

SEARCH FOR MICROBIAL INSECT GROWTH REGULATORS

II.[†] ALLOSAMIDIN, A NOVEL INSECT CHITINASE INHIBITORSHOHEI SAKUDA^{††}, AKIRA ISOGAI, SHOGO MATSUMOTO and AKINORI SUZUKI*

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Allosamidin, a novel insect chitinase inhibitor, was isolated from the mycelium of *Streptomyces* sp. It showed strong inhibitory activity against the chitinases of the silkworm, *Bombyx mori*, *in vitro*, and also insecticidal activity by preventing its ecdysis *in vivo*.

Chitin is a polymer of β -1,4 linked *N*-acetyl-D-glucosamine, whose natural occurrence is specialized and which is a main component of cuticle in insects. Its turnover is regulated mainly by two enzymes, chitin synthase for its synthesis and chitinase for its degradation, and it plays an important part in ecdysis. Therefore it has been thought that inhibitors of these enzymes should be good leads for designing new types of insect growth regulators²⁾. To the present time, polyoxin and nikkomycin are known as inhibitors of chitin synthase³⁾ and actually inhibit the insect ecdysis *in vivo*, while an effective inhibitor of insect chitinase has not yet been found. From this viewpoint we have been searching for insect chitinase inhibitors among the metabolites of actinomycetes and isolated the potent inhibitor, termed allosamidin, from the mycelial extract of *Streptomyces* sp. No. 1713. Allosamidin markedly inhibited the chitinases of the silkworm, *Bombyx mori*, *in vitro* and also prevented its ecdysis *in vivo*. In this paper we wish to report a bioassay method for detection of chitinase activity, and production, isolation and insecticidal activities of allosamidin. Structural elucidation of allosamidin and its inhibitory activities against insect chitinases purified from *Bombyx mori* and other chitinolytic enzymes have been reported elsewhere^{4,5)}.

Materials and Methods

Preparation of Insect Chitinase

In the chitinase assay the crude enzyme was used. The crude enzyme solution was prepared from the pupal alimentary canal of the silkworm, *Bombyx mori*, by the method of KIMURA⁶⁾. In brief, the alimentary canal and the gut contents were collected from the pupa just after pupation and homogenized in 50 mM citric acid - Na₂HPO₄ buffer (pH 5.0). The homogenates were centrifuged and the supernatant was mixed with ammonium sulfate to give 70% saturation. After centrifugation, the precipitate was dissolved with the same buffer at the concentration of 1.5 pupae per ml, and the solution thus obtained was stored at -80°C and used in the chitinase assay as the enzyme solution.

Chitinase Assay Method

The reaction of the chitinase assay was performed in the mixture containing the enzyme solution 0.1 ml, 100 mM citric acid - Na₂HPO₄ buffer (pH 5.0) 0.9 ml, water 0.8 ml, methanol 0.2 ml and the chromogenic substrate, γ -chitin red 5 mg⁷⁾. An inhibitor was dissolved in the water or methanol

[†] Part I: See ref 1.

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portion according to its solubility. In the course of screening among the metabolites of microorganisms, a culture filtrate or a mycelial methanol extract was added instead of the water or methanol portion. The reaction mixture was incubated at 35°C overnight in the vial (27.5×55 mm). The red color, of the supernatant obtained by centrifugation, was measured at 510 nm before ($OD_{510}B$) and after ($OD_{510}A$) incubation. The chitinase activity shown as the increasing value of the absorbance ($\Delta A = OD_{510}A - OD_{510}B$) during incubation, was approximately constant (+0.12~0.14) under these assay conditions without an inhibitor.

Production and Isolation of Allosamidin

The allosamidin-producing microorganism (No. 1713) was isolated from soil in Kanagawa Prefecture, Japan, and identified as *Streptomyces* sp.

Streptomyces sp. No. 1713 was cultured in a Bennet medium consisting of glucose 1.0%, peptone 0.2%, meat extract 0.1% and yeast extract 0.1% (pH 7.2) in a 600-liter jar fermentor. Fermentation was carried out at 26.5°C for 5 days under aeration (300 liters/minute) and agitation (100 rpm).

The culture broth (300 liters, pH 7.28) was filtered through Celite (Kanto Chemical, Japan) and the mycelial cake was extracted with methanol (70 liters) and 80% methanol (60 liters) successively. The extracts were combined and concentrated *in vacuo* to an aqueous solution (15 liters), which was then adsorbed on a charcoal (Charcoal, Activated, obtained from Wako Pure Chemical Ind., Japan) column (7.0×40 cm). After washing with water, the active principle was eluted with 50% ethanol. The eluate (7.5 liters) was concentrated *in vacuo* to a half volume and then applied to a column of Dowex-50 (H⁺ form, 8.4×9.0 cm). The column was washed with water and eluted with 1 M AcONH₄ (3 liters). The 1 M AcONH₄ eluate was adsorbed on a charcoal column (2.8×19 cm) for desalting and eluted with 50% ethanol (1 liter), which was then concentrated to a half volume. After being diluted 3-fold with water and adjusted to pH 3.9 with acetic acid, the active solution was applied to a SP-Sephadex C-25 column (2.8×30 cm) pre-equilibrated with 50 mM AcONH₄ - AcOH (pH 5.0) and the column was eluted with the same buffer. The eluting fractions were analyzed by bioassays and HPLC assays using an Asahipak ES-502C column (7.6×100 mm) and then the active eluate was combined and lyophilized. The active fraction thus obtained afforded 280 mg of allosamidin as white crystalline powder from water.

Assay Method of Insecticidal Activity

Larvae of *Bombyx mori* and *Leucania separata* were reared on artificial diet under controlled temperature and light conditions. Allosamidin was dissolved in 0.1 N acetic acid and the solution was injected to the haemocoel of the assay larvae. After injection, the test larvae were reared to the next molting stage and the effect of allosamidin on their development and ecdysis was observed. EI_{50} (50% ecdysis inhibition) values were defined as the minimal doses inhibiting the molting process in half of the test insects in an assay.

Results

Chitinase Assay

Fig. 1 shows a typical dose-response curve obtained in the chitinase assay at various concentrations of allosamidin. The value of the ΔA was decreasing as the inhibitory activity was increasing in the curve. Allosamidin completely inhibited the chitinases of the silkworm at the concentration of more than 1 μ g per ml in this assay system.

Production, Isolation and Characterization of Allosamidin

A typical time course of allosamidin production by *Streptomyces* sp. No. 1713 in a 500-ml Erlenmeyer flask containing 100 ml Bennet medium, which was shaken on a rotary shaker at 120 rpm and 26.5°C, is shown in Fig. 2. A chitinase inhibitory activity was not observed for 3 days' fermentation but suddenly appeared at day 4 correlated with change in the color of the culture broth from yellow

to brown, and reaching a maximum after 6 days' fermentation.

The isolation procedure of allosamidin is summarized in Fig. 3. Fig. 4 shows HPLC analysis of allosamidin. Characterization of allosamidin was shown in a separate paper⁴⁾.

Fig. 1. The effect of allosamidin on the chitinases of the silkworm, *Bombyx mori*.

* ΔA is defined as mentioned in Materials and Methods.

---: ΔA value of enzyme control without inhibitor.

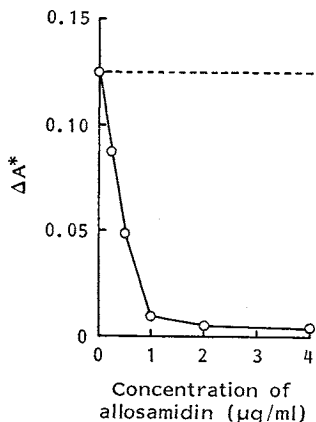


Fig. 3. Isolation procedure of allosamidin produced by *Streptomyces* sp. No. 1713.

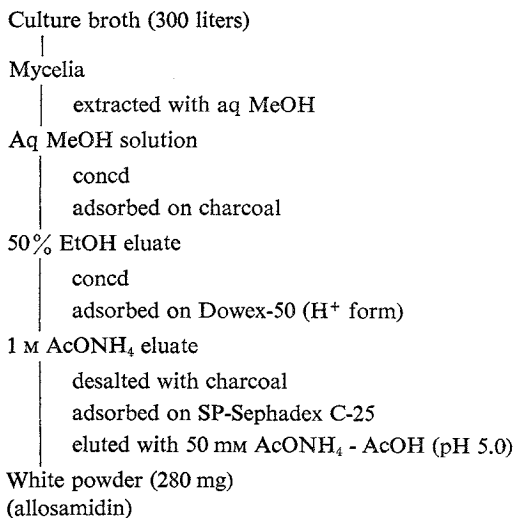


Fig. 2. The time course of the production of allosamidin by *Streptomyces* sp. No. 1713.

ΔA value was measured with the mycelial methanol extract of the culture broth at the dose of 0.25 ml equiv (indicated as ○). ●: pH.

---: ΔA value of enzyme control without inhibitor.

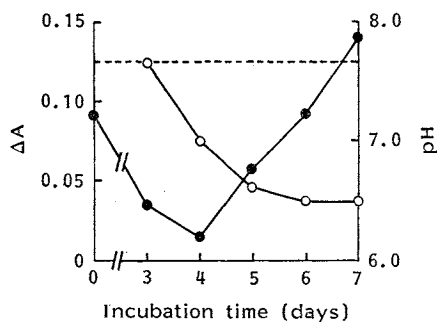


Fig. 4. HPLC analysis of allosamidin.

The peak indicated by arrow is allosamidin.

HPLC conditions: Column; Asahipak ES-502c (7.6×100 mm), mobile phase; 10 mM AcONH₄ - NH₄OH (pH 8.9), flow rate; 1.0 ml/minute, temperature; 40°C, detector; UV-210 nm, sample; one of the eluting fractions from SP-Sephadex C-25 column.

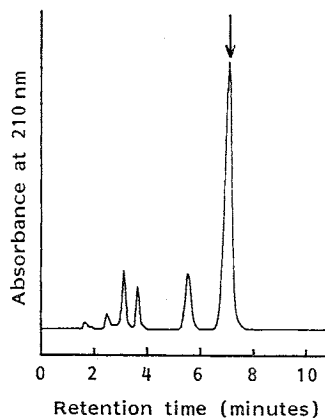


Fig. 5. The structure of allosamidin.

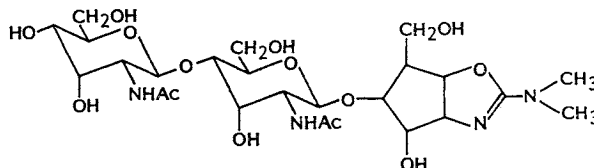
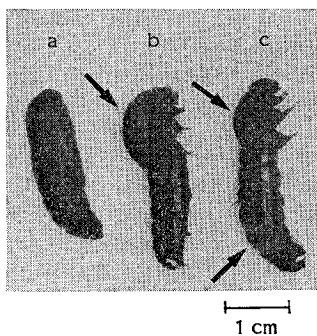


Fig. 6. The effect of allosamidin ($16 \mu\text{g}$) on the pupal ecdysis of *Leucania separata* larvae.

a: Normal pupa. Pupal cuticle was partially observed (\rightarrow), but the larval cuticle shedding was mostly inhibited.



and cyclopentane rings. *N*-Acetyl-*D*-allosamine is a C-3 epimer of *N*-acetyl-*D*-glucosamine and hitherto unknown in nature.

Insecticidal Activities of Allosamidin

Allosamidin showed an insecticidal activity by inhibiting the ecdysis. When allosamidin at a dose of more than $4 \mu\text{g}$ was injected to the 4th instar larva of the silkworm, *Bombyx mori*, at the feeding stage, all test larvae failed in larval ecdysis to the next stage and death followed. No effect of allosamidin on the development of the test larvae during the feeding stage to the molting stage was observed, except the growth delay which was induced when it was applied immediately after ecdysis. The ecdysis inhibitory activity of allosamidin was also tested in the 5th and last instar larvae of the common armyworm, *Leucania separata*. Allosamidin caused these larvae to fail in the larval and pupal ecdysis respectively (Fig. 6). The values of EI_{50} for larvae of *B. mori* and *L. separata* are listed in Table 1.

Discussion

Although it has been proposed that an insect chitinase inhibitor should be a promising candidate for a new type of insect growth regulator, an effective inhibitor has not yet been found. To the present time, some *N*-acetylglucosamine analogs have been reported as inhibitors of chitinases⁸⁾. The sugar analogs inhibited the chitinases from the tobacco hornworm, *Manduca sexta*, but they could only induce a slight growth delay by injection to the insect larvae⁸⁾. Avermectin was once suggested to inhibit a microbial chitinase⁹⁾, but that was denied later by the experiment using pure avermectin¹⁰⁾. It showed no inhibitory activity against the *M. sexta* chitinase¹¹⁾.

Because of the lack of an adequate screening system, no attempt has been made to search for chitinase inhibitors. We have now developed a simple screening system for chitinase inhibitors using the insect enzymes from the silkworm. Although the crude enzymes are used in the assay, it is sufficient to search for effective and specific inhibitors against the insect chitinases. Allosamidin strongly inhibited the chitinases of *Bombyx mori*, whereas the effect on the microbial chitinases of *Streptomyces griseus* and *Serratia marcescens* was very weak and no effect was observed on the plant chitinase of yam⁹⁾. The specificity of allosamidin for different types of chitinases suggests the necessity of using the insect enzymes to screen chitinase inhibitors as insect growth regulators. Allosamidin is the first compound isolated by using this assay system, demonstrating that insect chitinase inhibitors are possible insect growth regulators.

Table 1. Ecdysis inhibitory activity of allosamidin to larvae of *Bombyx mori* and *Leucania separata*.

Species	Larval stage of application	EI_{50} (μg)
<i>Bombyx mori</i>	4th instar	2
<i>Leucania separata</i>	5th instar	4
	Last instar	8

The structure of allosamidin was determined as shown in Fig. 5 by acid hydrolysis experiments and analysis of 2D NMR spectra of allosamidin and its derivatives⁴⁾. It is a unique pseudotrisaccharide consisting of *N*-acetyl-*D*-allosamine and a novel aminocyclitol derivative, termed allosamizoline, which has aminooxazoline

Acknowledgment

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