Macromolecular Insect Chitinase Inhibitors Produced by Fungi: Screening and Partial Characterization

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Culture broths of fungal strains were screened for novel insect chitinase inhibitors using the Spodoptera litura chitinase inhibitory assay. The culture filtrates of 5 strains showed potent and specific inhibitory activity against the insect chitinase. Partial characterization showed that the active compounds produced by these strains were water-soluble macromolecular compounds which had not been hitherto reported as chitinase inhibitors. These novel chitinase inhibitors are, therefore, expected to be potential agents for insect control.

Key words: insect chitinase inhibitor, insect growth regulator, Spodoptera litura, static culture, polysaccharide, protein.

INTRODUCTION
Chitinase is a key enzyme in the molting of insects and its inhibitors are expected to be biorational insect growth regulators. Allosamidins have been reported as chitinase inhibitors, but they have not yet been used commercially. Therefore, novel insect chitinase inhibitors are desired.

In previous studies, we have developed a Spodoptera litura chitinase inhibitory assay, which is a rapid and sensitive screening method for insect chitinase inhibitors. We have screened cultures of fungi using this assay. Fungal strains were grown in static culture to take advantage of their ability to produce diverse secondary metabolites. This paper reports on the screening of static cultures from 776 fungal strains and the characterization of insect chitinase inhibitors produced by 5 active strains.

MATERIALS AND METHODS
1. General
Allosamidin was a gift from Dr. S. Sakuda (The University of Tokyo). Chitinase solution from S. litura pupae was prepared as previously described. Colloidal chitin was prepared from chitin powder (Wako Pure Chemical Industries, Osaka, Japan) as previously described. Chitinase from Streptomyces griseus, glycosidases from

Turbo cornutus and Pronase P were purchased from Sigma (St. Louis, MO), Seikagaku Kogyo (Tokyo, Japan) and Kaken Kagaku (Tokyo, Japan), respectively. All other chemicals were commercially available.

2. Isolation of Fungal Strains
Fungal strains were isolated from soil samples or plant leaves. Each soil sample was divided into 3 parts which were separately exposed to three treatments as follows for selective isolation: (1) removal of bacteria (heating at 40°C for 24 hr), (2) induction of spore germination (soaking in 50% ethanol for 15 min), (3) removal of spores (decantation of water suspension). Treated soil samples were suspended in sterile distilled water and plated on the 4 different agar media described below, then incubated at 28°C.

Plant leaves were cut into 1 cm squares and surface-sterilized in 90% ethanol for 10 sec, followed by 1% sodium hypochlorite for 5 min, and rinsed in sterile distilled water. After sterilization, the pieces were divided into 4 equal squares and placed on 4 different agar media, which were incubated at 22°C.

Four different agar media were prepared by adding 15 g/l agar to PSM, SD, YpsSs and Glc-Cz liquid media. PSM liquid medium was prepared as previously described. SD liquid medium (pH 5.6): glucose 40.0, Polypepton (Nihon Pharmaceutical, Tokyo, Japan) 10.0 (g/l water). YpsSs liquid medium (pH 5.6): starch 15.0, yeast extract (Nacalai Tesque, Kyoto, Japan) 4.0, K.HPO4, 1.0, MgSO4·7H2O 0.5 (g/l water). Glc-Cz liquid medium (pH 5.8-6.0): glucose
36.0 NaNO₃ 2.0, KH₂PO₄ 1.0, MgSO₄•7H₂O 0.5, KCl 0.5, FeSO₄•7H₂O 0.01 (g/l water). In order to suppress the growth of bacteria and actinomycetes, chloramphenicol (100 mg/l) was added to the media. Rose bengal (50 mg/l) was added except for the Glc-Cz agar medium to minimize the size of fungal colonies. Isolated strains were maintained in slant tubes of PSM agar medium.

3. Cultivation for Screening
Mycelia (2-5 mm square) of each isolated strain grown on PSM agar slant were scraped from the slant surface and added to 10 ml of PDM liquid medium (pH 5.5-6.5: peeled potato 300, glucose 20.0, malt extract (DIFCO, Detroit, MI, USA) 2.0 (g/l water)) in a test tube (ø25 mm). An inoculated medium was placed at a 20° angle to give a large surface and incubated statically at 28°C for 21 days.

4. Assay of Chitinase Inhibitory Activity
Inhibitory activity against chitinase from S. litura was determined as previously described.⑨ Inhibitory assay against Streptomyces griseus chitinase was carried out in a similar manner to the inhibitory assay against S. litura chitinase, except that 111 mM sodium acetate buffer (pH 5.0) was used instead of 111 mM citrate-phosphate-borate buffer (pH 7.0).

5. Screening of Fungal Cultures
Culture filtrates and cell extracts of isolated strains were screened for insect chitinase inhibitory activity. The culture broth of 776 fungal strains grown by static culture in PDM liquid medium were filtered through cotton to separate the cells from the supernatant. The cells were soaked in acetone-methanol (1:1) at room temperature for 3-4 days, and the extract was concentrated to dryness. The dried extract was suspended in 25 μl of methanol. To the suspension, 475 μl of water was added with stirring. A portion (200 μl) of the suspension of cell extract and culture filtrate was tested for S. litura chitinase inhibitory activity.

6. Fermentation for the Selection of Highly Productive Strains
Mycelia (2-5 mm square) of each active strain grown on PSM agar slants were scraped from the slant surface and added to 10 ml of PDM, SD, YpsS, YE and Glc-Cz liquid media in test tubes (ø25 mm). An inoculated medium was incubated statically at 28°C for 21 days. Culture filtrates were tested for inhibitory activity against S. litura chitinase. YE liquid medium (pH 5.6): glucose 20.0, yeast extract (Nacalai Tesque, Kyoto, Japan) 5.0, KH₂PO₄ 0.5, K₂HPO₄ 0.5, MgSO₄•7H₂O 0.5 (g/l water).

7. Large-Scale Fermentation
Active strains were cultured on a reciprocal shaker (320 strokes/min) in 10 ml of fermentation medium at 28°C until cells reached a high density (for 3-8 days). The culture broth was transferred to a 700-ml flat-bottom (8 cm × 18 cm) flask containing 150 ml of each optimized medium. The inoculated medium was incubated statically at 28°C for 12-36 days. The culture broth was harvested every 3 days and the culture filtrate was tested for S. litura chitinase inhibitory activity.

8. Characterization of Active Compounds Produced by Active Strains
Molecular weights of active compounds were estimated using Centricon ultrafiltration membrane cartridges (3, 10, 30, 100 kDa-cut off for globular proteins, Millipore, MA). To evaluate thermal stability, the culture filtrates of active strains were heated at 100°C for 10 min. Culture filtrates were also treated with Pronase P and glycosidases. The pronase reaction mixture contained Pronase P (1 mg/ml), culture filtrates (~400 μl), 5 mM Tris-HCl buffer (pH 7.8) and 1 mM CaCl₂. The glycosidase reaction mixture contained glycosidases (1 mg/ml), culture filtrates (~400 μl) and 5 mM acetate buffer (pH 4.7). The reaction mixtures were incubated at 37°C for 24 hr. Ionic characters of active compounds were determined by ion exchange chromatography. SP-Toyopearl 650 M (Tosoh Co., Tokyo) or Dowex 50W × 8 (Dow Chemical Co., Midland, MI) and Q-Sepharose XL (Amersham Pharmacia Biotech, Uppsala, Sweden) or AG1 × 8 (Bio-Rad, Richmond, CA) were used as cation and anion exchangers, respectively.

RESULTS AND DISCUSSION

1. Screening of Fungal Cultures
Seven hundred and seventy-six fungal strains were isolated from soil samples or plant leaves by the methods described in Materials and Methods. After these isolated strains were cultivated statically in test tubes containing PDM liquid medium, both their culture filtrates and cell extracts were tested for S. litura chitinase inhibitory activity. The activity was found only in culture filtrates, not in cell extracts. Culture filtrates of 27 strains showed inhibition of 70-100%.

The productivity of the inhibitors for these 27 strains was tested using the culture filtrates recultivated in 5 different media, PDM, SD, YpsS, YE and Glc-Cz. The 5 strains, TNPT116-Cz, F76, F77, AKF46 and HUF45, were selected as active strains. The strains TNPT116-Cz, F76, F77, AKF46 and HUF45 exhibited the highest production levels of chitinase inhibitors using YpsS, PDM, YE, SD and SD among 5 different media used, respectively.

The incubation time for the highest level of production of chitinase inhibitors by these 5 active strains was determined using 700 ml flat-bottom culture flasks containing each optimized medium described above. The strains TNPT116-Cz, F76, F77 and AKF46 showed the strongest inhibitory activity after 24, 24, 30 and 33 days of cultivation, respectively (Fig. 1, Table 1). The strains F77 and AKF46 showed another production maximum after 21 days of
cultivation, indicating the possibility of the production of additional chitinase inhibitors. Strain HUF45 did not show a stable productivity under the static conditions, but exhibited stable and strong activity under the shaking conditions. The highest activity was obtained by cultivation at 28°C and 123 strokes/min for 7 days in a 500 ml Sakaguchi-flask containing 40 ml of SD medium (Table 1).

2. Characterization of Active Compounds

The active compounds produced by the 5 active strains were partially characterized and compared with known inhibitors. The culture filtrates of these 5 active strains cultivated under the optimized conditions exhibited strong inhibitory activity against insect chitinase (ID₅₀ as a volume of culture filtrate: < 20 μL), but had no activity against Streptomyces griseus chitinase (ID₅₀ as a volume of culture filtrate: > 200 μL) (Table 1). These filtrates, therefore, exhibited over 10 times more inhibitory activity against insect chitinase than against microbial chitinase. Allosamidin, a potent chitinase inhibitor, exhibited an IC₅₀ of 50 and 410 nM against S. litura and S. griseus chitinases, respectively, in our assay system. These results suggested that the active compounds produced by these 5 fungal strains had a higher selectivity against insect chitinase than allosamidin.

The physicochemical characters of these compounds were further examined by the following methods: solvent fractionation, ultrafiltration, enzyme treatment, best treatment and ion exchange chromatography. Active compounds of all 5 active strains were found to be water-soluble by the solvent fractionation with ethyl acetate followed by 1-

![Graph showing the production of insect chitinase inhibitors by fungal strains over days](image)

**Fig. 1. Large-scale production of insect chitinase inhibitors by fungal strains, TNPT116-Cz (●), F76 (○), F77 (□) and AKF46 (▲), in static culture. ID₅₀ is expressed as a volume of culture filtrate.**

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<th>Table 1. Cultivation conditions and chitinase inhibitory activity of five strains</th>
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<td><strong>Optimized culture conditions</strong></td>
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<tr>
<td>Media</td>
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<td>TNPT116-Cz</td>
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*As a volume of culture filtrate.

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<th>Table 2. Comparison and characterization of active substances produced by fungal strains</th>
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<td><strong>Molecular weight</strong></td>
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<td><strong>Protein P</strong></td>
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<td>TNPT116-Cz</td>
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*As a globular protein.

N.E.: could not be evaluated (due to sensitivity to heat treatment at 100°C for 10 min) for the inactivation of Protein P.
butanol. They were also found to be macromolecules by the fractionation with ultrafiltration. The estimated molecular weights of the active compounds produced by strain AKF46 and other strains ranged from 3000 to 10,000 and from 30,000 to 100,000 as globular proteins, respectively (Table 2). Since the water-soluble and macromolecular properties suggested that the active compounds were proteins or polysaccharides, the culture filtrates were treated with Pronase P and glycosidases from *Turbo cornutus*. The active compounds of the strains except for F77 were inactivated by glycosidase treatment, but not by pronase treatment (Table 2). The active compound of strain F77 was resistant to glycosidase treatment, but completely inactivated by heat treatment at 100°C for 10 min, while those of other strains were resistant to heat (Table 2). Based on the behavior toward both anion- and cation-exchange resins, the active compounds of strains F77, AKF46 and HUF45, and TNPT116-Cz and F76 were found to be amphoteric, anionic and nonionic, respectively (Table 2).

These results suggested that strains TNPT116-Cz and F76 produced neutral polysaccharide inhibitors, and that strains F77, AKF46 and HUF45 produced proteinous, acidic oligosaccharide and acidic polysaccharide inhibitors, respectively. Since the insect chitinase inhibitors reported so far were low molecular weight compounds (MW < 1000),1-6 all of the 5 active strains were considered to produce novel inhibitors. These structurally different inhibitors may have different modes of action, hence, they are expected not only to be potential agents for insect control and preventing pesticide resistance, but also to become helpful tools in the study of the roles and biological functions of chitinase.

The present study indicates the structural diversity of insect chitinase inhibitors, which suggests various mechanisms of chitinase inhibition. Furthermore, it is implied that insect chitinase inhibitors have additional biological functions in their fungal producers. Further purification and structural elucidation of the active compounds are in progress.

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REFERENCES