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ORIGINAL ARTICLE

Toxic activity of a protein complex purified from *Xenorhabdus nematophila* HB310 to *Plutella xylostella* larvae

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Abstract *Xenorhabdus nematophila*, a Gram-negative proteobacterium belonging to the family Enterobacteriaceae and associated symbiotically with soil entomopathogenic nematodes, *Steinernema carpocapsae*, is pathogenic to a wide range of insects. A protein complex with insecticidal activity was isolated from the cells of *X. nematophila* HB310 strain using methods of salting out and native polyacrylamide gel electrophoresis (PAGE). Seven polypeptides ranging 50∼250 kDa were well separated from the protein complex (named Xnpt) by sodium dodecyl sulfate (SDS)-PAGE, five of which are identified as XptA2, xptC1, XptB1, GroEL and hypothetical protein by matrix-assisted laser desorption-time-of-flight mass spectrometry (MALDI-TOFMS). Xnpt showed high oral virulence to larvae of diamondback moth (DBM), *Plutella xylostella* L. (Lepidoptera, Plutellidae) as its median lethal concentration (LC_{50}) against second and third instar larvae were 331.45 ng/mL and 553.59 ng/mL at 72 h, respectively. The histological analysis of Xnpt-fed DBM larvae showed extensive histopathological effects on the midgut. Biochemical analysis indicated that Xnpt markedly inhibited the activities of three important enzymes in the midgut. Overall, our data showed that the protein complex isolated from *X. nematophila* HB310 induced the antifeedant and death of insects by destroying midgut tissues and inhibiting midgut proteases activities.

Key words insecticidal toxin, midgut, *Plutella xylostella*, proteases, *Xenorhabdus nematophila*

Introduction

The diamondback moth (DBM), *Plutella xylostella* (Lepidoptera, Plutellidae) is known worldwide as a serious pest of cruciferous vegetables. The extensive application of insecticides has led to the development of resistance in DBM to a number of insecticides including *Bacillus thuringiensis* (Bt) formulations under field conditions (Mohan & Gujar, 2000; Zhao *et al*., 2006). For this reason, new types of toxins, particularly those with different modes of action, are desired, so that effective pest management can be achieved.

Xenorhabdus and *Photorhabdus* bacteria are Gram-negative bacteria symbiotic with entomopathogenic nematodes (Steinernema, Heterorhabditis), which delivers the bacteria into the hemocoel of the target insects (Adams & Nguyen, 2002). In the hemocoel, the bacteria produce a variety of metabolites to enable them to colonize and reproduce in the insect host (Webster *et al*., 2002). The bacteria also produce toxins that are responsible for killing the insect host after it is released into the insect hemocoel upon nematode invasion (ffrench-Constant *et al*., 2007). Recently, a new family of oral insecticidal toxins

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produced by *Xenorhabdus* and *Photorhabdus* bacteria has been identified (ffrench-Constant *et al*., 2001). These oral insecticidal toxins are high molecular weight protein complexes. The genes encoding the toxin complexes (Tcs or Xpt) described in *Xenorhabdus* and *Photorhabdus* have no similarity to the Bt δ-endotoxins (Cui *et al*., 2003; ffrench-Constant *et al*., 2003; Waterfield *et al*., 2005, 2007). It implies that these bacteria have the potential to be developed as insecticidal agents.

Two insecticidal toxin complexes from *X. nematophila* have been described which show oral activity against a broad range of insect pests. Each complex involves three proteins: XptA1/XptB1/XptC1 and XptA2/XptB1/XptC1 (Morgan *et al*., 2001; Cui *et al*., 2003; Sergeant *et al*., 2003). These proteins have high similarity in amino acid sequence to Tcs proteins of *P. luminescens*, and full insecticidal activity requires the combination of at least three gene products. XptA1 or XptA2 are the key components for the toxicity of the XptA/B1/C1 complex (Cui *et al*., 2003; Sergeant *et al*., 2003). Recent research indicates that the three-Xpt-component toxin system may have a different mode of action compared with Bt δ-endotoxins (Lee *et al*., 2007). Lee *et al*. (2007) have demonstrated that the XptA1 protein remains intact at high pH, and processing is not required for the protein to interact with target membranes. However, the details of the mode of action of the Xpt (Tcs) toxins from *Xenorhabdus* and *Photorhabdus* are still unclear.

Xenorhabdus nematophilas strain HB310 is symbiotically associated with a strain of the entomopathogenic nematode *Steinernema carpocapsae* isolated from the soil in Hebei Province, China. Primary studies showed that this bacterial strain had a high insecticidal activity when fed to a range of pest species, especially to DBM (Li *et al*., 2004). A high molecular weight protein complex named Xnpt was purified from the intracellular protein extract of the bacterium, which shows insecticidal activity in DBM larvae. However, little is known about the components of Xnpt. The relationships between insect gut environments and toxicity of *Xenorhabdus* insect toxins have not been addressed in earlier work. Therefore, the major objective of the present research was to explore the insecticidal mechanism of *Xenorhabdus* insect toxins. Here, the peptides of the toxin complex were identified by matrix-assisted laser desorption-time-of-flight mass spectrometry (MALDI-TOFMS). With oral delivery, the toxicity of the peptides, the histopathology effect, and the impact on some midgut proteases of DBM larvae were also investigated. The primary digestive proteinases of lepidopterans, including *P. xylostella*, are serine proteinases, such as trypsin and chymotrypsin (Christeller *et al*., 1992; Rymerson & Bodnaryk, 1995). These protease activities in midgut were often used as indicators to test the digesting activity of the insect (Christeller *et al*., 1992; Terra *et al*., 1994). Serine proteases are also important in *B. thuringiensis* protoxin activation (Oppert *et al*., 1996). We mainly checked the changes of some serine proteases in the midgut of DBM larvae after feeding of the toxin.

Materials and methods

Insects and bacteria

A population of DBM, collected from a crucifer field in Baoding, Hebei Province, China, was continuously maintained in the laboratory for more than 50 generations. The adults and larvae were reared as previously described (Shelton *et al*., 1991). Briefly, adults were fed with 10% sucrose solution. Larvae were reared on 1-week-old organically pot-grown radish seedlings (*Raphanus sativus* L.) at 25°C and 65% relative humidity under a 16 h light photo phase.

Xenorhabdus nematophila HB310 strain used in this study was isolated and stored in the Pest Biocontrol Laboratory (PBL), Agricultural University of Hebei, China.

Purification of Xnpt

To purify the insect toxin from *X. nematophila* strain HB310, the bacteria were incubated in Luria-Bertani (LB) broth for 48 h at 28°C on a rotary shaker at 250 r/min. The 2 L bacteria (6×10^8 cells/mL) were centrifuged and washed three times with phosphate buffered saline (PBS). After resuspension in 300 mL of PBS buffer, the bacterial cells were lysed by sonication on ice and the lysate was centrifuged at $10\,000 \times g$ for 15 min at 4°C. The supernatant was filtered through a 0.22 nm filter. The total intracellular proteins were isolated by precipitation with 85% saturated ammonium sulfate and concentrated by using a Centriprep 100 ultrafiltration device with a molecular mass cutoff of 100 kDa (Millipore Corporation, Bedford, MA, USA). The Xnpt were separated by 6% native polyacrylamide gel electrophoresis (PAGE) using the Bio-Rad model 491 Prep Cell apparatus (Bio-Rad, Benicia, CA, USA). The fractions were monitored by a UV detector and collected using a fraction collector, then were concentrated by using a Centriprep 100 device. The oral insecticidal activities of these fractions to DBM larvae were determined using the leaf dip method (Sayyed *et al*., 2000). The purified protein toxin was analyzed by native-PAGE, and sodium dodecyl sulfate (SDS)-PAGE. Protein

concentrations were determined using bicinchoninic acid protein assay reagent (Pierce, Rockford, IL, USA) and bovine serum albumin (BSA) as the standard.

Xnpt identification

To identify the purified protein complex, the purified Xnpt was first separated by SDS-PAGE and protein bands were individually excised from the gel and digested with trypsin. Peptide sequencing was performed with an Autoflex mass spectrometer (Bruker Autoflex, Karlsruhe, Germany). The results of the amino acid sequence were queried against entries for protein databases in National Center for Biotechnology Information (NCBI) using the Mascot protein search program (Matrix Science Ltd, London, UK).

The toxicity of Xnpt to DBM

To test the toxicity to DBM, Xnpt were serially diluted in distilled water containing 0.3% Tween 80 for bioassays with second- or third-instar larvae using the leaf dip method (Sayyed *et al.*, 2000). Each leaf disk (diameter = 16 mm) was immersed in a test solution for 10 s. Control leaf disks were immersed in distilled water with 0.3% Tween 80. After brief drying, the leaf disks were placed in Petri dishes (9 cm diameter). Ten larvae were placed in each dish and incubated at 25°C, and mortality was determined every 24 h after the treatment. Each protein concentration was replicated four times. Median lethal concentration (LC_{50}) was determined by probit analysis (Finney, 1971).

The anti-feeding activity of Xnpt against DBM larvae

To determine the anti-feeding activity of Xnpt against DBM larvae, bioassay was performed using a conventional leaf disk method described by Abdelgaleil *et al*. (2003). Briefly, three leaf disks (diameter = 1.6 cm) of cabbage (*Brassica oleracea* L.) were immersed in 500 ng/mL Xnpt solution or distilled water with 0.3% Tween 80 for 10 s. After brief drying, three discs were separately allocated in one Petri dish (diameter = 9 cm). Five larvae were placed in each Petri dish as a treatment. The larvae were starved 5 h before testing. The area of the consumed leaf disk was calculated after 24 h by counting the square units of a squared paper (1 mm^2 per square), which was placed under the leaf disks as a background. The antifeedant percentage was calculated with the formula: antifeedant (%) = $((C-T)/C)$ 100, where C is the area of leaf discs consumed in the control and T is the area of leaf discs consumed in the treatment. Each treatment or control was replicated six times.

Histopathology

Most of the documented insecticidal toxins from bacteria possess histopathological effects on the midgut (Endo *et al*., 1980; Yu *et al*., 1997). To determine whether Xnpt has a similar effect, third instar larvae of DBM were fed with leaves immersed in 500 ng/mL toxin solution or distilled water with 0.3% Tween 80. The larvae were starved for 6 h before the feeding. The midguts of the larvae were removed and then fixed with 4% formaldehyde in PBS 24 or 48 h post-feeding. The fixed larvae were then embedded in paraffin, and 6 μm sections were cut. The sections were stained with eosin and hematoxylin and mounted with glycerol for microscope imaging.

The effects of Xnpt on protease activity

To determine the effect of Xnpt on the protease activity in the midgut of DBM, third instar larvae were fed with cabbage leaf dishes which were immersed in different concentrations of Xnpt solution or distilled water as previously described. The bodies of 10 larvae were ground up in a pre-chilled mortar, then homogenized in 4 volumes (mL/g) of cold distilled 0.15 mol/L NaCl solution on ice. Homogenates were centrifuged for 15 min at 6 000 × g. The supernatant was collected to determine the protein concentration using bicinchoninic acid protein assay reagent (Pierce), and used as enzyme extract to test enzyme activity. This work was replicated three times.

Total protease activity of the midgut was determined at pH 10.5 using azocasein (Sigma, St Louis, MO, USA; product no. A2765) as the substrate. To determine the total protease activity, 0.9 mL enzyme extract (0.2 mol/L, pH 10.5 Gly-NaOH buffer) was mixed with 0.9 mL (20 mg/mL) azocasein and incubated at 30°C for 2 h. The digestion was stopped by 1.8 mL 20% (w/v) tichloroacetic acid, and undigested azocasein was precipitated by addition of an equal volume of 20% trichloroacetic acid followed by incubation at room temperature for 1 h. The precipitated proteins were removed by centrifugation at 10 000 × g, 4°C for 10 min. The supernatant was collected, and its optical absorbance at 366 nm was measured with a UV-1800 Spectrophotometers (SHIMADZU, Shimadzu Corporation, Tokyo, Japan). The rate of proteolysis of azocasein was expressed in mOD336 (milli optical density at 336 nm)/min/mg of BDM protein. One unit of activity (U) was defined as a change of one absorbance unit.

Active alkaline trypsin-like enzyme activity in the larvae homogenate was determined using Na-benzoyl-L-arginine *p*-nitroanilide (BApNA; Sigma) as the substrate. Forty microliters of 20 mg/mL BApNA was mixed with 0.15 mL of enzyme extract in 1.35 mL Gly-NaOH (0.1 mol/L, pH 10.5), and incubated at 28°C for 20 min. The enzymatic reaction was stopped by adding 0.5 mL 30% acetic acid. The optical absorbance was recorded at 406 nm with a UV-1800 Spectrophotometers (SHIMADZU, Shimadzu Corporation). For all assays, blank controls were prepared by precipitating the substrate with trichloroacetic acid prior to the addition of enzyme extract.

Weak alkaline trypsin-like enzyme activity in the larvae homogenate was determined using Na-p-tosyl-L-arginine methyl ester (TEME; Sigma) as the substrate. Forty microliters of 2 mmol/L TEME in 0.15 mol/L NaCl solution was mixed with 0.15 mL of enzyme extract in 1.35 mL Tris-HCl (0.2 mol/L, pH 8.5). The enzymatic reaction at 28°C was monitored by recording the optical absorbance at 248 nm for 60 min.

The determination of chymotrypsin-like enzyme activity was similar to the method used for the weak alkaline trypsin-like assays except 1 mmol/L Na-benzoyl-L-tyrosine ethyl ester (BTEE) was used as the substrate. Forty microlites of 1 mmol/L BTEE in 10% methanol was mixed with 0.15 mL of enzyme extract in 1.35 mL Gly-NaOH. The enzymatic reaction at 28°C was monitored by recording the optical absorbance at 256 nm for 60 min. The experiment was replicated three times for each enzyme activity. The molar extinction coefficients for BApNA ($\varepsilon = 8\,800$), BTEE ($\varepsilon = 964$), and BApNA ($\varepsilon = 8\,800$) were used to calculate the protease activities. The unit of enzyme activity is defined as the amount of enzyme which produces 1 μmol *p*-nitroaniline/min/mg protein.

Each of the above experiments was replicated three times. Data in text are presented as mean + standard deviation of the mean.

Results

Identification of the protein complex from X. nematophila *strain HB310*

At the primary research stage, we found that the crude protein extract from *X. nematophila* HB310 strain using 85% ammonium sulfate precipitation method had a high insecticidal activity. To identify the active component, we further purified the protein using native -PAGE. One protein complex showed oral insecticidal activity to BDM larvae and was a single band on the native-PAGE (Fig. 1A) but separated into eight bands by SDS-PAGE (Fig. 1B). Here we named the protein complex as Xnpt.

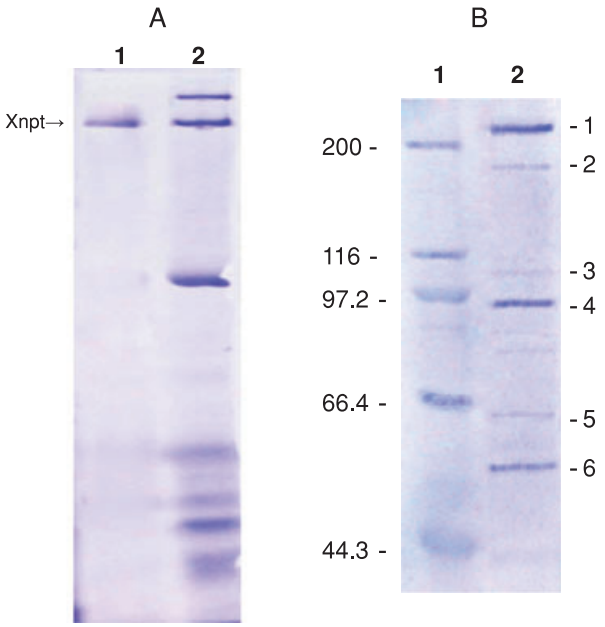

Fig. 1 Xnpt identification. A. native-polyacrylamide gel electrophoresis (PAGE) analysis of purified Xnpt toxin and crude extracted intracellular protein. Lane 1: The arrow indicates one single band named Xnpt, which was isolated from the precipitation of intracellular protein. Lane 2: Multi-bands of crude intracellular protein. B. Sodium dodecyl sulfate (SDS)–PAGE analysis of Xnpt. Lane 1: The molecular mass marker shown in kDa. Lane 2: The Xnpt complex was separated in to six bands.

To identify each peptide in Xnpt complex, the separated bands on SDS-PAGE were cut for sequencing analysis using MALDI-TOFMS. Five protein bands were identified by searching the Bioinformatic database of amino acid sequences (Table 1). Band 1, band 2, band 3 and band 5 (Fig. 1B) were identified as XptA2, XptC1, XptB1 and GroEL, respectively. Band 6 was defined as hypothetical protein. However, band 5 was not identified. It might be a mixture of two proteins.

The insecticidal activity of Xnpt on DBM

The bioassays were performed using the leaf dip method against DBM larvae. Xnpt showed high oral insecticidal activity. The LC_{50} to second instar and third instar DBM larvae were 331.45 ng/mL and 553.59 ng/mL at 72 h, respectively (Table 2).

The antifeeding activities of Xnpt were tested against the second instar and third instar larvae of DBM by the diet-non-choice method. The results (Table 3) showed

Table 1 Identification of proteins by matrix-assisted laser desorption-time-of-flight mass spectrometry peptide mass fingerprinting.

Band no.	Isoelectric point/ molecular weight	Score	Sequence Coverage%	Matched number	Accession no.	Protein name
1	5.19/284 733	540	38	80/99	gi 14041733	XptA2 protein
2	5.04/157 617	159	39	43/95	gi 14041732	XptC1 protein
3	7.05/110 257	120	28	39/95	gi 14041731	XptB1 protein
4	5.95/151 931	77	24		gi 29366735 +	Mixture 1
	4.77/390 838	71	15		gi 57116237	
5	4.78/57 554	195	58	28/86	gi 169244313	GroEL
6	6.09/55 719	89	45	14/86	gi 147859294	hypothetical protein

Table 2 Lethal concentration (LC_{50}) of Xnpt on the second and third instar larvae of diamondback moth.

Larvae	LC_{50} (ng/mL) at 3d	Dose-mortality probit	SE	95% fiducial limits
Second instar	331.45	$y = 2.2466 + 1.0924x$	0.0796	231.42–474.72
Third instar	553.59	$y = 1.9621 + 1.1074x$	0.0756	393.58–778.65

Table 3 Antifeeding activity of Xnpt (500 ng/mL) against diamondback moth larvae at 24 h.

Instars	Feeding area (mm^2)		Antifeedant (%)
	Control	Treatment	
Second	140.63 ± 5.52	60.20 ± 3.53*	57.19
Third	271.76 ± 8.42	156.05 ± 6.8*	42.58

*Significance compared to the control at $P < 0.05$ (Duncan's test).

significant antifeeding activities of Xnpt to the test larvae ($P < 0.01$, Duncan's test). The feeding area of larvae fed with the toxin-treated leaf disk was significantly less than that of larvae fed the control leaf disk. Antifeedant percentages of Xnpt to the second instar and third instar larvae of DBM were 57.19% and 42.58%, respectively.

The effect of Xnpt on midgut protease activities

After ingestion of the Xnpt (concentration of 500 ng/mL), the activities of total protease, weak alkaline trypsin-like enzyme, active alkaline trypsin-like enzyme, chymotrypsin-like enzyme in the toxin-treated larvae were significantly lower than those in the control larvae 6 h post-ingestion (Fig. 2A–D). We found that the effect to protease activities increased steadily when the toxin concentration increased from zero to 1 000 ng/mL (Fig. 3). In conclusion, Xnpt reduced the activities of these proteases in both concentration-dependant and time-dependant manners.

Histopathology

Histopathological observations of the DBM larvae midgut cross-sections showed extensive damage to the epithelium, which indicates that the midgut tissue is a primary site of action by Xnpt protein (Fig. 4). Sections from larvae that had been fed on toxin for 24 h showed that the epithelium cells of the anterior midgut swell apically and begin to extrude large cytoplasmic vesicles into the gut lumen (Fig. 4B, D). At 48 h, degeneration of the epithelium cells continued. The apical vesicles, cell nuclei and disrupted cellular debris were shed into the gut lumen (Fig. 4E). The larvae died at 72 h, and desquamation of the epithelial layer was complete. The basal membrane was the only tissue left (Fig. 4F). Longitudinal section at 24 h after ingested toxin showed obvious damage to the gut epithelium in the anterior region of the midgut. There was less cell disruption in the posterior region (Fig. 4B). Midgut epithelial cells from control larvae were closely associated with one another, showing no damage (Fig. 4A, C).

Discussion

We have identified the key peptides in Xnpt complex from a native strain of *X. nematophila* HB310, which

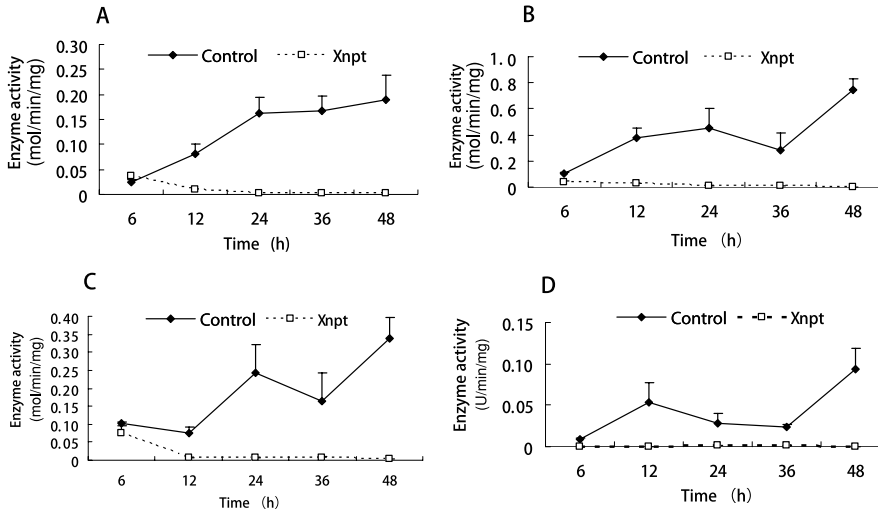

Fig. 2 The time course of enzyme activities of several proteases in midgut of diamondback moth (DBM) larvae after treated with Xnpt or control. A. The active alkaline trypsin-like enzyme activity. The enzyme activity was determined using Na-benzoyl-L-arginine *p*-nitroanilide (BApNA) as the substrate. B. The weak alkaline trypsin-like enzyme activity. The enzyme activity was determined using Na-p-tosyl-L-arginine methyl ester (TEME) as the substrate. C. The chymotrypsin-like enzyme activity. The enzyme activity was determined using Na-benzoyl-L-tyrosine ethyl ester (BTEE) as the substrate. D. The total protease activity. The enzyme activity was determined using azocasein as substrate.

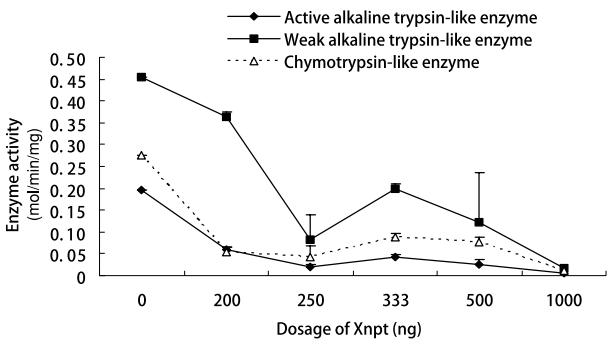

Fig. 3 The relationship between protease activities and dosages of Xnpt 24 h post-feeding.

are taxic to *P. xylostella* larvae. They are identified as XptA2, XptB1 and XptC1. This result is consistent with finding of Sergeant *et al.* (2003) that XptA2 is essential for insecticidal activity against *P. xylostella*.

The disruption of the xptC1, xptB1, or xptA2 genes reduced the activity of *X. nematophila* PMFI296 mutants against *P. xylostella* by more than 30-fold. However, when lysate from an *E. coli* expressing xptA2 was mixed with a lysate of *E. coli* expressing xptB1 and xptC1 no activity toward *P. xylostella* was observed (Sergeant *et al*., 2003). Therefore, Sergeant *et al*. (2003) considered that there should be other unidentified insecticidal toxin genes in *X. nematophila* PMFI296, which requires XptB/XptC proteins for activity and is responsible for activity toward *P. xylostella*. In our results, we found other peptides in the Xnpt complex. Although the functions of the other peptides in the same complex are unclear, one peptide, GroEL may play a role in toxicity against *P. xylostella* larvae as suggested by Joshi *et al*. (2008). The function of this peptide together with the other unknown peptides is being investigated.

Our histopathological study shows that the action target of the toxin complex is the midgut epithelium in DBM, which acted in the same fashion as tca against *Manduca sexta* (ffrench-Constant & Bowen, 1999) and δ-endotoxins and Vip3A from *B. thuringiensis* (Kinsinger *et al*., 1979; Endo *et al*., 1980; Yu *et al*., 1997; Blackburn *et al*., 1998; Aronson *et al*., 2001); interference in midgut function by disrupting the gut cells seems to be the strategy adopted by the most effective insecticidal proteins. Because the Xpt toxin system shows broad-spectrum insecticidal activities differing in specialization from δ-endotoxins, they may have a different mode of action from the Bt δ-endotoxins. In the case of Bt, the δ-endotoxins are produced as protoxins and form a crystalline inclusion that is solubilized during passage through the insect gut. The high pH conditions in the midgut and the action of proteases solubilize the protein and produce a smaller active toxin (Bravo *et al*., 2007).

The digestive enzymes in midgut, including protease, are secreted by midgut columnar epithelial cells. When the

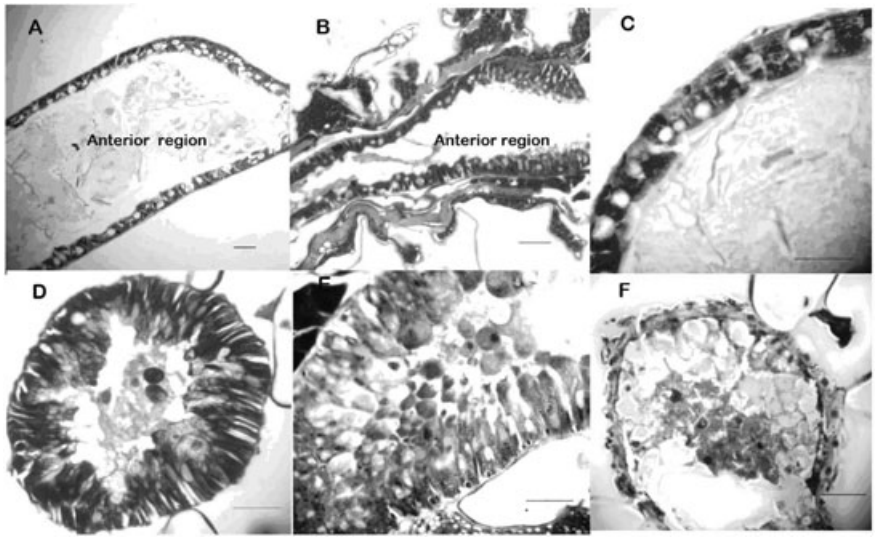

Fig. 4 The histopathological images of the midgut of diamondback moth (DBM) larvae fed with 500 ng/mL Xnpt toxin. A, Longitudinal section of control. B, Xnpt-treated midgut 24 h post-feeding. C, Cross section of control. D, Xnpt-treated midgut 24 h post-feeding. E, Xnpt-treated midgut 48 h post-feeding. F, Xnpt-treated midgut 72 h post-feeding.

columnar epithelial cells are destroyed by the Xnpt complex, the digestive enzyme secretion will be influenced. Therefore it is possible that Xnpt complex indirectly inhibits the protease activities by destroying columnar epithelial cells.

In conclusion, we have purified and identified the key components of an Xnpt complex from a native strain of *X. nematophila* HB310, which is highly toxic to the larvae of BDM. The action mode is similar to the toxin complex identified from *P. luminescens*. In searching sprayable alternatives for chemical insecticides, this toxin complex has great potential in control of BDM pest.

Acknowledgments

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