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Promotion of hyphal growth and underlying chemical changes in *Antrodia camphorata* by host factors from *Cinnamomum camphora*

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Abstract

The aim of this research was to investigate the hyphal growth-promoting factors (HGFs) of *Antrodia camphorata* from the host-related species, *Cinnamomum camphora* (CC) and the underlying chemical produced. The HGF was identified in the polysaccharide fraction of CC at levels ranging from 80 to 320 mg L^{-1}, and it maximally stimulated growth to 5.50 g L^{-1} during a 14-day culture period compared to that of the control of 2.88 g L^{-1}. We also investigated the nature and chemical composition of the CC polysaccharide. Herein, size-exclusion column chromatography followed by high-performance anion-exchange chromatography after complete hydrolysis of the CC polysaccharide was performed to derive its molecular weight and sugar composition. The M_w values of the CC polysaccharide were determined to be 728.2, 187.5, 28.7, 7.5, and 1.9 kDa. Compositional analysis of the CC polysaccharide showed that galactosamine, mannose, and glucose were the major monosaccharides. Time-course studies of mycelial extracts of cultures revealed that prolonged incubation with the water-soluble extracts of CC resulted in an increase in the relative amounts of two lanostane-type compounds, i.e., dehydrosulphurenic acid and 15α-acetyl-dehydrosulphurenic acid, which are found in the fruiting bodies of *A. camphorata*. This finding offers the possibility of the reliable production of this medicinal fungus under laboratory conditions compared to its limited slow growth in nature.

Keywords: *Antrodia camphorata*; *Cinnamomum camphora*; Polysaccharides

1. Introduction

Antrodia camphorata, a medicinal mushroom native to Taiwan and commonly known as 'niu-chang chih' or 'jang-jy,' is traditionally used for the treatment of toxication caused by food, alcohol, or drugs, as well as for anti-aging, anti-hepatoma, diarrhea, abdominal pain, hypertension, skin itching, and cancer (Tsai and Liaw, 1985). Chemical ingredients found in *A. camphorata* include sesquiterpene lactone, steroids, and triterpenoids (Chen et al., 1995; Cherng and Chiang, 1995; Chiang et al., 1995; Cherng et al., 1996; Yang et al., 1996). Zhankuic acid A (a type of steroid acid) was reported to have cytotoxic effects against P388 murine leukemia and was anticholinergic as well as antiserotonergic in pig ileum preparations (Chen et al., 1995). The aqueous extract of *A. camphorata* mycelia also exhibited significant cytotoxicity against leukemia HL-60 cells but not against cultured human endothelial cells (Hseu et al., 2002). In our previous study, extracts from cultured mycelia of *A. camphorata* displayed vasorelaxation (Wang et al., 2003) and anti-inflammatory activities (Shen et al., 2004). Polysaccharides from cultured *A. camphorata* mycelia show anti-hepatitis B virus activity (Lee et al., 2002).

A. camphorata is an indigenous fungus that parasitizes the inner cavity wood of the endemic camphor tree species, *Cinnamomum kanehirae* (*Cinnamomum*). Due to the limited distribution of the host plant and the slow growth rate of the fungus, the mass production of the fungus through in vitro culture systems for pharmaceutical usage has been attempted. However, there is limited documentation concerning the mass production of this species. It would therefore be worthwhile searching for hyphal growth-promoting factors (HGFs) in natural products. According to documentation, an HGF for

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Ustilago violacea was isolated from a water-soluble fraction of *Silene alba* (Kokontis and Ruddat, 1986). In a previous study, we cultured the parasitic hypha of *A. camphorata* in the presence of water-soluble wood extracts from the host and four host-related species (*C. kanehirae*, *Cinnamomum micranthum*, *Cinnamomum osmophloeum*, *Cinnamomum camphora*, and *Cinnamomum kotoense*). We showed that the *C. camphora* (CC) water extract exhibited a higher level of growth-promoting activity than that of *A. camphorata*'s natural host (Shen et al., 2004). Furthermore, because of its almost unlimited availability on the island of Taiwan, it was chosen as an alternative species on which to culture *A. camphorata* in vitro. In this study, we first show the purification and characterization of the HGF of *A. camphorata* from *C. camphora*. We were also interested in examining the chemical constituents in the mycelial extracts of *A. camphorata* incubated with the HGF.

2. Materials and methods

2.1. Liquid culture of A. camphorata

The *A. camphorata* isolate (strain B85) obtained from Taitung County, southeastern Taiwan, was a generous gift from Dr. T.T. Chang (Division of Forest Protection, Taiwan Forest Research Institute, Taipei). *A. camphorata* was subcultured and maintained in essentially the same manner as previously reported (Lee et al., 2002). Briefly, *A. camphorata* was inoculated at the center of a Petri dish containing 39 g L^{-1} potato dextrose agar (PDA) and incubated at 28 °C for 19 days before being transferred to liquid culture. The basal medium, denoted as the control medium, for liquid culture contained 24 g L^{-1} potato-dextrose broth (PDB) and 20 g L^{-1} glucose at pH 5.6. The various CC extracts were added to the basal medium at the corresponding dosages and incubated for another 14 days. Following incubation, mycelia were rapidly washed with 1 L of NaCl (250 mM). An aspirator-suction system was used to remove any contaminating culture medium. Samples were then lyophilized and stored at 4 °C.

2.2. Preparation of the water-soluble extract from CC

The crude water-soluble extract of CC was prepared by incubating 40 g of ground wood with 500 mL of hot water at 80 °C for 3 h and then repeating this again. The combined water-soluble fraction from the above extraction was collected after filtration. The extract was either used fresh or was lyophilized for storage.

2.3. Partitioning extracts for the HGF

The lyophilized crude water-soluble extract (15 g) of CC was homogenized in 1 L H_2O and partitioned against ethyl acetate (2:3 v/v). The organic layer (denoted as the EA-layer) was evaporated and weighed. The aqueous fraction was further partitioned against *n*-butanol (3:2 v/v). The organic phase was evaporated and denoted as the BuOH layer. The aqueous fraction was lyophilized and denoted as the H_2O layer.

2.4. Extraction of polysaccharide from the CC-partitioned H_2O layer

Polysaccharide was extracted in essentially the same manner as previously reported (Zhang et al., 1994). Briefly, the CC-partitioned H_2O layer (2 g) was dissolved in 50 mL H_2O. Four volumes of 99% ethanol were added and allowed to stand at 4 °C overnight. The precipitate produced was collected by centrifugation at 9000 rpm for 10 min at 4 °C and was denoted the polysaccharide (PS). After centrifugation, the supernatant was rich in oligosaccharides and monosaccharides and was denoted the non-polysaccharide (NPS).

2.5. Fractionation of the polysaccharide fraction by Sephadex LH-20 filtration chromatography

After having been dissolved in water, 6.5 g of the polysaccharide fraction was fractionated by gel filtration on a Sephadex LH-20 column (7×100 cm) and eluted with water at a flow rate of 3.5 mL min^{-1}. Fractions (42 mL $tube^{-1}$) were collected and assayed for hexose by the phenol–sulfuric acid method (Zevenhuizen et al., 1980), and the absorbance at 488 nm was recorded. The fractions were then subdivided and combined to form four groups: fraction A (10–38), fraction B (39–58), fraction C (59–74), and fraction D (75–160).

2.6. Size-exclusion chromatography (SEC) of the CC polysaccharide

SEC-quaternary signal detection was performed with the Viscoteck TDA 301 system (Houston, TX) which contains a refractive index detector (RI, concentration detector). The eluent was water with a flow rate of 1.0 mL min^{-1}. All solutions with a polysaccharide concentration of 1 mg mL^{-1} were filtered with a 0.2-μm filter (Whatman, Middlesex, UK) before injection into the SEC column ($G4000PW_{XL}$, 7.8×300 mm, Viscotek). A calibration curve was constructed using an authentic standard, Sodex P-82 series (Showa Denko America, NY, USA) containing polymaltotriose with molecular weights of 78.8×10^4, 40.4×10^4, 21.2×10^4, 4.73×10^4, and 1.18×10^4 Da. The TriSEC software program (Viscotek) was used for the acquisition and analysis of the Viscotek data.

2.7. Hydrolysis of the polysaccharide

One milligram of polysaccharide was hydrolyzed with 6N HCl at 80 °C in a heating block for 6–8 h. The mixture was cooled and evaporated to remove the acid, resuspended in milli-Q water and passed through a Millipore-GX nylon membrane before analysis.

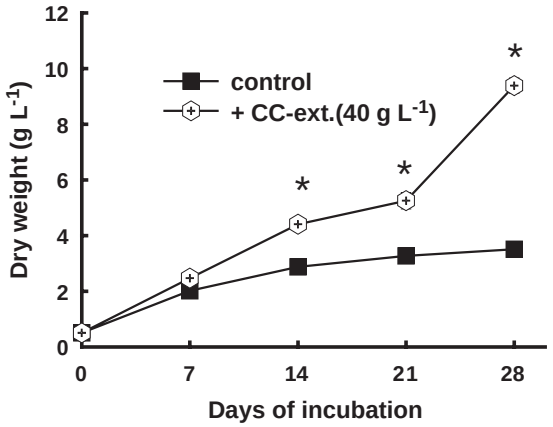

Fig. 1. Effect of 40 g L^{-1} of the *C. camphora* (CC) water extract on the mycelial growth of *A. camphorata* B85. The parasitic hypha of *A. camphorata* was cultured in the presence of 24 g L^{-1} potato-dextrose broth (PDB) and 20 g L^{-1} glucose (control) or with the addition of 40 g L^{-1} of the water-soluble fraction prepared from the wood of CC to study their growth-promoting effects estimated by the dry weight (g L^{-1}) at the end of 7-, 14-, 21-, and 28-day culture periods. Data are presented as the mean ± S.D. ($n=4$). *$p<0.05$ vs. the control (PDB + glucose only).

2.8. Compositional determination of the CC polysaccharide

Monosaccharides of CC-polysaccharide hydrolysates were separated on a high-performance anion-exchange chromatographic (HPAEC) system (Dionex, Sunnyvale, CA) and 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.

2.9. Preparation of ethanolic extracts from cultured mycelia of *A. camphorata*

Fifty milligrams of lyophilized mycelia was extracted with 1 mL ethanol (99%) at room temperature three times for 6 h each. The combined ethanolic extract was then evaporated to dryness and resuspended in ethanol at a concentration of 1 mg mL^{-1}.

2.10. High-performance liquid chromatography (HPLC)

HPLC was performed on an Agilent (Santa Clara, CA, USA) 1100 series chromatograph. The detecting wavelength was set at 243 nm. Separations were obtained with a reversed-phase column (Cosmosil $5C_{18}$-AR-II, 250 × 4.6 mm, Kyoto, Japan) eluted at a flow rate of 0.8 mL min^{-1} with a linear solvent gradient elution system composed of eluents A, B, and C (A: 0.0085% H_3PO_4 in H_2O; B: 100% methanol; C: 100% acetonitrile) according to the following profile: 0–20 min, 99.8% A, 0.2% B; 20–40 min, 99.8%→88% A, 0.2%→4% B, 0%→8% C; 40–70 min, 88%→50% A, 4%→15% B, 8%→35% C; 70–90 min, 50%→4% A, 15%→32% B, 35%→64% C; 90–115 min, 4%→0% A, 32%→20% B, 64%→80% C; 115–125 min, 20%→10% B, 80%→90% C; 125–135 min, 10%→0% B, 90%→100% C; and 135–140 min, 100% C. Dehydrosulphurenic acid and 15α-acetyl-dehydrosulphurenic acid were purified as in our previous report (Shen et al., 2003) and were used as external standards to identify the active components in these extracts.

2.11. Statistical analysis

Data are presented as the mean ± standard error (S.E.), and n represents the number of experiments. In the bar graphs, the S.E. values are indicated with error bars. Statistical analyses were carried out using Student's unpaired t-tests when applicable. p values of <0.05 were considered to indicate statistical significance.

3. Results

3.1. HGF of *A. camphorata* from the water-soluble extract of CC

We cultured the parasitic hypha of *A. camphorata* in the presence of the water-soluble extract from the wood of CC in

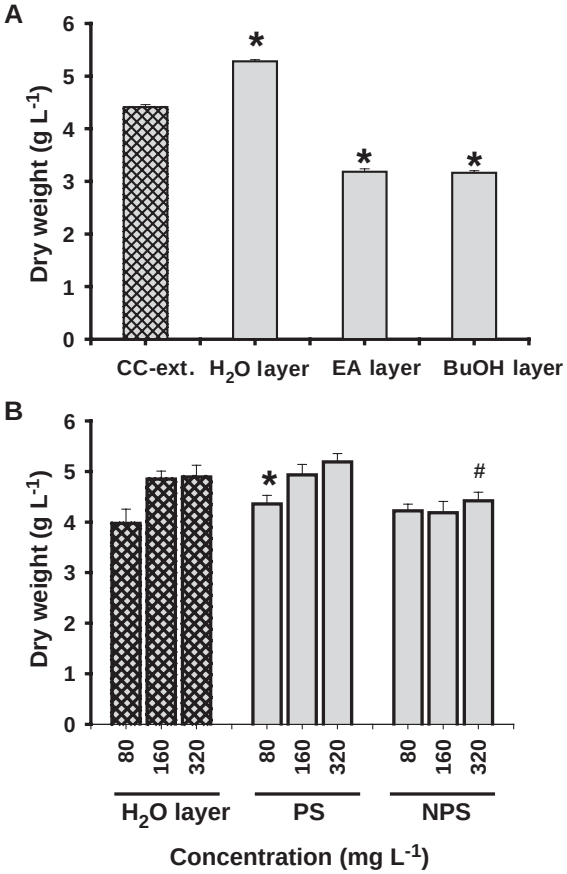

Fig. 2. Partitioning of hypha growth-promoting factor (HGF) from the *C. camphora* (CC) water extract. (A) The parasitic hypha of *A. camphorata* was cultured in the control medium with the addition of 40 g L^{-1} of the water-soluble fraction prepared from the wood of CC or with each of 160 mg L^{-1} partitioned CC water extracts to evaluate the growth of *A. camphorata* B85. Mycelia were incubated at 28 °C for 14 days. Data are presented as the mean ± S.D. ($n=4$). *$p<0.05$ vs. the CC ext. (B) Effect of the polysaccharide (PS) and non-polysaccharide (NPS) fractions from the water extracts of CC on the growth of *A. camphorata* B85. Mycelia were incubated at 28 °C for 14 days. Data are presented as the mean ± S.D. ($n=4$). *$p<0.05$ vs. the H_2O layer; $^{\#}p<0.05$ vs. the PS fraction at the corresponding concentration.

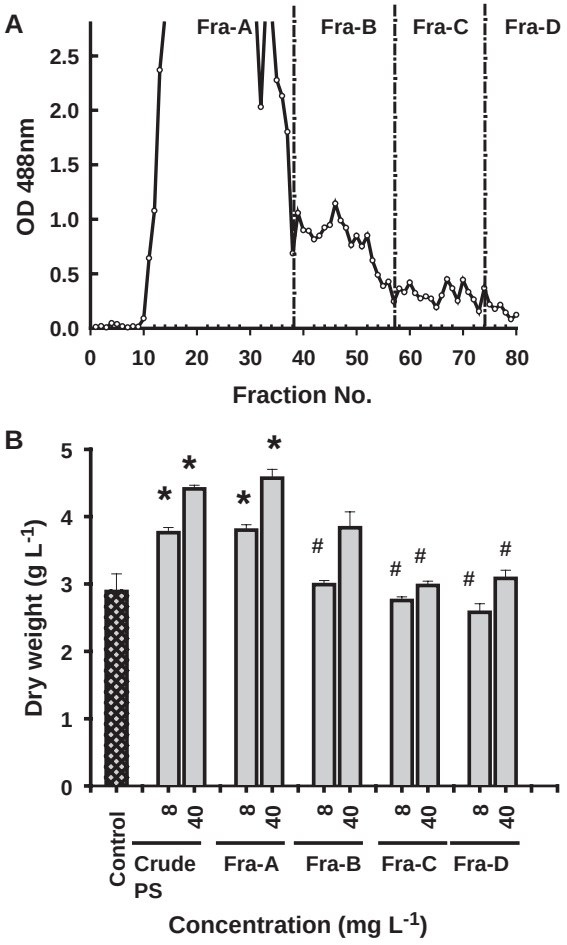

Fig. 3. Fractionation of hypha growth-promoting factor (HGF) from *C. camphora* (CC) polysaccharide (PS). (A) Fractionation of CC polysaccharide by Sephadex LH-20 filtration chromatography. (B) Effect of the CC PS fractions a–d on the growth of *A. camphorata* B85. Mycelia were incubated at 28 °C for 14 days. Data are presented as the mean ± S.D. ($n=4$). *$p<0.05$ vs. the control, $^{\#}p<0.05$ vs. the crude PS fraction.

the control medium to study the growth of mycelia in comparison with that of the control. As shown in Fig. 1, the water-soluble extract of CC (40 g L^{-1}) markedly promoted the growth of hypha of *A. camphorata* with maximal dry weights of 4.4, 5.2, and 9.4 g L^{-1}, at 14, 21, and 28 days, respectively.

3.2. Partitioning of the HGF from the CC water extract

To isolate the HGF from the CC water-soluble extract, partitioning and fractionation of the extract were performed as described in Materials and methods. After partitioning, three layers were formed: the EA layer, the BuOH layer, and the residual H_2O layer. Comparisons were made between the culture grown on control medium with the addition of the CC water extract or with the partitioned extracts (Fig. 2A). The results showed that the culture grown on the medium with the addition of the H_2O-layer extract exhibited significant growth promotion compared to that of CC-water extract with 14-day dry weights of 5.28 and 4.41 g L^{-1}, respectively. The culture grown on medium containing the EA- or BuOH-layer extracts exhibited inhibited growth compared with that of medium containing the CC water extract. The partitioned H_2O layer of CC was further precipitated with 80% ethanol to obtain the polysaccharide (PS) and non-polysaccharide (NPS) fractions. The PS fraction of CC at the low level of 80 mg L^{-1} maximally stimulated growth to 4.53 g dry weight L^{-1} compared to that of the CC H_2O layer. Cultures grown with the PS fraction (320 mg L^{-1}) showed significant growth promotion compared to those grown with the NPS fraction, with 14-day dry weights of 5.50 and 4.42 g L^{-1}, respectively (Fig. 2B).

3.3. Fractionation of HGF from CC polysaccharides

The HGF-containing PS fraction was subsequently fractionated by Sephadex LH-20 filtration chromatography and

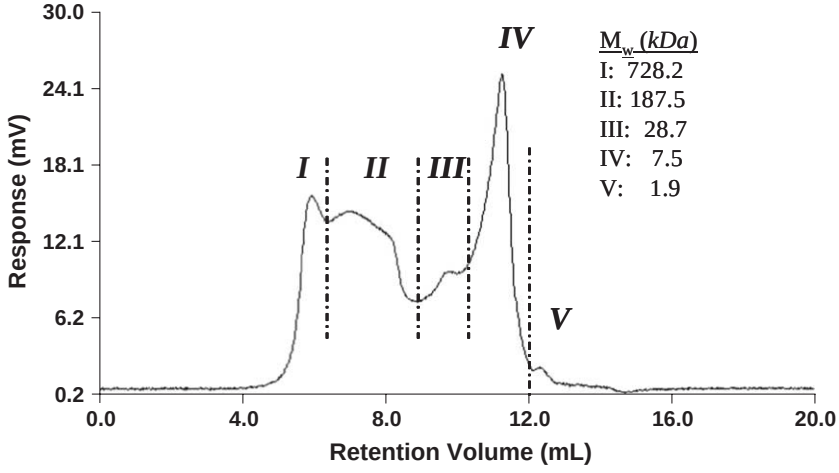

Fig. 4. SEC-HPLC profile of *C. camphora* (CC) polysaccharide (PS). A PS solution in milli-Q water was diluted to give a concentration of 1 mg mL^{-1} and was filtered with a 0.22-μm filter (Millipore) before injection into the SEC column ($G4000PW_{XL}$, 7.8 × 300 mm). The eluent was deionized water at a flow rate of 1 mL min^{-1}.

Table 1
Characteristics of crude polysaccharide (2.5 μg) isolated from *C. camphora*

Neutral sugars	Concentration (ng)	Percent crude polysaccharide
myo-Inositol	65.5	2.62
Sorbitol	47.8	1.91
Fucose	38.8	1.55
Galactosamine (GalN)	132.9	5.32
Galactose	30.8	1.23
Glucose	79.9	3.20
Mannose	274.1	10.96
Fructose	1.8	0.07

eluted with H_2O (Fig. 3A). Fraction A (10–38) retained the growth promotion activity in a dose-dependent manner as compared to the crude polysaccharide, with maximal growth of 4.57, and 4.41 g L^{-1}, respectively (Fig. 3B). On the contrary, fractions C (59–74) and D (75–80) showed significant growth-inhibitory activities compared to that of the crude polysaccharide.

3.4. Molecular mass determination of the CC polysaccharide

Fig. 4 shows the size-exclusion chromatographic plot of the CC polysaccharide in the aqueous solution at 25 °C. The CC polysaccharide contained more than five polysaccharide fractions. The M_w values of the five polysaccharides of fractions I to V were, respectively, determined to be 728.2, 187.5, 28.7, 7.5, and 1.9 kDa.

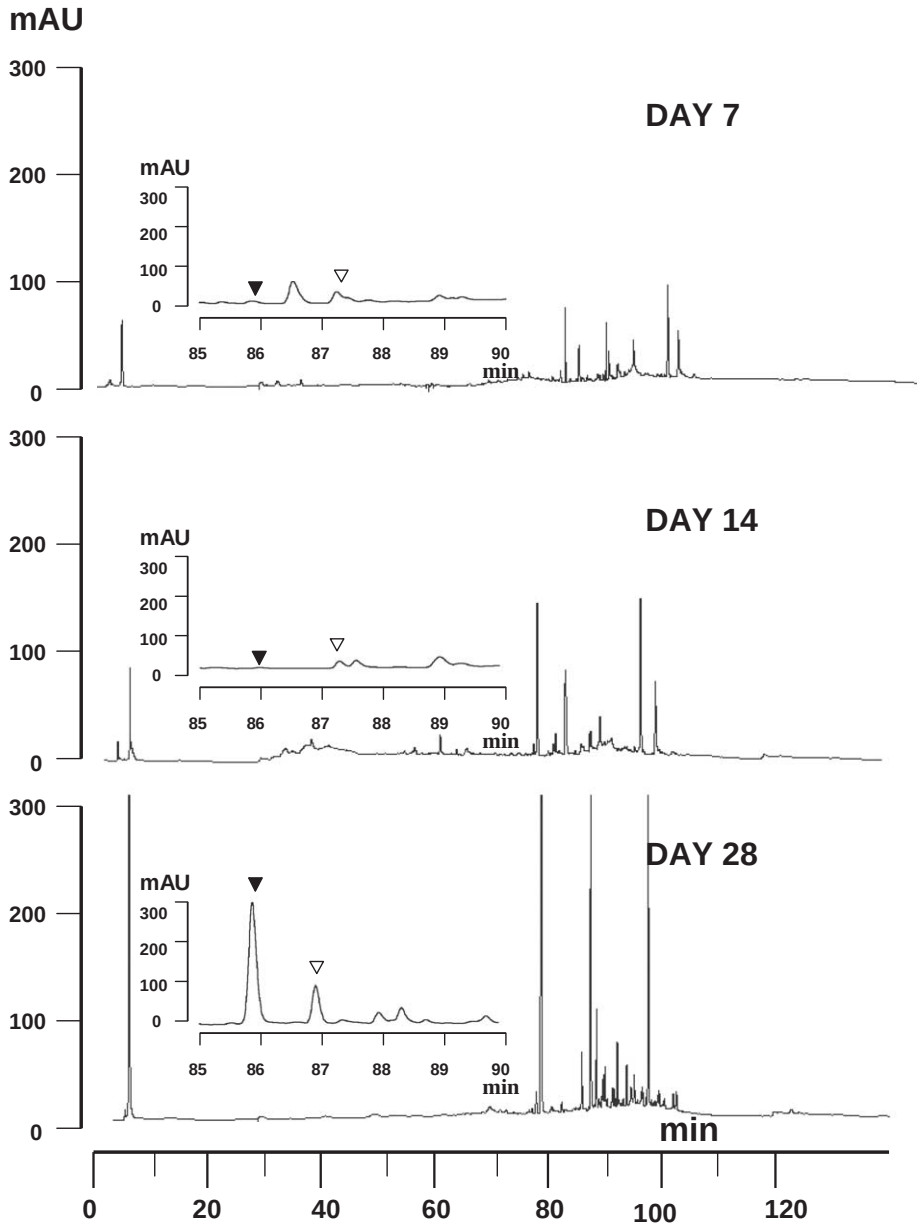

Fig. 5. Chromatograms of the components in the *A. camphorata* cultured with the water-soluble fraction of *C. camphorata* (CC). The ethanolic extracts (20 μg/20 μl), prepared as described in Materials and methods, were injected directly into an HPLC system with a linear solvent gradient (flow rate of the mobile phase: 0.8 mL min^{-1}; wavelength of the UV detector: 243 nm). The inset on the left-hand side of each chromatogram shows the HPLC profile between 85 and 90 min. Two compounds, i.e., dehydrosulphurenic acid (▼) and 15α-acetyl-dehydrosulphurenic acid (▽), were identified by comparison with external standards.

Table 2
Time-course study of the relative contents of dehydrosulphurenic acid and 15α-acetyl-dehydrosulphurenic acid in the mycelia of *A. camphorata*

Ethanolic extracts from mycelia of *A. camphorata*	Dehydrosulphurenic acid (mg g^{-1} crude extract)	15α-Acetyl-dehydrosulphurenic acid (mg g^{-1} crude extract)
7 days	17.7±1.4	12.6±1.2
14 days	6.8±0.4	10.8±1.0
28 days	90.6±1.0	26.7±0.6

The relative content of dehydrosulphurenic acid and 15α-acetyl-dehydrosulphurenic acid in ethanolic extracts isolated from mycelia of *Antrodia camphorata* cultured in media containing *Cinnamomum camphora* water-soluble extract (40 g L^{-1}) for 7, 14, and 28 days, respectively, were detected by a HPLC system as described in "Materials and methods". Data are expressed as the means±SEM of replicated experiments performed on different days.

3.5. Compositional analysis of the HGF-containing CC polysaccharides

Compositional analysis of the sugar moieties of the HGF-containing CC polysaccharides is shown in Table 1. Mannose was the dominant sugar component of the polysaccharide mixture and represented 10.96% of the total polysaccharides. Other major sugar species were *myo*-inositol, galactosamine, and glucose, respectively, comprising 2.62%, 5.32%, and 3.20% of the total polysaccharides. It has been reported that a mannose-rich polysaccharide plays an important role in yeast endoplasmic reticula (Handford et al., 2003) and represents a major component in the cell walls of gymnosperm species (Buckeridge et al., 2000; Lundqvist et al., 2002). Our results suggest that the mannose-rich polysaccharide of CC possesses growth-associated activity in *A. camphorata*. Further studies are needed to validate its specific active components.

3.6. Components in the ethanolic extracts of CC-cultured mycelia of A. camphorata

Results of a time-course study investigating the bioactive components in the ethanolic extracts isolated from the mycelia of *A. camphorata* are illustrated in Fig. 5. We compared the HPLC profiles of the ethanolic extracts from mycelia of *A. camphorata* cultured with the water-soluble extracts of CC (40 g L^{-1}) for 7, 14, and 28 days. Two lanostanol-type compounds, i.e., dehydrosulphurenic acid (at 85.8 min) and 15α-acetyl-dehydrosulphurenic acid (at 87.5 min), previously isolated from the fruiting bodies of *A. camphorata* (Handford et al., 2003), were identified and may be involved in its anti-inflammatory actions (Shen et al., 2004). The relative contents of the compounds in these extracts are listed in Table 2. The maximal values of dehydrosulphurenic acid and 15α-acetyl-dehydrosulphurenic acid were reached in 28-day-old mycelia with 90.6±1 and 26.7±0.6 mg g^{-1} of the crude extract, respectively.

In conclusion, the purification of the hypha growth-promoting factors for *A. camphorata* from alternative sources of natural products is the first attempt for a fungus species to the best our knowledge. From previous studies, we screened for growth-promoting activity of *A. camphorata* among five host-related species (including the host plant itself) in the genus *Cinnamomum* (Shen et al., 2004), and *C. camphora* (CC) was chosen as the alternative source. In this report, CC polysaccharide was isolated for maximal growth promotion to 5.50 g dry weight L^{-1} in the 14-day culture period. The sizes of five polysaccharides of the CC polysaccharide fraction were determined to be 728.2, 187.5, 28.7, 7.5, and 1.9 kDa. Compositional analysis showed that galactosamine, mannose, and glucose were the major monosaccharides of the CC polysaccharide fraction.

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