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A 50-Kilodalton Cry2A Peptide Is Lethal to *Bombyx mori* and *Lymantria dispar*

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The Cry2Aa3 gene was introduced into asporogenic *Bacillus thuringiensis*, and the synthesized protoxin killed *Bombyx mori* and *Lymantria dispar* larvae. Chymotrypsin hydrolyzed the linkages between 49Tyr/Val50 and 145Lys/Ser146 in the protoxin, and 50- and 58-kDa fragments were generated, respectively. Both peptides killed the larvae of both insects.

Recently, the use of *Bacillus thuringiensis* toxins (Bt toxins) has been threatened by the emergence of Bt toxin-resistant insects (8); therefore, different types of Bt toxins having the same target insects have been sought. Cry2A, which was found in *B. thuringiensis* subsp. *kurstaki* HD-1 (20), has only two of the five typical conserved amino acid sequences (AAS) (14). This small number of conserved regions suggests that the relationships between Cry2A and other Cry toxins are limited; indeed, the AAS homology between Cry2Aa and Cry1Aa is as low as 20%. However, interestingly, the three-dimensional structures of these two proteins are very similar (10, 12). The similarity in the structures suggests that the mechanism by which Cry2Aa kills is highly similar to the killing mechanisms of many other Cry toxins; on the other hand, the low sequence homology suggests that Cry2Aa has minor characteristics that differ from those of Cry1Aa. Understanding the various characteristics of Cry2Aa is necessary for the effective use of Cry2Aa toxin.

Cry2Aa has dual activity against *Diptera* and *Lepidoptera* (19) but the efficacies against these two orders do not seem to be equal, because activity against *Helicoverpa armigera* (50% lethal concentration [LC_{50}], 1.57 μg/ml) was different from that of Cry1Ac (0.24 μg/ml) and the killing activity quickly increased, suggesting that Cry2Aa kills the target insect faster than Cry1Ac (3). Cry2Aa is active even against insects that are resistant to Cry1A (3), and all of these characteristics indicate the usefulness of Cry2A in preventing the emergence of resistant insects. The weak activity of Cry2A relative to that of Cry1Ac could be solved with gene technology in the future (6). Alternative uses of Cry1A and Cry2A to solve the problems with resistance have been suggested (18). To use Cry2A effectively, it is necessary to elucidate the killing mechanism of the Cry2A toxin. In this study, we demonstrate that the Cry2Aa 50-kDa peptide (P50) lacking the N termini α3 in domain I kills *Bobyx mori* and *Lymantria dispar* larvae.

Solubilization and purification of the Cry2Aa protoxin. *B. thuringiensis* asporogenic strain BT51 was transformed with pBC16.1 carrying the full-length *cry2Aa3* gene (13). The toxin crystals in BT51 were precipitated, suspended, and sonicated on ice at 20 kHz for 5 min, with 1-min rest intervals after each 1 min of sonication. The sequential treatment was repeated three more times, and finally the toxin was suspended with a small volume of distilled water. A suspension containing 1 mg of protein was incubated with 1 ml of 50 mM glycine-NaOH, pH 13, for 5 min at 37°C and centrifuged to recover the soluble toxin. The toxin was purified by fast-performance liquid chromatography (FPLC) with a HiLoad column (Superdex 200 pg; GE Healthcare Bio-Sciences), and a single major symmetrical peak was collected (termed the Cry2Aa protoxin). The amount of protein was quantified based on the absorbance at 280 nm or by the Bradford method (4).

Digestion of Cry2Aa protoxin with midgut fluid. One-day-old fifth-instar *B. mori* larvae of the Shunrei × Shogetsu hybrid strain which had been reared on a diet from Nosan Kogyo Co. (Yokohama, Japan) (1) were reared for one more day after molding and then kept for two more days without feeding. Midgut fluid (MF) was collected from *B. mori* larvae that had been shocked electrically, and the fluid was centrifuged at 100,000 × *g* to recover the native MF. One ml of MF was mixed with 0.5 mg of Cry2Aa protoxin in 50 mM Tris-HCl, pH 10 (Tris buffer 10) containing 50 mM NaCl at 25°C with gentle shaking for 120 min. The digested protein was analyzed with SDS-PAGE as described by Laemmli (11), and the proteins were stained with Coomassie brilliant blue (CBB). The Cry2Aa protoxin disappeared within 1 min, and 58-kDa (P58) and P50 fragments appeared (Fig. 1, left panel).

Western blotting of the digests was performed with Cry2Aa antiserum using a Hybond-P polyvinylidene difluoride membrane (GE Healthcare Bio-Sciences) (Fig. 1, right) (17). Cry2Aa protoxin, P58, and P50 were detected. No band was detected in the MF (Fig. 1, right, lane MJ). P50 and P58 seemed to be resistant to digestion with MF, because the intensities of each peptide were the same after 120 min and 30 min of digestion.

Digestion of Cry2Aa protoxin with chymotrypsin. We previously demonstrated that Cry2Aa crystals from BT51 were very trypsin resistant but were susceptible to chymotrypsin, although the reasons for this pattern of resistance and susceptibility are

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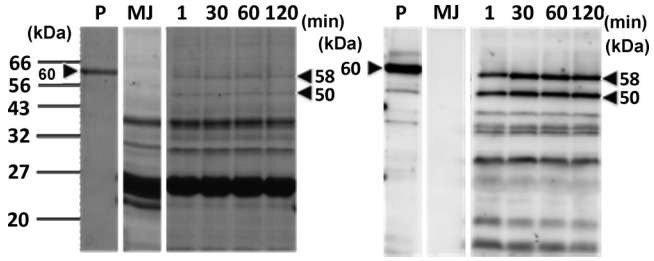

FIG 1 SDS-PAGE and Western blotting of Cry2Aa protoxin digested with midgut fluid. Purified Cry2Aa protoxin was digested with *B. mori* MF at 25°C for 1, 30, 60, and 120 min, and the digests were analyzed by SDS-PAGE. (Left) Migration profile of the digests stained with Coomassie brilliant blue. (Right) Western blotting of the digests. P, solubilized Cry2Aa protoxin; MJ, MF.

unknown. In this study, Cry2Aa protoxin was digested with α-chymotrypsin (pancreas, type II; Sigma-Aldrich Chemicals) at a 1:50 (wt/wt) ratio with gentle shaking for 2 h at 37°C and profiled using SDS-PAGE (Fig. 2, left panel) and Western blotting (Fig. 2, right). P60 and P58 were present, and P58 appeared to be chymotrypsin resistant. However, when the Cry2Aa protoxin was treated with 5-fold-concentrated chymotrypsin for 60 min, P58 almost completely disappeared (Fig. 2, left and right), whereas P50 was still present after the 120-min digestion. These data suggest that P58 may be a precursor of P50, which may be chymotrypsin resistant.

We used chymotrypsin at a pH of 13, but the optimum pH for its activity is 7.5; therefore, Cry2Aa protoxin was digested at pH 7.5. As expected, a 2-h treatment almost completely digested P60 and P58 and only P50 remained along, with several smaller fragments (data not shown).

Purification of P50. Cry2Aa protoxin was digested with chymotrypsin at pH 7.5, and the resulting fragments were separated chromatographically with FPLC. The major peak obtained was shown to be the 50-kDa toxin, based on SDS-PAGE and Western blotting (data not shown). Unfortunately, it was difficult to obtain a large amount of purified P58 due to contamination by the P60 protoxin.

N-terminal amino acid sequence of P50. The N-terminal AAS of P50 was determined with a PPSQ-20 peptide analyzer (Shimadzu, Japan). Although the purification of P58 was difficult, a small amount of P58 was obtained from the SDS-PAGE gel. The AAS of 11 residues from P58 and P50 were VAPVVGTVSSF and SITSSVNTMQQ, respectively; these sequences correspond to the region from the valine at residue 50 to the phenylalanine at residue 60 and from the serine at residue 145 to the glutamine at residue 155 of Cry2Aa protoxin, respectively (12). The Y/V and L/S linkages that were hydrolyzed match chymotrypsin recognition sites and were located just before the starting point of the α-1 and α-4 helices, respectively, in domain I.

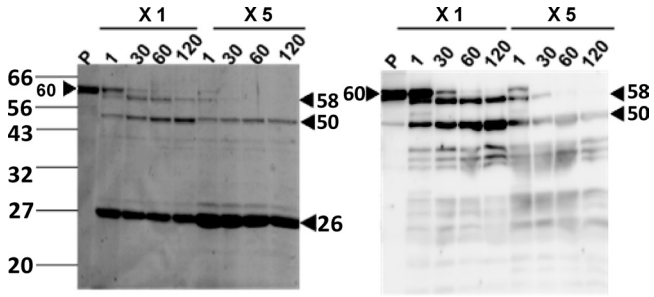

FIG 2 SDS-PAGE and Western blot analysis of digests of Cry2Aa protoxin treated with chymotrypsin. Purified Cry2Aa protoxin was digested with chymotrypsin for various periods, and the digests were analyzed by SDS-PAGE. The proteins were detected with CBB staining (left) and with anti-Cry2Aa antiserum (right). The numerals shown above each lane represent the digestion period. Lanes marked as ×5 indicate the toxin was digested with 5-fold-concentrated chymotrypsin. P, Cry2Aa protoxin.

TABLE 1 Insecticidal activities of Cry2Aa protoxin and P50 against *B. mori* and *L. dispar*[a]

Species and expt	Cry2Aa protoxin		P50	
	LD_{50} or LC_{50}[a] (95% CL)[a]	Slope (SE)	LD_{50} or LC_{50} (95% CL)[a]	Slope (SE)
B. mori				
Expt 1	0.41 (0.19–0.62)	5.07 (1.59)	0.36 (0.21–0.63)	4.99 (1.43)
Expt 2	0.38		0.35	
L. dispar				
Expt 1	0.26 (0.02–1.22)	2.57 (0.45)	0.13 (0.10–0.17)	4.69 (1.10)
Expt 2	0.25 (0.18–0.34)	3.11 (0.62)	0.14 (0.10–0.19)	3.89 (0.84)

[a] For *B. mori*, the activity reported is the LD_{50} (reported as microgram per larva), while the *L. dispar* activity reported is the LC_{50} (in units of nanogram per square centimeter of leaf). CL, confidence limit.

Bioassay with Cry2Aa protoxin and P50. The activities of Cry2Aa protoxin and P50 were estimated. Purified P58 was not available, as mentioned above. The purity of the toxins was confirmed by SDS-PAGE and Western blotting. Toxin in 20 μl of Tris buffer 10 containing 6% sucrose (final concentration) was administered orally to 10 2-day-old fourth-instar *B. mori* larvae at each concentration, and the 50% lethal dose (LD_{50}) was calculated based on mortality on day 7 using a probit model (9). The assay was performed twice.

L. dispar eggs were collected from the field, and larvae were reared on azalea leaves. The leaves were cut into 15- by 25-mm fragments and soaked in 37.5 μl of Tris Buffer 8.3 containing the toxin at various concentrations. After drying the toxin, the leaves were given to 12 larvae in a 12-well titer plate for 10 days at 25°C (16), and the LC_{50} was calculated (9) in two different experiments on different days.

LD_{50} values for the protoxin and P50 against *B. mori* were 0.40 and 0.36 μg/larva, respectively (Table 1). The LC_{50} of the protoxin and P50 against *L. dispar* were 0.26 and 0.14 ng/cm^2 leaf, respectively.

The insect receptor protein for Cry2Aa has been suggested to be different from that for Cry1A toxins (5, 7, 15), and this difference explains why Cry2A kills even Cry1A-resistant insects (18). The use of Cry2A has been applied to prevent the development and spread of resistant insects (5), and Cry1Ac and Cry2Ab have been combined into the GM cotton Bollgard II (http://www.monsanto.co.jp/biotech/development/insect.shtml). The usefulness of Cry2A in a Bt toxin formulation seems to be clear, and it is important to further investigate the mechanism by which Cry2A kills insects.

In this study, we demonstrated that chymotrypsin completely digested the 60-kDa Cry2Aa protoxin, yielding P50 via a P58 intermediate. The P58 and P50 fragments were generated by removing 49-mer and 116-mer peptides, respectively, from the N termi-

nus of the Cry2Aa protoxin, and the former and latter peptides contained the N termini α-0 helix and N termini α-3 helix, respectively.

P50 is resistant to chymotrypsin, and both Cry2Aa protoxin and P50 killed *B. mori* and *L. dispar*. The activities of these two toxins against *B. mori* were almost the same, but P50 exhibited a 2-fold-higher activity against *L. dispar* than the protoxin. If these results are correct, then the α-0∼α-3 helix region must not be necessary for the protein to exert its toxic effects against both insects. Furthermore, the removal of this region was effective in increasing the insecticidal activity against *L. dispar* but not against *B. mori* (Table 1). This suggests two possibilities: (i) the roles of the α-0∼α-3 region for the toxic activity are different in *B. mori* and *L. dispar*, or (ii) the MF of *L. dispar* cannot correctly process the Cry2Aa protoxin to P50. If the former is true, P50 must be easier to insert into epithelial membranes in *L. dispar* than in *B. mori*. If the latter is correct, the correctly processed protoxin will kill *L. dispar* as effectively as P50. However, in either case, it should be emphasized again that the N termini α-3 region is not necessary to kill either insect. If the bioassay of P58 and the digestion of Cry2Aa protoxin with *L. dispar* MF yield positive results, the reason why P50, which lacks the N termini α-3 region, can kill *L. dispar* and *B. mori* must be investigated in the future.

Previously, the 50-kDa Cry2Aa1 was reported to exhibit no toxicity against *L. dispar* (>5,000 ng/cm^2) (2), but we showed that P50 killed *L. dispar* effectively at a level of 0.14 ng/cm^2. When comparing these toxicities, however, it is important to consider the differences, if they exist, between the toxins Cry2Aa3 and Cry2Aa9 (used by the previous study group) and between the Asian type of *L. dispar* and the European type of *L. dispar* (used by the other group).

REFERENCES

1. **Asano S, Jr, et al.** 1993 Evaluation of biological activity of *Bacillus thuringiensis* test samples using a diet incorporation method with diamondback moth, *Plutella xylostella* (Linnaeus) (Lepidoptera: Yponomeutidae). Appl. Entomol. Zool. **28**:513–524.
2. **Audtho M, Valaitis AP, Alzate O, Dean DH.** 1999. Production of chymotrypsin-resistant *Bacillus thuringiensis* Cry2Aa1 delta endotoxin by protein engineering. Appl. Environ. Microbiol. **65**:4601–4605.
3. **Babu BG, Udayasuriyan V, Mariam MA, Sivakumar NC, Bharathi M.** 2002. Comparative toxicity of Cry1Ac and Cry2Aa delta-endotoxins of *Bacillus thuringiensis* against *Helicoverpa armigera* (H.). Crop Prot. **21**: 817–822.
4. **Bradford MM.** 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.
5. **Carmen SH, Adri VV, Nadine B, Jeroen VR, Juan F.** 2008. Specific binding of *Bacillus thuringiensis* Cry2A insecticidal proteins to a common site in the midgut of *Helicoverpa* sp. Appl. Environ. Microbiol. **74**:7654–7659.
6. **Chandi CM, et al.** 2007. Prediction-based protein engineering of domain I of Cry2A entomocidal toxin of *Bacillus thuringiensis* for the enhancement of toxicity against lepidopteran insects. Protein Eng. Design Select. **20**:599–606.
7. **English L, et al.** 1994. Mode of action of Cry IIA: a *Bacillus thuringiensis* delta-endotoxin. Biochem. Mol. Biol. **24**:1025–1035.
8. **Ferré J, Van Rie J.** 2002. Biochemistry and genetics of insect resistance to *Bacillus thuringiensis*. Annu. Rev. Entomol. **47**:501–533.
9. **Finney DJ.** 1971. Probit analysis, 3rd ed, p 318. University Press, Cambridge, England.
10. **Grochulski P, et al.** 1995. *Bacillus thuringiensis* Cry1A (a) insecticidal toxin: crystal structure and channel formation. J. Mol. Biol. **254**:447–464.
11. **Laemmli UK.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**:680–685.
12. **Morse RJ, Yamamoto T, Stroud RM.** 2001. Structure of Cry2Aa suggests an unexpected receptor binding epitope. Structure **9**:409–417.
13. **Sasaki J, et al.** 1997. Characterization of a cry2A gene cloned from an isolate of *Bacillus thuringiensis* serovar Sotto. Curr. Microbiol. **35**:1–8.
14. **Schnepf E, et al.** 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol. Mol. Biol. Rev. **62**:775–806.
15. **Shahid K, Riazuddina S, Gould F, Dean DH.** 2000. Determination of receptor binding properties of *Bacillus thuringiensis* δ-endotoxins to cotton bollworm (*Helicoverpa zea*) and pink bollworm (*Pectinophora gossypiella*) midgut brush border membrane vesicles. Pest. Biochem. Physiol. **67**:198–216.
16. **Shirai Y, Takahashi M.** 2005. Effects of transgenic Bt corn pollen on a non-target lycaenid butterfly, *Pseudozizeeria maha*. Appl. Entomol. Zool. **40**:151–159.
17. **Shitomi Y, et al.** 2006. A novel 96-kDa aminopeptidase localized on epithelial cell membranes of *Bombyx mori* midgut, which binds to Cry1Ac toxin of *Bacillus thuringiensis*. J. Biochem. **139**:223–233.
18. **Tabashnik BE, et al.** 2002. Control of resistant pink bollworm (*Pectinophora gossypiella*) by transgenic cotton that produces *Bacillus thuringiensis* toxin Cry2Ab. Appl. Environ. Microbiol. **68**:3790–3794.
19. **Widner WR, Whiteley HR.** 1989. Two highly related insecticidal crystal proteins of *Bacillus thuringiensis* subsp. *kurstaki* possess different host range specificities. J. Bacteriol. **171**:965–974.
20. **Yamamoto T, McLaughlin RE.** 1981. Isolation of a protein from the parasporal crystal of *Bacillus thuringiensis* var. *kurstaki* toxic to the mosquito larva, *Aedes taeniorhynchus*. Biochem. Biophys. Res. Commun. **103**: 414–421.