

A Stable Crude Chitinase Solution from *Spodoptera litura* Pupae and A Search for Its Inhibitors

Kazuyoshi Kawazu, Shigehiko Ohnishi, Hiroshi Kanzaki, and Akio Kobayashi*

Laboratory of Bioresources Chemistry, Faculty of Agriculture, Okayama University, Okayama 700, Japan

Z. Naturforsch. **51c**, 738–742 (1996); received June 3/June 28, 1996

Chitinase Inhibitory Assay, Screening, Loculoascomycete Metabolites

A convenient method for preparing a stable chitinase solution from a pest insect, *Spodoptera litura*, and a bioassay method for insect chitinase inhibitors using this enzyme solution were developed. Allosamidin, the first insect chitinase inhibitor, exhibited an IC_{50} of 0.2 μM in the present *Spodoptera litura* chitinase assay, whereas allosamidin was reported to exhibit an IC_{50} of 0.7 μM in the *Bombyx mori* chitinase assay. Thus, the present assay using *Spodoptera litura* chitinase was 3.5-fold more sensitive than the *B. mori* chitinase assay. Screening of 300 supernatants of microbial cultures for chitinase inhibitors using the *Spodoptera litura* chitinase assay found 13 active supernatants.

Introduction

For sustainable agricultural production, the development of selective and biorational insect growth regulators which are environmentally gentle is desired.

Molting, a physiological phenomenon characteristic of insects, is one of the targets of selective regulation of insects. Compounds inhibiting molting are expected to be ineffective against non-molting creatures. These compounds may be found more precisely and intensively by an inhibitory assay of molting enzymes than by an *in vivo* assay of insect ecdysis inhibition.

During molting, insects use chitinase to degrade chitin of the old cuticle to *N*-acetylglucosamine, from which they simultaneously synthesize the chitin of a new cuticle by chitin synthase. Therefore, inhibitors of chitinase or chitin synthase should inhibit insect cuticle formation. Several compounds, such as nikkomycin and polyoxins, are known as chitin synthase inhibitors, but only allosamidin and its related compounds (Isogai *et al.*, 1989; Nishimoto *et al.*, 1991; Sakuda *et al.*, 1986, 1987a, 1987b) are known as chitinase inhibitors. To find novel

insect chitinase inhibitors, a number of materials need to be screened efficiently by a simple bioassay using a stable insect chitinase solution. Sakuda *et al.* (1986) developed an insect chitinase inhibitory assay using *Bombyx mori* chitinase and used it to isolate allosamidin, the first insect chitinase inhibitor. The chitinase solution used in the assay was prepared from laboriously excised alimentary canals of *Bombyx mori* pupae (Sakuda *et al.*, 1987a).

In the present study, we have developed a simple procedure for the preparation of a stable crude chitinase solution from the whole body of a pest insect, *Spodoptera litura*, which was not used before as a source of chitinase. We also describe a convenient and rapid screening method for chitinase inhibitors using this enzyme solution.

Materials and Methods

Chemicals

Colloidal chitin was prepared from powdered chitin treated with conc. H_2SO_4 (Jeuniaux, 1958, 1966). Allosamidin was a gift from Dr. S. Sakuda. All other chemicals were commercially available.

Spodoptera litura for enzyme preparation

Eggs of *Spodoptera litura* were supplied by the Research Center of Mitsubishi Chemical Cooperation, Yokohama, Japan. Larvae were reared on an artificial diet, Insecta LF (Nihon-Nousan Kogyo,

* Present address: Department of Biotechnology Faculty of Engineering, Osaka University, Yamada-oka, Suita, Osaka 565, Japan

Reprint requests to Prof. Dr. K. Kawazu. Telefax: +81-86-254-0714.



Yokohama, Japan) at 25 °C under 16-h light and 8-h dark conditions. Insects were collected at various stages as follows.

a) Sixth-instar larvae just after exuviation: Fifth-instar larvae were transferred to 9-cm petri dishes (one larva per dish) containing the artificial diet on a piece of filter paper (No.2, Advantec Toyo Kaisha, Tokyo, Japan). The growth of the larva was checked every morning and evening. The sixth-instar larva just after exuviation was detected by a new slough on the filter paper.

b) Sixth-instar larvae in the wandering stage: These were the larvae that were leaving the diet and getting into the peat moss (Canadian Supreme Products, New Brunswick, Canada).

c) Pharate pupae: These were sixth-instar larvae that had shrunk in body length to about 2.5 cm in the peat moss.

d) Young pupae: These were the pupae just after pupation.

After collection the above insects were stored at -80 °C until preparation of the enzyme solution.

Preparation of a stable S. litura chitinase solution

Preparation of the enzyme solution was carried out at temperatures between 0 and 4 °C. Ten g of the insect tissue was homogenized in a buffer (see below) containing 0.01% phenylthiourea in a mortar. The homogenate was filtered through gauze and the filtrate was centrifuged at 20,000 x g for 30 min. The supernatant could be used directly as the enzyme solution, but ultracentrifugation at 200,000 x g for 15 min was found to make the solution more stable.

Chitinase assay

The chitinase activity against colloidal chitin was determined by a turbidimetric method (Yabuki *et al.*, 1986) with some modifications. In this method, the degradation of colloidal chitin was monitored by a decrease in light scattering at 610 nm. The reaction mixture was composed of 32 µl of a 1.0% colloidal chitin suspension and 368 µl of the enzyme solution in a 25 mM buffer. An enzyme-free mixture and a substrate-free mixture served as controls. Before and after incubation for 60 min at 37 °C, the A_{610} values were measured with a spectrophotometer (Ubest-30, JASCO, Japan), and the decrease in A_{610} after incubation was cal-

culated. The decrease in A_{610} of the mixture was corrected by subtracting the corresponding decreases in A_{610} of the two controls.

To determine a suitable buffer for the enzyme extraction, enzyme solutions were prepared by homogenizing young pupae in buffers of various pH values (25 mM citrate buffer of pH 5.0, 25 mM citrate-phosphate-borate buffer of pH 7.0, or 25 mM borate buffer of pH 9.0). The homogenates were centrifuged at 20,000 x g for 30 min and the supernatants were tested for activity.

To determine the optimum pH of the enzyme reaction, the chitinase activity was measured in citrate-phosphate-borate buffers of different pHs. The enzyme activity in buffers having a pH higher than 6.5 was determined by the method described above. Below pH 6.5, the activity was determined by the increase in the amount of reducing sugar, because the activity could not be determined by the turbidimetric method due to deep turbidity of the mixture. The amount of reducing sugar in the supernatant of the reaction mixture before and after incubation for 60 min at 37 °C was determined by Schales' procedure with some modifications (Imoto and Yagishita, 1971) as follows. The mixture was centrifuged at 10,000 x g for 15 min. One hundred µl of the supernatant was mixed with 200 µl of 1.37 mM $KFe(CN)_6$ in 0.5 M Na_2CO_3 in a test tube and heated in boiling water for 15 min. After cooling, A_{420} of the mixture was measured. The difference (ΔA_{420}) between the A_{420} values of the mixture and a blank was determined. The decrease in ΔA_{420} of the mixture after incubation was corrected by subtracting the corresponding decreases in ΔA_{420} of enzyme-free and substrate-free controls.

The amounts (µ equivalents) of reducing sugars yielded by the chitinase reaction were read from a standard curve of known µ equivalents versus ΔA_{420} values of *N*-acetylglucosamine, a representative of reducing sugars liberated from chitin. One unit of the chitinase activity was defined as the amount of the enzyme that yielded 1.0 µ equivalent of reducing sugar per minute.

In the turbidimetric method, a decrease in A_{610} of 0.278 corresponded to 0.1 unit of the chitinase activity.

Protein was assayed by the Bio-Rad assay method based on the Coomassie brilliant blue dye binding procedure (Bradford, 1976) in which bo-

vine serum albumin served as the standard protein. Specific activity is expressed as unit activity per mg of protein.

Assay method of chitinase inhibitory activity

Chitinase inhibitory activity was determined by the aforesaid turbidimetric method with colloidal chitin as substrate (Yabuki *et al.*, 1986). The test mixture contained 200 μ l of the enzyme solution (0.28 units, in 50 mM citrate-phosphate-borate buffer of pH 7.0), 32 μ l of a 1.0% colloidal chitin suspension, and 168 μ l of an aqueous solution of a test material. If the test material was insoluble in water, it was dissolved in methanol (less than 40 μ l) and the same volume of methanol was added to the control. The absorbance of the test mixture was measured at 610 nm (A_{610}) before and after incubation for 60 min at 37 °C. Percent inhibition was calculated by the equation

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

where A, B, C, and D are the decreases in the absorbance (A_{610}) of the test mixture, the enzyme solution, the colloidal chitin suspension, and the control mixture containing no test material, respectively.

Fermentation of microorganisms

One hundred and fifty strains of *Loculoascomycetes* were cultured at 28 °C for 7 days on a rotary shaker at 210 rpm in 2 different media, A and B. Medium A contained 4.0% glutinous starch syrup, 2.0% soyafLOUR, 1.0% Pharmamedia, 0.5% Sun-grain, 0.3% soybean oil, 0.5% CaCO_3 , 0.001% $\text{FeSO}_4 \cdot \text{H}_2\text{O}$, 0.001% $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, and 0.0001% $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$. Medium B contained 2.0% corn starch, 0.5% Pharmamedia, 0.1% Staminol, and

1.0% CaCO_3 . The supernatants of the culture broths were tested for chitinase inhibitory activity.

Results and Discussion

Establishment of measurement conditions for enzyme activity

Since the optimum pH value of chitinase varied with its sources, the most suitable pH value for experiments using *S. litura* chitinase was examined. The highest activity was detected when citrate-phosphate-borate buffer of pH 7.0 was used as an extraction buffer (Table I). Furthermore, the optimum pH of the chitinase reaction was pH 7.0 (Fig. 1). These results indicated that the citrate-phosphate-borate buffer of pH 7.0 was suitable for both extraction and reaction of the chitinase. Therefore, this buffer was adopted for the subsequent experiments.

The chitinase activity in the supernatant decreased to 10% of its original value after 24 hr at 4 °C. However, ultracentrifugation of the superna-

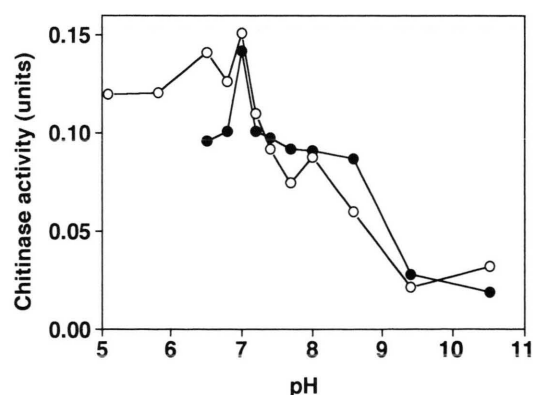


Fig. 1. Dependence of *S. litura* pupa chitinase activity on pH. Activity was determined by turbidimetric (●) and colorimetric (○) methods.

Table I. Activities of crude chitinase extracts in various buffers.

Buffers used	Turbidimetric method	Colorimetric method	
	Total activity (units)	Total activity (units)	Specific activity (units/mg protein)
Citrate buffer (pH 5.0)	—**	31.2	1.34
CPB* buffer (pH 7.0)	47.2	45.9	1.73
Borate buffer (pH 9.0)	29.6	23.1	0.87

*: Citrate-Phosphate-Borate buffer. **: Not detected by the turbidimetric method due to deep turbidity of the reaction mixture.

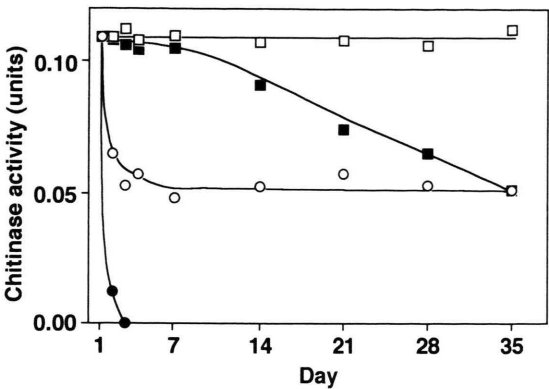


Fig. 2. Stability of crude *S. litura* pupa chitinase. Centrifuged crude enzyme solution stored at 4 °C (●), centrifuged crude enzyme solution stored at -80 °C (○), ultracentrifuged crude enzyme solution stored at 4 °C (■), ultracentrifuged crude enzyme solution stored at -80 °C (□).

tant maintained the activity for more than a week even at 4 °C. The enzyme solution could be more effectively stored at -80 °C; 100% activity was retained for at least one month (Fig. 2).

The most suitable developmental stage of *S. litura* for the enzyme preparation was also studied. Among the various stages tested (see Materials and Methods), chitinase activity per head was the highest in the young pupa (Table II). Thus, young pupae were used as a material for a stable enzyme solution with high activity.

Table II. Chitinase activity of *S. litura* at various stages.

Stage	Chitinase activity (units/head)	Specific activity (units/mg protein)
Sixth-instar larva just after exuviation	ND	—
Sixth-instar larva in the wandering stage	ND	—
Pharate pupa	0.92	7.97×10^{-2}
Young pupa	3.49	4.16×10^{-1}

ND: not detected.

Inhibitory assay against S. litura pupa chitinase

The present chitinase inhibitory assay using colloidal chitin as a substrate could be performed in a short time (less than 1 hour). In contrast, a previous chitinase inhibitory assay using γ -chitin red as

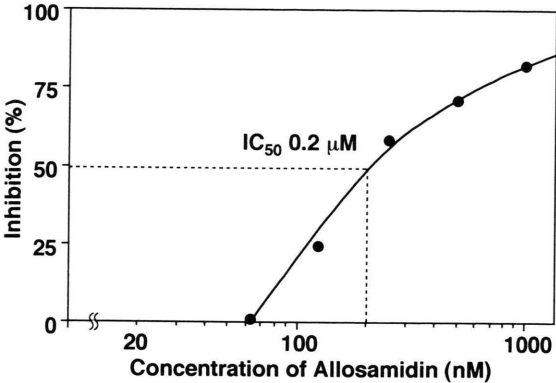


Fig. 3. Inhibitory activity of allosamidin against *S. litura* pupa chitinase.

a substrate (Sakuda *et al.*, 1987b) needed to go overnight because of the sedimentary nature of this substrate.

The IC_{50} of allosamidin against *S. litura* chitinase was 0.2 μ M (Fig. 3). Comparison of this value with that obtained against *B. mori* chitinase (0.7 μ M) (Sakuda *et al.*, 1987a) showed that the present *S. litura* chitinase inhibitory assay was 3.5-fold more sensitive than the *B. mori* chitinase inhibitory assay.

Therefore, the present assay would possibly detect more compounds than the *B. mori* chitinase inhibitory assay.

Screening of microbial cultures for insect chitinase inhibitors

Assays of supernatants of 300 cultures of 150 microbial strains (100 μ l each) revealed 13 samples

Table III. Inhibitory activity of the microbial cultures.

Strain	Medium	Inhibitory activity (ID ₅₀)
L 1226	A	46 μ l
L 1272	B	88 μ l
L 1386	A	45 μ l
L 1432	B	87 μ l
L 1456	B	77 μ l
L 1458	B	84 μ l
L 1472	A	69 μ l
L 1488	B	69 μ l
L 1510	A	50 μ l
L 1512	A	59 μ l
L 1512	B	98 μ l
L 1518	B	99 μ l
L 1520	B	73 μ l

with more than 70% inhibitory activity. The 50% inhibitory dose (ID_{50}) values for these active supernatants are listed in Table III. These results demonstrate that the feasibility of using the present assay system for screening for chitinase inhibitory activity.

Acknowledgments

This work was supported partly by the Chemical Materials Research and Development Foun-

dation. The authors are grateful to Dr. S. Sakuda, Department of Applied Biological Chemistry, Division of Agriculture and Agricultural Life Science, The University of Tokyo, for generously providing allosamidin. The authors are also indebted to Dr. T. Mikawa and Mr. Y. Kohyama, the Research Center of Mitsubishi Chemical Cooperation, for the cultivation of *Loculoascomycetes* and for supplying the *Spodoptera litura* eggs, respectively.

- Bradford M. (1976), A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.
- Imoto T. and Yagishita K. (1971), A simple activity measurement of lysozyme. *Agric. Biol. Chem.* **35**, 1154–1156.
- Isogai A., Sato M., Sakuda S., Nakayama J., and Suzuki A. (1989), Structure of demethylallosamidin as an insect chitinase inhibitor. *Agric. Biol. Chem.* **53**, 2825–2826.
- Jeuniaux C. (1958), Recherches sur les chitinases. I. Dosage néphélométrique et production de chitinase par des *Streptomyces*. *Arch. Int. Physiol. Biochim.* **66**, 408–427.
- Jeuniaux C. (1966), Chitinases. *Methods Enzymol.* **8**, 644–650.
- Nishimoto Y., Sakuda S., Takayama S., and Yamada Y. (1991), Isolation and characterization of new allosamidins. *J. Antibiotics* **44**, 716–722.
- Sakuda S., Isogai A., Matsumoto S., Suzuki A., and Koseki K. (1986), The structure of allosamidin, a novel insect chitinase inhibitor, produced by *Streptomyces* sp. *Tetrahedron Lett.* **27**, 2472–2478.
- Sakuda S., Isogai A., Makita T., Matsumoto S., Koseki K., Kodama H., and Suzuki A. (1987a), Structures of allosamidins, novel insect chitinase inhibitors, produced by actinomycetes. *Agric. Biol. Chem.* **51**, 3251–3259.
- Sakuda S., Isogai A., Matsumoto S., and Suzuki A. (1987b), Search for microbial insect growth regulators II. Allosamidin, a novel insect chitinase inhibitor. *J. Antibiotics* **40**, 296–300.
- Yabuki M., Mizushima K., Amatatsu T., Ando A., Fujii T., Shimada M., and Yamashita M. (1986), Purification and characterization of chitinase and chitobiase produced by *Aeromonas hydrophila* subsp. *anaerogenes* A52. *J. Gen. Appl. Microbiol.* **32**, 25–38.