Serratia marcescens の生産するプロディギオシンがハスモンヨトウに対する Bacillus thuringiensis Cry1C 内毒素の殺虫活性を増大させる

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Original Article

Prodigiosin Produced by *Serratia marcescens* Enhances the Insecticidal Activity of *Bacillus thuringiensis* delta-Endotoxin (Cry1C) against Common Cutworm, *Spodoptera litura*

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Synergistic effects of chitinases A, B and C1, chitin binding protein and prodigiosin from *Serratia marcescens* on the insecticidal activity of delta-endotoxin, Cry1C, of *Bacillus thuringiensis* against the common cutworm, *Spodoptera litura*, were investigated. Only prodigiosin showed a potent synergistic activity with Cry1C on both the lethal and growth inhibitory activity. In the previous paper (Asano *et al.*, 1999) a synergistic effect in the supernatants of *S. marcescens* culture on the insecticidal activity of Cry1C was described. The supernatants from *S. marcescens* and partially purified prodigiosin showed a similar synergistic activity on the insecticidal activity of Cry1C. The content of prodigiosin in the supernatants was estimated as 10% according to the absorbance at 467 nm where prodigiosin showed a peak and the content seemed to be enough to explain the synergistic activity of the supernatants on the activity of Cry1C.

Key words: synergism, *Serratia marcescens*, *Bacillus thuringiensis*, Cry1C, delta-endotoxin, prodigiosin, *Spodoptera litura*.

INTRODUCTION

Insecticidal crystal proteins, delta-endotoxins, from *Bacillus thuringiensis* have been utilized worldwide as one of most representative biopesticides which have been adapted for the integrated pest management as alternatives of chemical pesticides. On the other hand, the endotoxins are known to posses an entomocidal specificity against insect species.[1] Among lepidopterous insects, *Spodoptera* and *Mamestra* are generally known as a tolerant group toward the delta-endotoxin.[1–3] To control those tolerant insects using delta-endotoxins of *B. thuringiensis*, several approaches have been tried such as 1) to increase toxin yield in *B. thuringiensis* culture, 2) to stabilize toxins under environmental conditions by formulation techniques, 3) to seek for novel and more effective isolates or toxins of *B. thuringiensis*, 4) to modify the toxins so that it may be more active by gene technology, and 5) to use some synergistic substances enhancing the activity of toxins. We have been interested in using the novel compounds which synergistically enhances the activity of delta-endotoxins of *B. thuringiensis*. We found a new factor which synergistically enhanced the activity of *B. thuringiensis* in the supernatant of various *B. thuringiensis* cultures.[4–6] The factor was seemed to be an ionic whose molecular weight was about 1000.[6]

Recently we have reported another unique synergistic factor in the supernatants from *Serratia marcescens* culture, which synergistically enhanced the insecticidal activity of CrylC against *Spodoptera litura*.[7] In the present study, chitinases, chitin binding protein and prodigiosin which were produced by *S. marcescens* were investigated to see whether they synergistically act with Cry1C against *S. litura*.

MATERIALS AND METHODS

1. *Bacteria and Preparation of the Compounds from Supernatants of Culture*

B. thuringiensis delta-endotoxin (Cry1C) was provided by Mycogen. Corp., San Diego, USA. *S. marcescens* 2170[8] was cultured in a yeast extract supplemented

with minimal (YEM) medium[9] containing 0.5% (W/V) colloidal chitin at 30°C for 2 days. The culture broth was centrifuged at 10,000×*g* for 10 min. The supernatants were saturated with ammonium sulfate at 80% (W/V) and the materials salted out were precipitated. The precipitates were dissolved in distilled water, dialyzed against distilled water and lyophilized. This product was described as the supernatants hereater. Chitinase A, C1 and chitin binding protein were prepared according to the method of Suzuki *et al.*[10]

2. Purification of Chitinase B

Chitinase B was produced in *E. coli* DH5alpha transformed with pMCB7 harboring the chitinase B gene. The transformant *E. coli* was cultured in LB broth containing 100 μg/ml ampicillin and 0.4 mM IPTG, Isopropyl-1-thio-β-D-thiogalactopyranoside, at 30°C for 24 hr. The cells were precipitated by a centrifugation and sonicated 8 times with 15 seconds intervals at 20 kHz, 40 W. The soluble fraction was recovered in 20–40% ammonium sulfate saturation and dialyzed against 2 mM phosphate buffer, pH 6.0. The dialysates were applied to the column chromatography of hydroxyapatite equilibrated with 1 mM phosphate buffer, pH 6.0. The hydroxyapatite (Seikagaku Kogyo Co. Ltd., Tokyo Japan) was allowed to stand over night in 1 mM phosphate buffer, pH 6.0 and fine particles were removed by decantation several times. The resin inside a column was equilibrated with 3 to 5 column volumes of the same phosphate buffer. One mg of crude soluble fraction was applied to 1 ml of the column and non adsorbed fraction was eluted with phosphate buffer and subjected further to chitin affinity column chromatography. The chitin EX (Funakoshi Co. Ltd., Hongo, Bunkyo, Japan) was equilibrated with 20 mM phosphate buffer, pH 6.0 and packed into a glass column (3.5 cm×13 cm). The sample was charged and the column was washed with 3 column volumes of 20 mM phosphate buffer containing 0.5 M NaCl. The column was further washed with 3 volumes of 20 mM sodium acetate buffer, pH 5.5 and the chitinase was eluted with 3 volume of 20 mM acetic acid. The flow rate of the loading buffer was 1 ml/min. The enzyme was dialyzed against 2 mM phosphate buffer, pH 6.0, and used for experiments.

3. Preparation of Prodigiosin

Serratia marcescens ATCC274 was grown in a stainless steel tray containing 1.5% agar medium with 0.5% Bacto-Peptone and 1% glycerol at 30°C for 48 hr. The bacteria on agar showing red pigmentation were harvested with a rubber spatula and suspended into physiological salt solution. The cells were spun down by centrifugation at 6000 rpm and red pigments were extracted by mixing with 95% ethanol. The extracts were concentrated and fractionated with a thin layer chromatography in a solvent mixture of chloroform: methanol: 5 M ammonia=80: 25: 4 (V/V). The prodigiosin (Fig. 1) was extracted from silica gel scraped by acetone. The purity of the prodigiosin was estimated as 95% according to the molecular extinction coefficient of authentic prodigiosin at 467 nm.

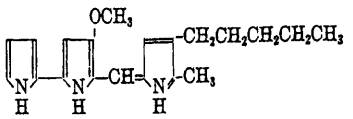

Fig. 1 Structure of prodigiosin.

4. Bioassays

Three kinds of lepidopterous insects were used for bioassays, *i.e.*, the common cutworm, *S. litura*, the diamondback moth, *Plutella xylostella*, and the smaller tea tortrix, *Adoxophyes honmai*. They were maintained using an artificial diet in our laboratory over 5–10 years. The Cry1C and test samples were diluted appropriately with distilled water and mixed with the artificial diet and fed to test insect as described previously.[7] No. of survivals and their instars were scored on the 7th day after treatment. The lethal activity of test samples was evaluated based on the rates of survivals to numbers of infested neonates, regardless of larval size and instars. For the growth inhibitory activity, a larval growth index Σ (no. of survivals×instars=cumulative instars of survivals) was used and per cent inhibition was calculated by comparing the index at treatments to controls.

RESULTS

1. Synergsitic Effects of Chitinases, Chitin Binding Protein (CBP) on the Activity of Cry1C against S. litura

S. marcescens produced several chitinases (A, B and C1) and chitin binding protein (CBP) in the culture medium during cell growth (Watanabe *et al.*, 1997). The chitinases A, B and C1 or CBP were mixed with 6 and 12 μg/g diet of Cry1C and the mixtures were given to neonates of *S. litura*. The supernatant was used as a positive control for the synergism with Cry1C.[7] Their biological activities were measured by mortality and growth inhibition on the 7th day after treatment (Table 1). Mortality by Cry1C alone was 4 and 21% at 6 and 12 μg/g diet, respectively. A combination of Cry1C and supernatants was 8% and 53% in mortality and, 24% and 51% in growth inhibition at lower and higher dose level of Cry 1C, respectively. When compared with the mortality and the growth inhibition by combination of Cry1C and supernatants, all kinds of chitinases with and without CBP could not be declared to posses the synergism as similar to that of supernatants.

Table 1 Insecticidal effects of mixture of chitinases, chitin binding protein, CBP and Cry1C against *Spodoptera litura*.

Test samples (μg/g diet)	Mortality and growth inhibition(%)[1]	
	Cry1C (μg/g diet)	
	6	12
Chitinase A 5	9 (3)	21 (1)
Chitinase B 5	7 (5)	24 (7)
Chitinase C 5	5 (3)	22 (5)
Chitinase A+B+C (1: 1: 1) total 7.5	8 (5)	18 (1)
Chitinase A+B+C+CBP (1: 1: 1: 2) total 12.5	6 (5)	21 (4)
Supernatant 10	8 (24)	53 (51)
Control (toxin alone)	4 (0)	21 (0)

[1] Fifty neonates were used for each treatment and controls. Survivals and developmental instars were read on the 7th day after treatment. Growth inhibition was calculated by comparing the larval growth index (Σ survivals×instars) between each mixture and the controls (Cry1C alone).

2. *Effects of the Prodigiosin in a Mixture of Prodigiosin and Cry1C on the Activity against S. litura*

S. marcescens produces a red pigment, prodigiosin and it secretes in the culture medium. Therefore to examine if prodigiosin posses the synergistic effects like supernatants do, prodigiosin was added at 1 μg/g diet with different doses of Cry1C, and the neonates of *S. litura* were fed with this mixture for 7 days (Table 2). The mortality and growth inhibition by Cry1C were 2–41% and 17–69%, respectively responding to doses. On the other hand, the addition of 1 μg/g diet of prodigiosin increased both mortality and growth inhibition higher than that of Cry1C alone (Table 2).

The synergistic activity by prodigiosin was reexamined more precisely with the combination of various concentrations of prodigiosin and Cry1C using *S.litura* (Table 3). The mortality of the insect by Cry1C alone at 0, 3, 6 and 12 μg /g diet were 0, 8, 29 and 56%, respectively. Prodigiosin was added at 0.125 to 2 μg per one gram of diet containing different concentration of the Cry1C cited above. Addition of prodigiosin over 0.25 μg/g diet enhanced the mortality of Cry1C at all combination rates of two components, responding to both doses. For example, when 12 μg /g diet of Cry1C alone gave 56% mortality, the similar mortality (59%) was obtained by a mixture of 3 μg/g diet of Cry1C and 2 μg/g diet of prodigiosin. It indicated that concentration of Cry1C could be reduced into one-fourth to get the same response by addition of prodigiosin. Besides, 100% mortality was obtained by a combination of 12 μg/g diet of Cry1C and 2 μg/g diet of prodigiosin (Table 3).

Table 2 Insecticidal effects of a mixture of prodigiosin and Cry1C against *Spodoptera litura*.

Cry1C (μg/g diet)	Mortality and growth inhibition(%)[1]	
	Cry1C alone	Cry1C+prodigiosin (1μg/g diet)
12	41 (69)	92 (97)
6	25 (50)	56 (79)
3	15 (35)	25 (56)
1.5	14 (31)	15 (42)
0.75	2 (17)	19 (33)
0	2 (0)	4 (0)

[1] Bioassays were done with three replicates (total 150 neonates per treatment and controls were used). Mortality and growth inhibition were read on the 7th day after treatment. Growth inhibition was calculated based on larval growth index (See footnotes in Table 1 and text).

Table 3 Effects of prodigiosin on the insecticidal activity of Cry1C at various concentration of prodigiosin and toxin against *S. litura*.

Prodigiosin (μg /g diet)	Mortality (%)[1]			
	Cry1C (μg/g diet)			
	0	3	6	12
2	0	59	94	100
1	0	35	78	99
0.5	0	26	49	90
0.25	0	25	34	75
0.125	0	11	29	54
0	0	8	29	56

[1] Mortality was read on the 7th day after treatment. Fifty larvae were used at each treatment and controls.

3. *Determination of Content of Prodigiosin in the Supernatants of S. marcescens Culture*

Prodigiosin was known to have a major absorption at 467 nm light.[12] The supernatants of *S. marcescens* culture which exhibited the synergism with Cry1C against *S. litura* showed a similar spectrum as that of prodigiosin. The absorption of 200 μg/ml of the supernatants at 467 nm light was almost equivalent to that of 20 μg/ml of prodigiosin (Data not shown). It indicated that the content of prodigiosin in the supernatants was about 10%.

4. *Insecticidal Activity of Prodigiosin against Three Lepidopterous Larvae*

Insecticidal activity of prodigiosin without Cry1C was examined using three lepidopterous neonates such as *S.*

Table 4 Larvicidal activity and growth inhibition of prodigiosin against three kinds of lepidopterous insects.

Prodigiosin (μg /g diet)	Mortality and growth inhibition (%)[1]		
	S. litura	*P. xylostella*	*A. honmai*
8	3 (34)	96 (99)	12 (9)
4	5 (23)	69 (80)	9 (5)
2	3 (7)	42 (50)	9 (3)
1	1 (0)	13 (17)	3 (0)
0.5	3 (0)	17 (6)	10 (3)
0	6 (0)	13 (0)	5 (0)

[1] Fifty neonates were used in each toxin concentration and the mortality was read on the 7th day after the treatment.

litura, *P. xylostella* and *A. honmai* by a diet incorporation method (Table 4). Of the three insects, *P. xylostella* was most sensitive to prodigiosin. The mortality and growth inhibition at 8 μg/g diet of prodigiosin were observed as 96 and 99%, respectively. *S. litura* was less sensitive. The mortality and the growth inhibition at 8 μg/g diet of prodigiosin in *S. litura* was as low as 3% and 34%, respectively. *A. honmai* was the least sensitive and no significant difference in both mortality and growth inhibition between treatment and controls was observed. This insecticidal specificity of prodigiosin was similar to those described previously using the supernatants of *S. marcescens* culture.[7]

DISCUSSION

Smirnoff[13] reported that a mixture of *B. thuringiensis* and chitinase caused more rapid and pronounced lethal activity against *Choristoneura fumiferana* than *B. thuringiensis* alone. Sneh *et al.*[14] also described a similar synergistic activity by chitinolytic bacteria on the activity of delta-endotoxin of *B. thuringiensis* against *S. littoralis*. We also reported in our previous paper, a synergism of the supernatants from *S. marcescens* culture on the insecticidal activity of Cry1C against *S. litura*.[7] *S. marcescens* is well known to secrete chitinases and chitin binding protein in culture medium.[8] Recently, Regev *et al.*[15] described a synergistic activity of the endochitinase from *S. marcescens* on the activity of Cry1C against *S. littoralis*. In the present study, we used purified chitinase A, B and C1 and chitin binding protein. But we failed to show a synergistic activity of those proteins on the insecticidal activity of Cry1C. Instead of these failures, a synergistic effect was obtained with the combination of prodigiosin and Cry1C as well as the mixture of supernatants of *S. marcescens* culture and Cry1C as previously reported.[7] Prodigiosin was secreted into culture medium from *S. marcescens*.[12] The supernatants of *S. marcescens* culture used in our study were slightly reddish and, therefore, the prodigiosin were thought to be in it to some extent. The partially purified prodigiosin and the supernatants from *S. marcescens* culture showed a high resemblance in absorption spectrum at a range of 200–700 nm and similar major absorption at around 467 nm was also observed (Data not shown). The intensity of absorbence of 200 μg/ml of the supernatants corresponded to that of the solution containing 20 μg/ml of prodigiosin. Therefore, the prodigiosin concentration in the supernatants used for experiments was estimated as 10%. If so, this concentration could explain the mortality and growth inhibition resulted by the addition of supernatants (Table 1). In the previous paper,[7] the supernatants of *S. marcescens* culture showed some lethal activity against *P. xylostella* at 20–80 μg/g diet but not against *S. litura* or *A. honmai*. In the present results the prodigiosin of 2–8 μg/g diet showed a similar insecticidal spectrum against the same insects. It suggests that the active substance in the supernatants was of the same component and composed of prodigiosin.

As we insisted several times in our previous papers[4–7] the synergistic effects strongly seemed to have a specificity toward both delta-endotoxin of *B. thuringiensis* and target insect species. Interestingly the synergism between prodigiosin and Cry1C was obviously observed in *S. litura* at 1 μg/g diet of prodigiosin (Tables 2 and 3) but not in *P. xylostella* at the same concentration of prodigiosin (Data not shown). The mechanism of synergism by prodigiosin and Cry1C has not been elucidated yet. Furthermore, in addition to the effects, the mechanism of the specificity itself need to be clarified to improve BT formulations.

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要　　約

Serratia marcescens の生産するプロディギオシンがハスモンヨトウに対する *Bacillus thuringiensis* Cry1C 内毒素の殺虫活性を増大させる

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Serratia marcescens の培養上清に存在し，*Bacillus thuringiensis* の δ-endotoxin (Cry1C) のハスモンヨトウに対する殺虫活性を増大する活性物質を明らかにする目的で，キチナーゼ (A, B 及び C1)，キチン結合蛋白 (CBP) 及び赤色色素プロディギオシン (prodigiosin) について協力作用の有無を調べた．その結果検定物質のなかではプロディギオシンのみに致死活性と幼虫発育阻害活性において顕著な協力作用が認められた．*S. marcescens* 培養上清と精製プロディギオシンの協力作用性は類似しており，さらに 467 nm の吸光度から推定した上清中のプロディギオシンの含有量で協力効果を十分説明できることから上清中の協力作用はプロディギオシンによるものと推論された．