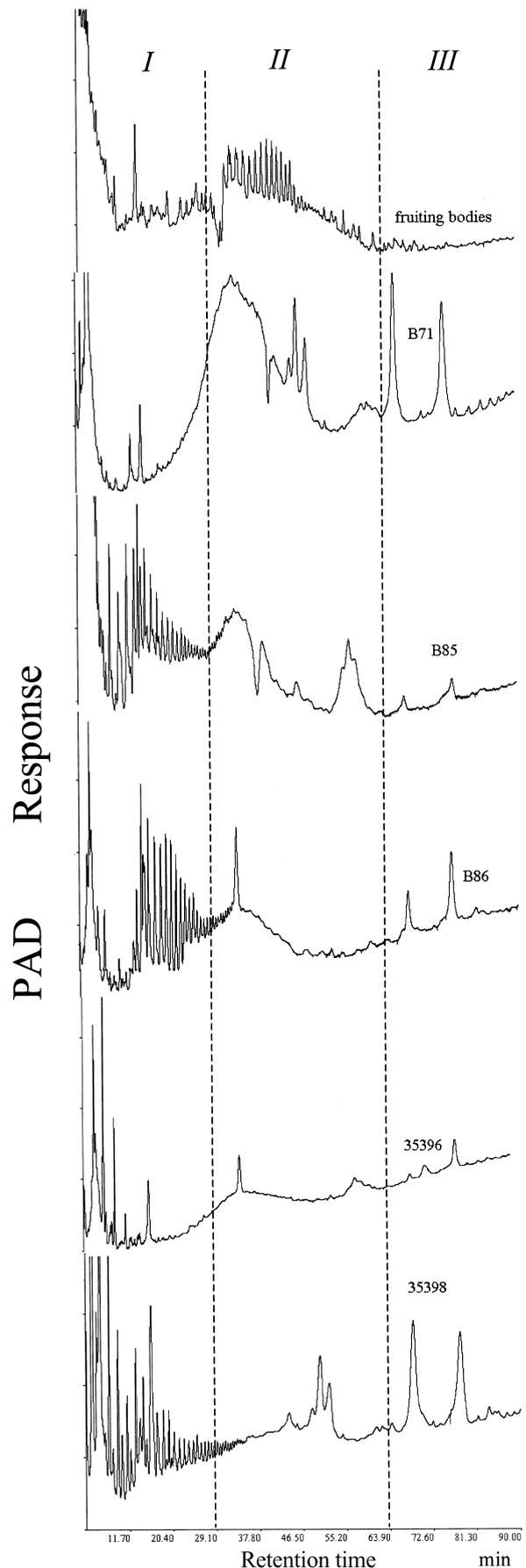


Fig. 2. Comparisons of polysaccharide profiles from fruiting bodies and from mycelium of different *A. camphorata* strains. The HPAEC analysis of polysaccharides was carried out in a linear gradient of 100–700 mM NaOAc in 90 mM NaOH for 90 min at ambient temperature.

Table 1
Characteristics of crude polysaccharide fractions isolated from *A. camphorata*

Strain	B71	B85	B86	35396	35398
Yield (%)	3.08	2.805	4.52	2.57	2.48
Total carbohydrate (mg g ⁻¹)	252	432	420	276	330
Protein (%)	49.8	33	44.4	42.6	45
Neutral sugars (μmol g ⁻¹)					
Myo-inositol	4.2	14.03	14.27	4.56	27.46
Sorbitol	0.68	4.66	5.1	1.08	10.47
Fucose	3.24	4.69	7.57	3.2	4.62
GalN	10.5	4.41	5.72	9.33	2.1
GlcN	26.64	0	0	21.23	28.545
Galactose	117.49	283.76	381.22	104	109.35
Glucose	146.74	35.6	63.9	137.32	209.56
Mannose	69.15	19.21	43.05	63.6	70.82
[α] _D (°)	30	151.8	67	29.2	19.2

A 26-day-old mycelium culture was harvested and used directly for polysaccharide extraction. The crude polysaccharide fractions were used for neutral sugar analysis. The amount of each neutral sugar is shown as μmol g⁻¹.

specific optical rotations are presented as means of at least two replicated extractions (Table 1). Yield and composition were species dependent. Of the five isolates, B85 and B86 had higher sugar contents than the others with total carbohydrate levels of 432 and 420 mg g⁻¹ of crude polysaccharides respectively. Galactose, glucose, mannose, glucosamine and galactosamine were the major sugar components in the tested strains. However, glucosamine was absent in strains B85 and B86.

3.4. Anti-HBsAg and anti-HBeAg activities

The anti-HBV activities of polysaccharides from *A. camphorata* (B71, B85, B86, 35396, and 35398) were evaluated in MS-G2 cells. The anti-HBsAg inhibition percentage values for the treatments are presented in Fig. 3. Strains B71, B85, B86, 35396, and 35398 display different levels of anti-HBsAg effects. At 50 μg ml⁻¹ the inhibition

percentage was 42.6 ± 2.6, 34.7 ± 1.5, 51.6 ± 2.0, 32.2 ± 3.8, and 38.1 ± 4.1, respectively. B86 polysaccharides were the most effective with a slightly higher inhibition than that of α-interferon at a dosage of 1000 U ml⁻¹ (50.4 ± 3.1). Polysaccharides from the other strains (B71, B85, 35396, and 35398) at 50 μg ml⁻¹ were more effective than α-interferon at a dosage of 250 U ml⁻¹. Only B86 and 35398 showed substantial anti-HBeAg activities (Fig. 4). The anti-HBeAg inhibition percentage at 50 μg ml⁻¹ concentration was 31.1 ± 6.0, and 31.6 ± 3.1 for B86 and 35398, respectively. Their effectiveness was lower than that of α-interferon at a dosage of 1000 U ml⁻¹ (46.0 ± 2.9), but higher than that of α-interferon at a dosage of 250 U ml⁻¹ (21.2 ± 2.3). No cytotoxic effects were observed for any of the treatments, as all AST values were lower than 25 I.U. l⁻¹ even at the maximum dosage of 50 μg ml⁻¹ (data not shown).

This study is the first report on the structural character-

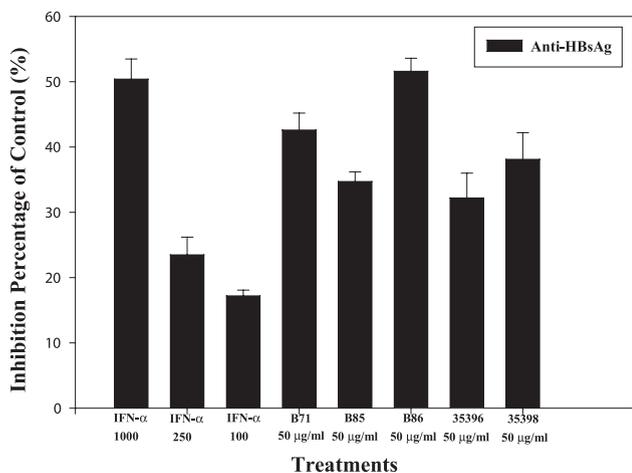


Fig. 3. Anti-HBsAg effect of α-interferon and polysaccharides isolated from cultured mycelia from five *A. camphorata* strains. Inhibition of viral multiplication as a percentage of the control was calculated as %inhibition = (OD_{control} - OD_{sample})/OD_{control}.

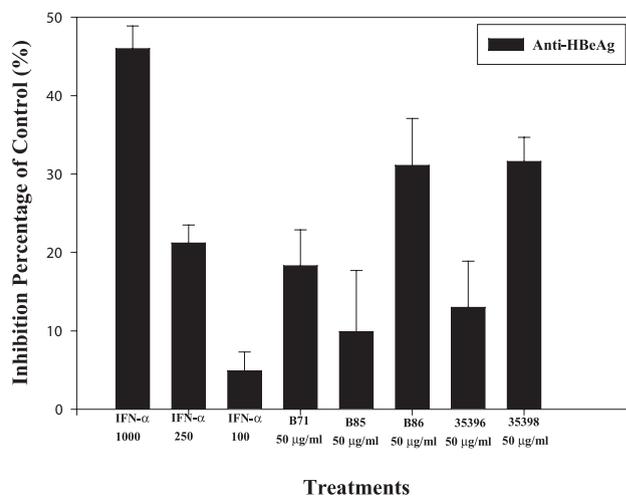


Fig. 4. Anti-HBeAg effect of α-interferon and polysaccharides isolated from cultured mycelia from five *A. camphorata* strains. %inhibition = (OD_{control} - OD_{sample})/OD_{control}.

ization of polysaccharides in the genus of *Antrodia*. Polysaccharide profiles obtained from different strains of *A. camphorata* were found to exhibit polymorphisms. Investigations on biologically active components of cultured *A. camphorata* mycelia have shown that the mushroom polysaccharides have anti-HBV activity. Anti-HBV activity has not been reported for polysaccharides from any other mushroom. Therefore, further studies on the relationship between specific polysaccharide fractions and their biological activities are necessary, and are in progress by our group.

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