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Phenochalasins, Inhibitors of Lipid Droplet Formation in Mouse Macrophages, Produced by *Phomopsis* sp. FT-0211

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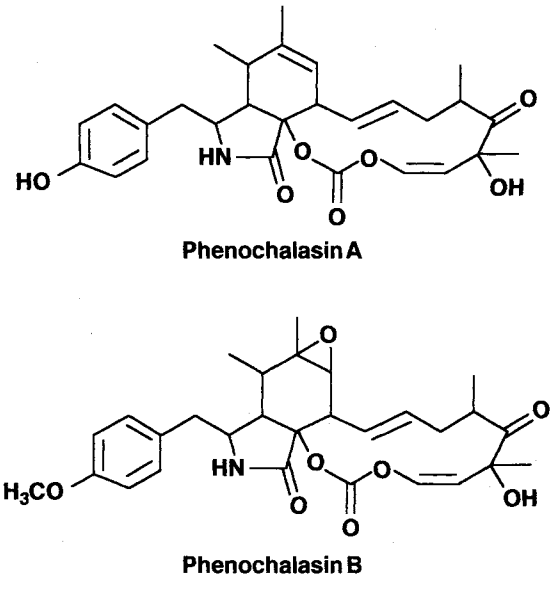
Phomopsis sp. FT-0211, a soil isolate, was found to produce inhibitors of lipid droplet formation in mouse peritoneal macrophages. Structurally related new compounds designated phenochalasins A and B were isolated from the fermentation broth of the producing strain by solvent extraction, ODS column chromatography and preparative HPLC. Phenochalasin A caused a dose-dependent reduction in the number and size of lipid droplets in macrophages without any cytotoxic effect at least up to 20 μM. On the other hand, phenochalasin B showed inhibition of lipid droplet formation with a severe cytotoxic effect on macrophages.

In the early stage of atherosclerogenesis, macrophages penetrate into the intima, efficiently take up modified low density lipoprotein (LDL), store cholesterol and fatty acid in the form of cholesteryl ester (CE) and triacylglycerol (TG) in the cytosolic lipid droplets, and are converted into foam cells, leading to the development of atherosclerosis in the arterial wall. Therefore, inhibitors of macrophage-derived foam cell formation would be expected to retard the progression of atherosclerosis[1~4].

A foam cell formation model was established using mouse peritoneal macrophages[5]. A number of lipid droplets were accumulated in cytosols when macrophages were cultured in the presence of negatively charged liposomes. Previously, we reported that fungal beauveriolides inhibit the lipid droplet formation[6,7]. From our continuing screening study, a fungal strain FT-0211 was found to produce inhibitors of lipid droplet formation in macrophages. Two structurally related compounds designated phenochalasins A and B[8] (Fig. 1), belonging to the cytochalasan family[9~15], were isolated from the culture broth. In this paper, the taxonomy of the producing strain, fermentation, isolation and biological properties of phenochalasins A and B are described.

Fig. 1. Structures of phenochalasins A and B.



Materials and Methods

Genaral Experimental Procedures

Fungal strain FT-0211 isolated from a sea water collected near Ponape Island was used for production of phenochalasins A and B. SSC-ODS-7515-12 (Senshu Sci. Co.) was used for column chromatography. HPLC was carried out using a Shimadzu (LC-7A) system. For determination of the amounts of phenochalasins A and B in culture broths, the samples (ethyl acetate extracts) dissolved in acetonitrile were analyzed by HPLC as follows: column, YMC packed column R-ODS-5, S-5, 4.6×250 mm; solvent, 46% acetonitrile; flow rate, 0.7 ml/minute; detection, UV at 200 nm. Under these conditions, phenochalasins A and B were eluted as peaks with retention times of 34 and 25 minutes, respectively.

Taxonomic Studies

The morphological properties were examined with a light microscope (Vanox-S AH-2, Olympus) or a scanning electron microscope (JSM-5600, JEOL). For identification of the fungus, cultures were observed after incubation at 25°C for 14 days on potato dextrose agar (PDA) (Difco).

Assay for Lipid Droplet Formation in Mouse Macrophages

Assay for lipid droplet formation in macrophages was carried out according to the method described previously[5]. In brief, primary mouse peritoneal macrophages (2.5×10^5 cells in GIT medium) in each well of a 96-well plastic microplate (Corning Co.) were incubated in a humidified CO_2 (5% v/v) atmosphere at 37°C for 2 hours. The medium was then replaced with 0.125 ml of Dulbecco's modified Eagle medium containing 8% (v/v) lipoprotein-deficient serum, penicillin (100 units/ml) and streptomycin (100 μg/ml). After another 2-hour preincubation, 1.25 μl of a sample in methanol, and 5.0 μl of liposomes (phosphatidylcholine 1.0 μmol, phosphatidylserine 1.0 μmol, dicetylphosphate 0.20 μmol and cholesterol 1.5 μmol suspended in 1.0 ml of 0.3 M glucose) were added to each well. After a 14-hour incubation, the cells were washed with PBS and then fixed by soaking in 10% formalin. Nuclei and intracellular neutral lipid droplets were then stained with hematoxylin and oil red O, respectively, and the stained cells were examined by light microscopy.

Other Biological Assays

Antimicrobial activity was tested using a paper disk (i.d. 6 mm, ADVANTEC) agar diffusion assay. Bacteria were grown on Mueller-Hinton agar medium (Difco), and fungi and yeasts were grown on potato broth agar medium. Antimicrobial activity was observed after a 24-hour incubation at 37°C for bacteria and after a 48-hour incubation at 27°C for fungi and yeasts.

Results

Taxonomy of the Producing Organism

The strain FT-0211 grew abundantly to form grayish white colonies, then gradually grayish brown to dark brown colonies with a diameter of 40~50 mm after incubation at 25°C for 14 days on PDA. The color of the reverse side of the colonies was gray to dark brown. The fungus produced subglobose to cepaeform pycnidia, which were immersed into the PDA medium. Pycnidia were an ostiole and neck, 400~600×300~500 μm in diameter and brown to dark brown in color (Fig. 2A). Two types of conidia were observed. One was hyaline, unicellular, filiform to hook form and 18~25×1.0~2.5 μm in size (Fig. 2B). The other type was hyaline, unicellular, elliptical to botuliform and 2.0~5.0×1.5~2.5 μm.

From these characteristics, the strain FT-0211 was considered to belong to a member of the genus *Phomopsis*[16,17] and named *Phomopsis* sp. FT-0211.

Fermentation

A slant culture of the strain FT-0211 grown on YpSs agar (soluble starch 1.5%, yeast extract 0.4%, K_2HPO_4 0.1%, $MgSO_4\cdot7H_2O$ 0.05%, agar 2.0%, and 50% sea water, pH 6.0) was used to inoculate a 50-ml tube containing 10 ml of the seed medium (glucose 2.0%, yeast extract 0.2%, $MgSO_4\cdot7H_2O$ 0.05%, Polypepton 0.5%, KH_2PO_4 0.1%, agar 0.1% and 50% sea water, pH 6.0). The tube was shaken on a reciprocal shaker at 27°C for 4 days. One-ml portions of the seed culture were inoculated into 500-ml Erlenmeyer flasks containing 100 ml of the same seed medium. The flasks were shaken on a rotary shaker at 27°C for 4 days. Two hundred ml of the seed culture was transferred into a 30-liter jar fermenter containing 20 liters of the production medium (potato dextrose broth 2.4% and 50% sea water, pH 6.0). The fermentation was carried out at 27°C. A typical time course of the fermentation is shown in Fig. 3. The production of phenochalasins was measured by HPLC. Phenochalasins A and B were detected in the culture broth at day 3 and 2 after inoculation, respectively, and their concentrations reached levels of 3.8 and 69 μg/ml,

Fig. 2. Scanning electron micrograph of *Phomopsis* sp. FT-0211 grown on PDA.

A, cross section of pycnidium (bar, 200 μm). B, conidia (bar, 10 μm).

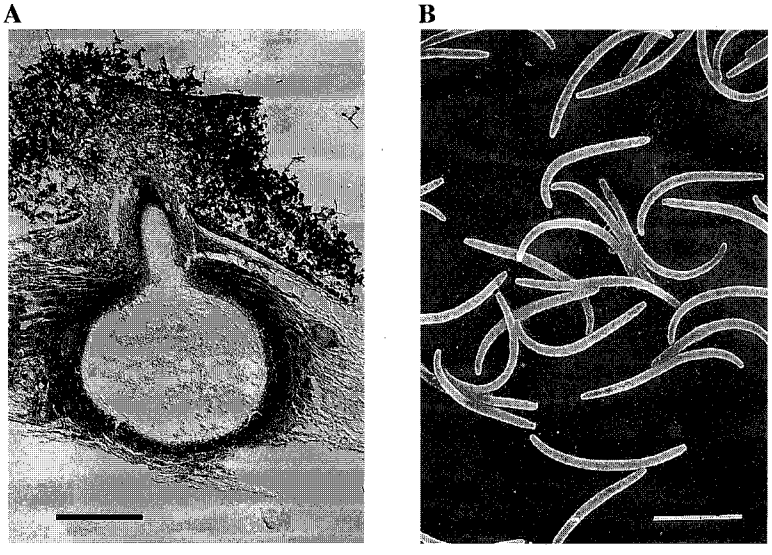


Fig. 3. A typical time course of phenochalasins production by *Phomopsis* sp. FT-0211.

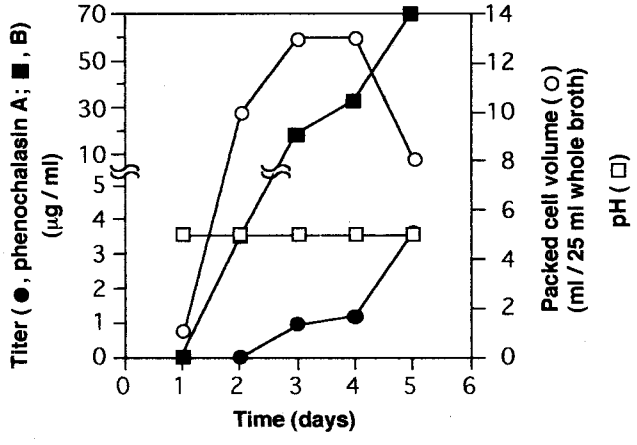
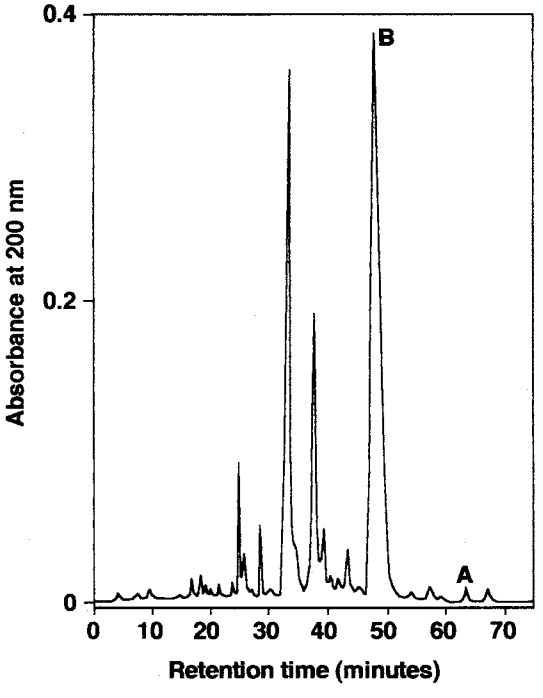


Fig. 4. A chromatographic profile of isolation of phenochalasins A and B by preparative HPLC.



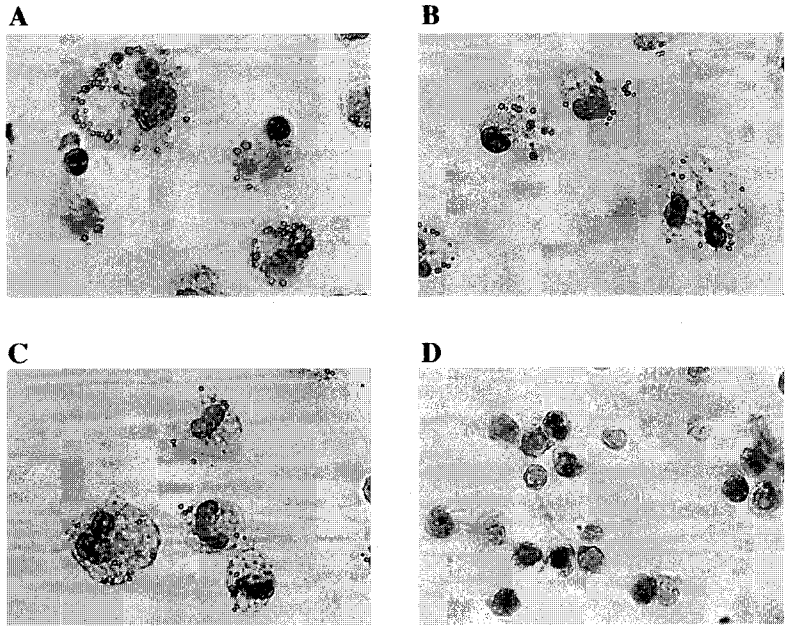
Coloumn, YMC-Pack D-ODS-5, 20×250 mm; solvent, 46% aq acetonitrile; detection, UV at 200 nm; flow rate, 6.0 ml/minute; sample, 100 μg of active materials (obtained through ODS column chromatography) dissolved in 50 μl CH_3CN was injected.

respectively, at day 5.

Isolation

To the 5-day old whole broth (20 liters) was added acetone (10 liters). After the acetone extracts were filtered and concentrated, the resulting aqueous solution was extracted with 20 liters of ethyl acetate. The extracts were dried over Na_2SO_4 and concentrated *in vacuo* to dryness to

Fig. 5. Effect of phenochalasins on lipid droplet formation in mouse peritoneal macrophages.



Macrophage monolayers (5×10^5 cells in 0.25 ml medium) grown in a tissue culture chamber (LAB-TEK 8-chamber, Nunc) were incubated with 10 μl of liposomes for 16 hours in the absence (A) or in the presence of 6 (B) or 20 μM (C) phenochalasin A or 3 μM phenochalasin B (D), respectively. Fixation and staining with oil red O and hematoxylin were then performed as described in the "Materials and Methods". Original magnification, $\times$200.

yield a brownish oil (15 g). The material suspended in 30% aq acetonitrile was subjected to an ODS column (750 g). The materials were eluted stepwise with aq acetonitrile solutions (30, 40, 50, 60, 70, 80, 90 and 100%, 2 liters each), and 500-ml fractions were collected. The fractions from 50% acetonitrile were pooled, concentrated and extracted with ethyl acetate to give a white powder (904 mg). The resulting active powder was purified by preparative HPLC (YMC-pack D-ODS-5, 20$\times$250 mm; 46% acetonitrile; UV at 200 nm; 6.0 ml/minute). Under conditions, phenochalasins A and B were eluted with retention times of 48.0 and 63.0 minutes, respectively (Fig. 4), each of which was concentrated *in vacuo* to dryness to give pure phenochalasins A (22 mg) and B (480 mg) as white powders.

Biological Properties

Effect of Phenochalasins on Lipid Droplet Formation in Macrophages

Under the culture conditions, mouse peritoneal macrophages accummulated a number of lipid droplets in the cytosols, which were observed microscopically after oil red O staining (Fig. 5A). In the presence of phenochalasin A, the drug caused a dose-dependent reduction in the size and number of lipid droplets in macrophages with no cytotoxic effect at least up to 20 μM (Fig. 5B and 5C), indicating that phenochalasin A inhibit the lipid droplet formation specifically. On the other hand, phenochalasin B showed inhibition of lipid doplet formation with drastic morphological changes of macrophages at 3 μM (Fig. 5D).

Other Biological Activities

No antimicrobial activity of phenochalasin A was observed at 10 μg/6 mm disk against the following microorganism; *Bacillus subtilis*, *Mycobacterium smegmatis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Micrococcus luteus*, *Staphylococcus aureus*, *Candida albicans*, *Saccharomyces cerevisiae*, *Pyricularia oryzae*, *Mucor racemosus* and *Aspergillus niger*. Phenochalasin B showed potent antimicrobial activity only against *Mucor racemosus*, with an 18 mm zone of inhibition at 10 μg/6 mm disk.

Discussion

More than 40 members of the cytochalasan family including cytochalasins and aspochalasins have been isolated from cultures of various fungal strains such as *Aspergillus* sp., *Phomopsis* sp., *Phoma* sp., *Helminthosporium* sp., *Chalara* sp. and *Chaetomium* sp.[18]. Cytochalasins B, D and E were shown to disrupt the actin cytoskeleton[19]. Phenochalasins (Fig. 1), novel members of the family, were isolated as inhibitors of lipid droplet formation in mouse peritoneal macrophages. Interestingly, phenochalasin A showed almost no cytotoxic effect (Fig. 5B and 5C) up to 20 μM, while phenochalasin B caused morphological changes of macrophages, which were round in shape and smaller in size (Fig. 5D). TABAS *et al.*[20] reported that cytochalasin D specifically inhibits atherogenic lipoproteins that stimulate cholesterol esterification in mouse macrophages, leading to a conclusion that the actin cytoskeleton plays an important role in CE synthesis in macrophage-derived foam cell formation. However, cytochalasin D showed very cytotoxic effects with drastic morphological changes of macrophages (data not shown), which were very similar to those of phenochalasin B. The difference in results might be due to the incubation time, that is, a short (3 hours) *vs.* long (14 hours in this paper) incubation. Also, the mode of action might be different between them. To confirm the specificity of phenochalasin A, the effects of other cytochalasins will be compared with those of phenochalasin A on lipid droplet formation in macrophages and on the morphology of macrophages.

Acknowledgments

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