# A Potent Insect Chitinase Inhibitor of Fungal Origin

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A water-soluble polysaccharide was isolated from the culture filtrate of a fungal strain, *Sphaeropsis* sp. TNPT116-Cz, as a novel insect chitinase inhibitor. It was purified to chromatographic homogeneity by ethanol precipitation, anion-exchange and gel filtration chromatography. Its molecular weight was estimated to be 16 kDa by gel filtration HPLC. Monosaccharide analysis showed that it contained glucose, galactose, *N*-acetylglucosamine and a deoxysugar. This polysaccharide showed potent and specific inhibitory activity against *Spodoptera litura* chitinase with an $IC_{50}$ value of 28 nM.

*Key words*: Chitinase Inhibitor, Extracellular Polysaccharide, *Sphaeropsis* sp.

## Introduction

Chitin is a structural component of the cuticle and the peritrophic membrane in the mid-gut of insects, and strict regulation of its metabolism is essential for the normal growth of insects. Chitinase is a key enzyme in degradation of chitin, and hence its inhibitors are expected to be biorational insect growth regulators or probes for studying the catalytic mechanism of chitinase and the chitinolytic system. Since only a few chitinase inhibitors such as allosamidins (Sakuda *et al.*, 1987) and less active compounds (Arai *et al.*, 2000; Izumida *et al.*, 1996; Kato *et al.*, 1995; Shiomi *et al.*, 2000; Tabudravu *et al.*, 2002) have been reported so far, novel chitinase inhibitors are desired.

We have been screening for insect chitinase inhibitors of fungal origin using the *Spodoptera litura* (common cutworm) chitinase inhibitory assay (Kawazu *et al.*, 1996; Nitoda *et al.*, 1999; Nitoda *et al.*, 2003). In the previous paper (Nitoda *et al.*, 2003), 5 fungal strains were found to produce at least 4 distinct novel inhibitors of high molecular weight in their culture filtrates. Among them, strain TNPT116-Cz showed the most stable and highest production. Results from preliminary fractionation and enzyme treatments of the culture filtrate suggested that the active compound produced by this strain was a water-soluble neutral polysaccharide. In this paper, the purification and characterization of the insect chitinase inhibitor produced by this strain is described.

## Materials and Methods

### *General experimental procedures*

$^1$H NMR spectra were recorded on a Varian VXR-500 instrument at 500 MHz. GC-MS analysis was carried out on JEOL Automass 20 coupled with Hewlett-Packard 5890 gas chromatograph (capillary column: J & W Scientific DB-1, 0.25 mm × 30 m, 0.25 $\mu$m film thickness). Helium was used as a carrier gas. A temperature program with an initial temperature of 100 °C holding for 2 min, then increasing to 200 °C at a rate of 20 °C/min with a final hold for 30 min was employed. Allosamidin was a gift from Dr. S. Sakuda (The University of Tokyo). Chitinase solution from *S. litura* pupae was prepared as previously described (Kawazu *et al.*, 1996). Chitinase from *Streptomyces griseus* (a soil actinomycete) was purchased from Sigma (St. Louis, MO, USA). All other chemicals were commercially available.

### *Identification of the strain TNPT116-Cz*

Fungal identification methods were based on the morphology of the fungal culture, the mechanism of spore production and the characteristics of the spores (Burnet and Hunter, 1987; Kiffer and Morelet, 2000; Kobayashi *et al.*, 1992; Sutton, 1980).

### *Assay method of chitinase inhibitory activity*

Inhibitory activity against chitinase from *Spodoptera litura* and *Streptomyces griseus* was determined as previously described (Kawazu *et al.*, 1996; Nitoda *et al.*, 1999).

### *Fermentation and purification*

The fungal strain TNPT116-Cz was maintained in slant tubes of a potato-sucrose-malt extract

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(PSM) agar medium as previously described (Nitoda *et al.*, 2003). A piece (2–5 mm square) of the mycelium from the mature slant culture of the strain TNPT116-Cz was inoculated into a test tube (∅ 25 mm) containing 10 ml of YpSs medium [starch 1.5%, yeast extract (Nacalai Tesque, Japan) 0.4%, $K_2HPO_4$ 0.1% and $MgSO_4 \cdot 7\ H_2O$ 0.05%, adjusted to pH 5.6 before sterilization] for preculture. The tube was incubated at 28 °C on a reciprocal shaker (320 strokes/min) for 4 days. This culture was inoculated into a 700-ml flat-bottom (8 cm × 18 cm) flask containing 150 ml of YpSs medium. The flask was incubated stationarily at 28 °C for 24 days.

Since the active compound was suggested to be a neutral polysaccharide, the fractionation was based on carbohydrate content determined by the resorcinol-sulfuric acid method (Monsigny *et al.*, 1988) in addition to *Spodoptera litura* chitinase inhibitory activity. The culture broth was filtered through Advantec Toyo filter paper No. 2 (Toyo Roshi Kaisha, Ltd., Tokyo, Japan) to remove mycelia. Four volumes of cold ethanol were added to the culture filtrate, and the mixture was kept overnight at 4 °C. The precipitated material was collected by centrifugation (60 min at 4600 × *g*) and resuspended in 80% ethanol. The suspension was kept overnight at 4 °C, and the precipitated material was collected by centrifugation (60 min at 4600 × *g*) and lyophilized to obtain crude active fraction. This fraction was dissolved in $H_2O$ and applied to a column of DEAE-Sephacel (Amersham Pharmacia Biotech, Uppsala, Sweden) in the borate form and eluted sequentially with water, 0.05 M and 0.08 M sodium borate, and 0.5 M NaOH. Carbohydrate-containing fractions were combined, dialyzed and assayed for chitinase inhibitory activity. The active fraction eluted with 0.05 M sodium borate was purified by repeated gel filtration on a Biogel-P100 (Bio-Rad, Hercules, CA, USA) column by eluting with $H_2O$.

### *Determination of molecular weight by gel filtration HPLC*

The purified polysaccharide was chromatographed on a TSKgel G4000 PWXL (7.8 × 300 mm, Tosoh, Tokyo, Japan) by eluting with $H_2O$ at a flow rate of 0.5 ml/min at 40 °C. The column was calibrated with a series of pullulans (molecular weights of $5.9 \times 10^3$, $2.3 \times 10^4$, $4.7 \times 10^4$, $1.1 \times 10^5$, and $2.1 \times 10^5$ Da, gifts from Hayashibara Biochemical Laboratories, Inc., Okayama, Japan). Eluted material was detected by a refractive index detector (HITACHI L-3300 RI Monitor).

### *Compositional analysis*

The purified polysaccharide was hydrolyzed in 2.0 M trifluoroacetic acid for 1.5 h at 115 °C. Alditol acetate derivatives prepared according to the method of Englyst and Cummings (1984) were analysed by GC-MS.

## Results and Discussion

### *Identification of the strain TNPT116-Cz*

Strain TNPT116-Cz was isolated from a leaf of *Cleyera japonica* (Theaceae) in Okayama, Japan. Morphological properties were examined after incubation at 25 °C up to 3 weeks on Bacto potato dextrose agar (PDA) (BD Diagnostic Systems), Bacto oatmeal agar (OA) (BD Diagnostic Systems), and 2% Bacto malt extract (BD Diagnostic Systems, Sparks, MD, USA) added with 1.5% agar (MEA). By the incubation at 25 °C for a week, the strain grew to form floccose white colonies with diameters of 65–70, 40, and 50–60 mm on PDA, OA, and MEA, respectively. Then, the strain on each medium began to form black conidioma. Neither reverse coloration nor soluble pigment production was observed. Microscopic observation showed that the strain produces unicellular, ovoid to oval shaped conidia blastically developed within a pycnidial conidioma. According to these morphological features, the strain was supposed to be a fungus belonging to the genus *Sphaeropsis* and named *Sphaeropsis* sp. TNPT116-Cz.

### *Purification and characterization of the insect chitinase inhibitor*

The polysaccharide (FPS-1) was obtained as a colorless amorphous powder by the purification from the culture filtrate, and a 400-fold increase in specific activity was achieved with 5.9% overall yield of activity. The molecular weight of FPS-1 was estimated as 16 kDa by gel filtration HPLC. GC-MS analysis on the alditol acetate derivatives of FPS-1 revealed major 3 peaks with retention times of 16.6, 16.8, and 22.6 min, values corre-

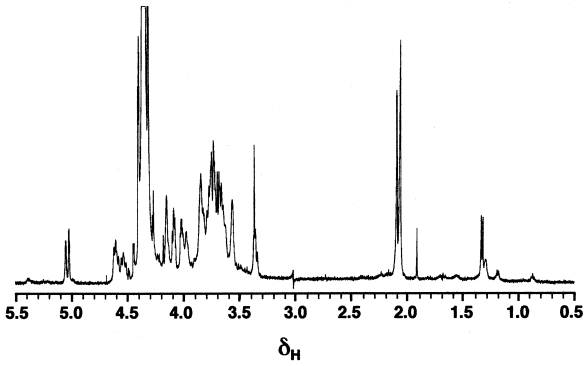


Fig. 1. $^1$H NMR (500 MHz, 70 °C) spectrum of FPS-1.

sponding to those for alditol acetate derivatives of authentic glucose (Glc), galactose (Gal), and *N*-acetylglucosamine (GlcNAc) standards, respectively. From the integrated peak area, FPS-1 was shown to contain GlcNAc, Glc, and Gal in the approximate ratio of 2:1:1 as major constituents. The $^1$H NMR spectrum of FPS-1 is shown in Fig. 1. This spectrum revealed at least two anomeric proton signals at $\delta$ 5.02 and 5.05. The singlets at $\delta$ 2.06 and 2.09 due to the acetyl group protons indicated the presence of two *N*-acetylglucosamine residues. The doublet at $\delta$ 1.33 ($J$ = 6.7 Hz) suggested that FPS-1 contained a deoxysugar residue. Although further structural analyses are required, these results indicated that FPS-1 was an unique polysaccharide never been reported as a fungal polysaccharide as far as we know.

FPS-1 inhibited *S. litura* chitinase with an $IC_{50}$ value of 28 nM (Fig. 2). Allosamidin, the most potent chitinase inhibitor, showed an $IC_{50}$ value of 50 nM in the same assay (Nitoda *et al.*, 1999). Therefore, FPS-1 has insect chitinase inhibitory activity comparable to that of allosamidin. FPS-1 showed no significant activity ($IC_{50}$ > 1.9 $\mu$M) in inhibitory assay against *Streptomyces griseus* chitinase, indicating that it is a specific inhibitor against insect chitinases.

Several polysaccharides have been reported to inhibit endoglycosidases. Amylostatins (Fukuhara *et al.*, 1982) and bacterial lipopolysaccharides (Ohno and Morisson, 1989) are inhibitors of $\alpha$-glucosidase and lysozyme, respectively. But chitinase-inhibiting polysaccharide has not been reported. In addition, chitinase inhibitors reported so far are low-molecular compounds ($M_r$ < 1000). Therefore, FPS-1 is a new type of chitinase inhibitor. Its inhibitory mechanism and biological role are now under investigation.

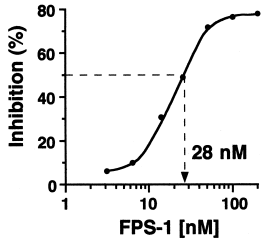


Fig. 2. Inhibitory activity of FPS-1 against *S. litura* chitinase. In the chitinase inhibitory assay, the degradation of colloidal chitin was monitored by a decrease in turbidity (absorbance at 610 nm). The test mixture was composed of 20 $\mu$l of the colloidal chitin suspension (6 mg/ml in $H_2O$), 180 $\mu$l of the enzyme solution in a 111 mM citrate/phosphate/borate buffer (pH 7.0), and 200 $\mu$l of an aqueous solution of FPS-1. The absorbance at 610 nm ($A_{610}$) of the test mixture was measured before and after incubation for 60 min at 37 °C. The amount of enzyme was adjusted so that the decrease in $A_{610}$ of the control mixture containing $H_2O$ instead of FPS-1 solution was 0.3. Inhibition in percent was calculated by the equation

$$\%\text{Inhibition} = 100 - (A/B) \times 100$$

where A and B are the decreases in $A_{610}$ of the test mixture and the control mixture, respectively. Assay results were represented as mean values of triplicate determinations. The rate of the non-inhibited control was 8.4 nmol/min·ml as the liberation of *N*-acetylglucosamine from colloidal chitin.

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