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Occurrence of OF4949II in the Fungal Mat formed by Surface Culture of *Aspergillus niger* F-580

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Aspergillus niger F-580, a potent producer of aminopeptidase M inhibitor, was isolated from the brown spots of plant leaves with a pathological trait. The inhibitory activity was found only in the fungal mat formed by surface culture of *Aspergillus niger* F-580, but not in the culture supernatant or cell pellet. The inhibitor was purified from the hot water extract of this fungal mat by using chromatographies on Diaion HP-20, DEAE-cellulose, Sephadex G-10 and YMC-ODS-AQ columns. The purified inhibitor was analyzed by UV, mass, and NMR spectroscopies, and identified as OF4949II, which had been isolated as an aminopeptidase B inhibitor from *Penicillium rugulosum* OF4949

Aminopeptidase M (microsomal leucine aminopeptidase, EC 3.4.11.2) hydrolyzes peptides containing L-methionine, L-alanine, L-leucine, or L-tyrosine as an N-terminal amino acid. It has been suggested that this enzyme might play an important role in the degradation of enkephalin (6) and the invasion of cancer cell (9, 10). Thus, the inhibitor of aminopeptidase M could be developed and used as pharmaceutically active agent with analgesic and antimetastatic effects

Aoyagi *et. al.* have suggested that aminopeptidase is located not only in cytosol but also on the the cell menbrane without being released extracellularly (1). The inhibitor of aminopeptidase can bind to the aminopeptidase located on the surface of cells, resulting in modulating the immune responses of the cell. The specific inhibitors of aminopeptidase, such as bestatin (14, 16), amastatin (2), arphamenines (13), probestin (3), actinonin (15), leuhistin (4) and OF4949 (11, 12), were known to enhance delayed-type hypersensitivity (DTH) and other cellular immune responses.

We screened for aminopeptidase M inhibitors that potentiate cellular immune responses or prevent enkepalin degradation and cancer metastasis in the products of fungi. A potent producer of aminopeptidase M inhibitor was selected and identified as *Aspergillus niger*. Here, we report that OF4949II, which had been previously isolated from *Penicillium rugulosum* OF4949 as an aminopeptidase B inhibitor (11), is also found in the fungal mat formed by the surface culture of a black fungus, *A. niger* F-580.

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MATERIALS AND METHOD

Chemicals

Chemicals employed were as follows: Diaion HP-20 from Mitsubishi Kasei, Japan; DEAE cellulose and L-leucine-*p*-nitroanilide from Sigma Chemical Co., U.S. A; Sephadex G-10 from Pharmacia Fine Chemicals, Sweden; YMC-ODS-AQ HPLC column from Yamamura Chemical Co., Japan.

Enzyme

Aminopeptidase M (Sigma Chemical Co.) from porcine kidney was used in this assay.

Assay for Aminopeptidase M and Inhibitory Activity

The activity of aminopeptidase M was determined colorimetrically by measuring the amount of nitroaniline that has been liberated when L-leucine-*p*-nitroanilide was used as a substrate. The reaction mixture (total 200 μl) contained 160 μl of 0.1 M Tris-HCl buffer (pH 7.0) containing 0.0125% L-leucine-*p*-nitroanilide, 20 μl of 1 mU enzyme and 20 μl of water or an aqueous solution containing a test compound. The reaction mixture was incubated at 37°C for 30 minutes. The absorbance of the liberated nitroaniline was measured by microplate reader at 405 nm. The percent inhibition was calculated by the formula (A-B)/A × 100, where A is the absorbance of nitroaniline

liberated by the enzyme without an inhibitor and B is that with an inhibitor. The IC_{50} value is the concentration of inhibitor at 50% of enzyme activity.

Production of Inhibitor

A loopful of spores of *A. niger* F-580 was inoculated and grown on potato dextrose agar media at 27°C for 4 days. One cm^2 of the above agar segment was inoculated into 200 ml of a production medium consisting of glucose 3%, soybean meal 2%, NaCl 0.2% and KH_2PO_4 0.15% (pH 6.5) in a 1000 ml Erlenmeyer flask and incubated without shaking at 27°C for 7 days.

Isolation of Inhibitor

The hot water extract of the cultured fungal mat was filtered through celite and the culture filtrate was adsorbed on Diaion HP-20 and eluted with 30% methanol. The eluate was directly adsorbed on DEAE cellulose (OH^- form) and eluted with 0.1% acetic acid. The eluate was concentrated to dryness under reduced pressure and further purified by gel filtration on a Sephadex G-10 column. The active fraction was subjected to HPLC on the column of YMC-ODS-AQ with a solvent of 15% methanol solution. The active fraction was concentrated and lyophilized to produce inhibitor as a white powder.

Instrumental Analyses of Inhibitor

UV spectrum was recorded on a Shimazu UV-260 spectrophotometer and the IR spectrum on a Laser Precision Analytical IFX-65S spectrophotometer. NMR spectrum was obtained on a Varian UNITY300. Mass spectrum was obtained on a Kratos Concept 1S mass spectrometer.

RESULT AND DISCUSSION

Inhibitor Producing Fungus

684 fungal strains were isolated from plant leaf brown spot showing a pathological trait, and strain F-580 was selected as a potent producer of aminopeptidase M inhibitor and identified as *Aspergillus niger* (7). The appearance of the conidial structure of this strain was shown in Fig. 1. The following characteristics of growth and morphology were observed. In Czapeck agar, colonies consisted of compact yellowish white basal felt mycelium with black conidial areas. Abundant sporulation was observed. The color and appearance of the reverse side was yellow and wrinkled, respectively. In malt extract agar, colonies spread broadly and loosely with dense sporulation. The black conidial structure and pale yellow reverse were observed. In potato dextrose agar, colonies spread broadly and compactly, grew rapidly and sporulated heavily. The color of conidial areas was black. Reverse color was pale yellow. Conidial heads were large, radiating, and late splitting into columns. Conidiophore stipes were smooth-walled, variable in length and 13-16 μm in diameter. Phialides (5-7×3-4 μm) borne on metulae. Metulae was 12-20×4-5 μm in size. Conidia were globose to subglobose in appearance and 4-5 μm in diameter with spins and ridges.

Production and Isolation of Inhibitor

When the strain *A. niger* F-580 was cultured in 250 ml Erlenmeyer flasks containing ordinary volumes (30-150 ml) of production medium on rotary shaker, aminopeptidase M inhibitory activity was not found in cuture supernatant or cell cake. Aminopeptidase M inhibitory activity occurred in the following culture conditions; surface or shaking culture in test tubes, shaking culture in Erlenmeyer flasks with a

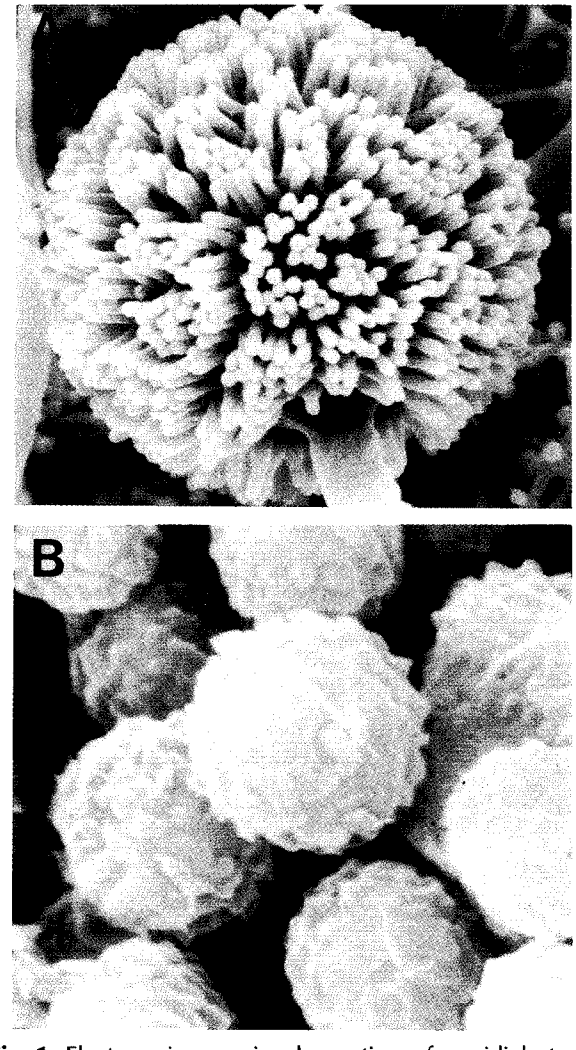

Fig. 1. Electronmicroscopic observation of conidial structure (A) and electronmicroscopic appearance of the conidiospore (B) of *Aspergillus niger* F-580.

large media volume (200 ml in 250 ml Erlenmeyer flask), and static surface culture in Erlenmeyer flasks. Our trials for the production of the inhibitor in a jar fermentor experiment by using *A. niger* F-580 were not successful. In the inhibitor producing culture conditions, this strain formed a wrinkled fungal mat on the surface of the liquid culture medium. Most of aminopeptidase M inhibitory activity was found in the water extract of this fungal mat. The occurrence of aminopeptidase M inhibitor in the in the fungal mat of *A. niger* F-580 suggested that the formation of mat is critical for the production of inhibitor by *A. niger* F-580 since cell cake or pellet formed in the submerged culture does not contain the aminopeptidase inhibitor. To elucidate the relationship between aminopeptidase M inhibitor and fungal mat, further research is required.

From the hot water extract of the fungal mat of *A. niger*, the inhibitor was isolated by the procedure shown in Fig. 2.

Physicochemical Properties of the Inhibitor

The inhibitor isolated from *A. niger* was soluble in water and dimethylsulfoxide, slightly soluble in methanol, and insoluble in ethanol, butanol, acetone, ethylacetate, chloroform, benzene and n-hexane. It was positive for ninhydrin, $KMnO_4$ and iodine color reaction, but not for the Sakaguchi reaction. In the color reaction with ferric chloride this inhibitor was positive. The UV absorption maxima of the inhibitor were at 209, 272 and 279 nm in neutral solution, with a bathochromic shift to 218, 243 and 297 nm in alkaline solution, suggesting the presence of a phenolic hydroxyl group (Fig. 3). The molecular mass was decided from electron scattering mass spectrometry to be m/z 471(M^-) (Fig. 4). The ^{1}H-NMR spectrum and ^{1}H, ^{1}H-NMR spectrum were given in Figs. 5 and 6, respectively.

In the ^{1}H-NMR and ^{1}H, ^{1}H-homocosy NMR data for inhibitor in D_2O, the signals at 6.94, 6.74 and 5.87 ppm indicated the presence of a tri-substituted benzene ring, and signals at 7.47, 7.24, 7.08 and 6.95 ppm indicated the presence of a di-substituted benzene ring. The signals at 4.48, 3.37 and 2.66 ppm showed an AMX system, and those at 3.70, 2.94 and 2.82 ppm an ABX system, which suggest the pre-

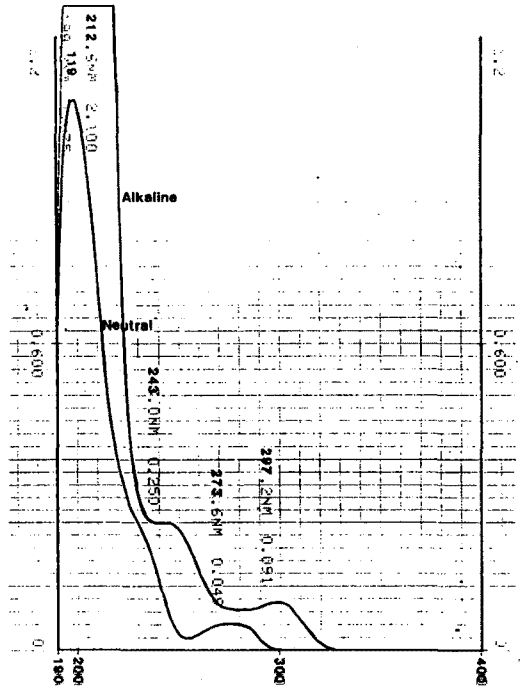

Fig. 3. UV spectrum of aminopeptidase M inhibitor produced by *Aspergillus niger* F-580.

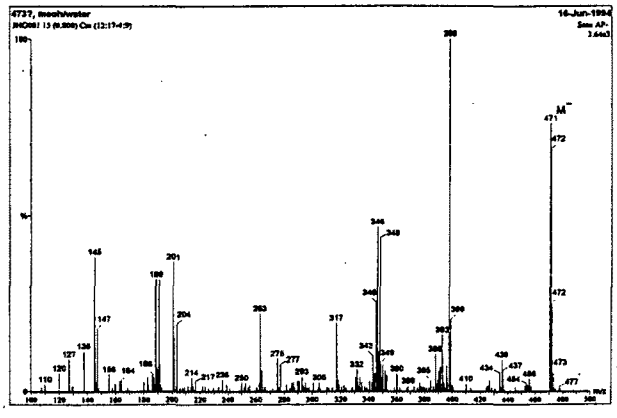

Fig. 4. Mass spectrum of aminopeptidase M inhinbitor produced by *Aspergillus niger* F-580.

Hot water extract of fungal mat
↓
Diaion HP-20 chromatography
↓ 30% methanol elution
DEAE celullose chromatography
↓ 0.1% acetic acid elution
Sephadex G-10 gel filtration
↓
HPLC on YMC-ODS-AQ column
↓ 15% methanol elution
White powder

Fig. 2. Purification procedure of aminopeptidase M inhibitor produced by *Aspergillus niger* F-580.

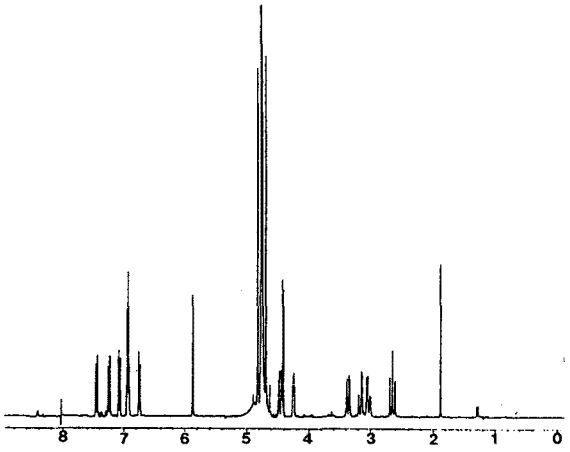

Fig. 5. ^{1}H-NMR spectrum of aminopeptidase M inhibitor produced by *Aspergillus niger* F-580.

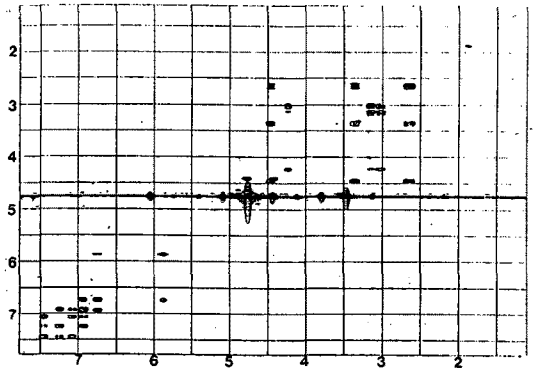

Fig. 6. ^{1}H, ^{1}H-homocosy NMR spectrum of aminopeptidase M inhibitor produced by *Aspergillus niger* F-580.

sence of two similar carbon chains both with C-CH_2-CH-C.

From the results of spectroscopic analyses of the aminopeptidase M inhibitor produced by *A. niger* F-580, the inhibitor was assumed to be OF4949II, which had been isolated from *Penicillium rugulosum* OF4949 as an aminopeptidase B inhibitor. *P. rugulosum* produces 4 types of OF4949, I, II, III and IV. However, *A. niger* F-580 is supposed to produce only OF4949II.

The black aspergilli are worldwide in distribution and widely used in industry for the production of organic acids, enzymes and other products (5). *A. niger*, on a variety of substrates, has been investigated in detail and shows promise for production of proteins for human and animal consumption and for improving protein-poor diets (5, 8). The results of this investigation indicate that the potential production of OF 4949II by this common fungus could give some beneficial or some detrimental effects on human health if such strains are used as starters, for example, in food industry. Further research on the effect of orally administrated OF4949II on human health is necessary.

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