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A SPECIFIC BINDING PROTEIN FROM TENEBRIO MOLITOR FOR THE INSECTICIDAL TOXIN OF BACILLUS THURINGIENSIS SUBSP. TENEBRIONIS

Carol J. Belfiore, Ratna K. Vadlamudi, Yehia A. Osman* and Lee A. Bulla, Jr.[1]

Department of Molecular Biology, University of Wyoming, P. O. Box 3944, Laramie, WY 82071
*Institute for Agricultural and Genetic Engineering, 9 Gamaa St., Giza 12619, Egypt

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SUMMARY Biopesticides based on the bacterium Bacillus thuringiensis have attracted wide attention as safe alternatives to chemical insecticides. In this paper, we report, for the first time, the identification of a single binding protein from a coleopteran insect, Tenebrio molitor, that is specific for the *cryIII* toxin of B. thuringiensis. The protein appeared as a single band of 144 kDa on radioligand and immunoblots of total proteins extracted from brush border membrane vesicles of the midgut of T. molitor. Radiolabelled *cryIIIA* toxin bound to the protein with a K_d value of 17.5 nM and could be specifically blocked by unlabelled toxin but not by toxins from other subspecies of B. thuringiensis. This study lays the groundwork to clone the *cryIIIA* toxin binding protein and to determine the molecular mechanism(s) of toxin action.

Bacillus thuringiensis is a Gram-positive spore-forming bacterium that produces a parasporal crystal during the sporulation process. The crystalline material is glycoprotein and possesses insecticidal properties specific to three orders of insects (1). Insect host specificity is dependent on the presence and expression of a particular *cry* gene(s) of B. thuringiensis. For example, the *cryI* gene product is toxic to lepidopteran insects (moths); that of *cryII* to lepidopteran and dipteran insects (such as mosquitoes and black flies); *cryIII* to coleopteran insects (beetles); and *cryIV* to dipterans, exclusively (2).

Toxicity appears to involve similar mechanisms among the affected orders of insects. Initial symptoms of toxicity include mouth and gut paralysis and cessation of feeding (3, 4), as well as disruption of K^+ transport across the epithelial cell membrane (5-7). Manifestations of toxicity are evidenced by disruption of epithelial cell ultrastructure and cell lysis (4, 8, 9). The proposal by Knowles and Ellar (10) that formation of pores in the luminal plasma membrane precedes osmotic cell lysis suggests that the toxin interacts with the plasma membrane to initiate toxic effects. Indeed, recent determination of the tertiary structure of the *cryIII* toxin of B. thuringiensis subsp. tenebrionis (11) indicates a potential membrane-spanning domain which probably is highly conserved among all the various *cry* gene products.

There have been several reports on the interaction of *cryI* toxins with brush-border membrane vesicles (BBMV) of lepidopteran insects (12-20). However, to our knowledge, no

[1]To whom correspondence should be addressed. FAX:(307)766-3875.

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such information has been presented for coleopteran insects. In this paper, we report, for the first time, the identification of a single binding protein from a coleopteran (beetle) insect, T. molitor, for the insecticidal toxin of B. thuringiensis subsp. tenebrionis.

MATERIALS AND METHODS

Bacterial Strains, Growth Conditions, Parasporal Crystal Isolation and Toxin Purification Bacillus thuringiensis subsp. tenebrionis (BTT) and israelensis (BTI) were obtained from R. M. Faust, Beltsville Agricultural Research Center, Beltsville, MD. Bacillus thuringiensis subsp. berliner (BTB) was obtained from M.-M. Lecadet, Institut Pasteur, Paris, France. Growth of BTT, BTI and BTB and isolation of their parasporal crystals were accomplished as described by Vadlamudi et al. (13), Hurley et al. (21) and Tyrell et al. (22), respectively. Purification of *cryIA(b)* and *cryIVD* toxins was accomplished according to Vadlamudi et al. (13). Crystals of *cryIIIA* were solubilized in 3.3 M NaBr, pH 7.0, 50 mM phosphate buffer containing 1 mM PMSF. Insoluble papain beads were activated as described by Louvard et al. (23), and added to the solubilized *cryIIIA* toxin to effect cleavage to a 67 kDa toxin. Cleavage was arrested by addition of Na-p-tosyl-L-lysine chloromethyl ketone (TLCK) to 1.25 mg/ml and 0.2 volume of pH 10.0 Na_2CO_3. Cleaved toxin was purified by passage over Sephadex G-75 equilibrated with 50 mM Na_2CO_3, pH 10.0, containing 1 mM EDTA.

Insect Larvae and Reagents Mealworm larvae (T. molitor) and artificial diet were obtained from Carolina Biological Supply Co., Burlington, NC. Reagents, except specified otherwise, were obtained from Sigma Chemical Co., St. Louis, MO. The BCA protein reagent was purchased from Pierce, Rockford, IL, and Immobilon-P membrane from Millipore, Bedford, MA. Peroxidase-conjugated anti-rabbit IgG was purchased from Kirkigaard & Perry Laboratories, Gaithersburg, MD. Sephadex G-75 and CL4B protein A Sepharose beads were obtained from Pharmacia, Piscataway, NJ; acrylamide from Eastman Chemical Company, Rochester, NY and Na[^{125}I] from Amersham Corporation, Arlington Heights, IL.

Radioiodination *CryIIIA* toxin was radioiodinated using a variation of the procedure described by Fraker and Speck (24). Toxin (50 μg) was incubated with 500 μCi ^{125}I in a glass tube coated with 5 μg of 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril for 15 min at 25°C. The reaction was stopped by addition of 100 μl 0.5 M NaI in 50 mM Na_2CO_3 and labelled toxin was purified by passage over Sephadex G-50. Specific activity ranged from 8 to 16 μCi/μg toxin protein.

Preparation of BBMV BBMV were prepared from larval midgut tissue of T. molitor by the procedure of English and Readdy (25). Protease inhibitors (5 μg/ml pepstatin, 10 μg/ml antipain, aprotonin, leupeptin and iodoacetamide, and 1 mM PMSF) were present during homogenization. The final pellet was suspended in 320 mM sucrose and was used immediately or was frozen in liquid nitrogen and stored at -70°C. Protein concentration was determined by the BCA method (26).

Ligand Binding Assays Binding assays were carried out as described by Vadlamudi et al. (13) in TBS (50 mM Tris and 0.9% NaCl at pH 8.0) containing 0.5% BSA. Nonspecific binding was assessed as radioactivity bound in the presence of unlabelled *cryIIIA* toxin (3 μM). Binding in the presence of varying amounts of unlabelled *cryIIIA*, *cryIA(b)* or *cryIVD* toxin was measured and the data were analyzed by Scatchard analysis (27).

Radioligand Blotting Polyacrylamide gel electrophoresis was performed according to the procedure of Laemmli (28). Fifty μg of BBMV protein were electrophoresed (7%, SDS) and transferred to Immobilon-P membranes as described by Vadlamudi et al. (13). Lanes containing BBMV protein were incubated for 90 min at 25°C in TBS (pH 8.0) containing 5% non-fat dry milk powder, 5% glycerol and 0.5% Tween 20 to block nonspecific binding. Membrane strips then were incubated for 30 min at 25°C in blocking buffer containing $2x10^5$ cpm/ml of radiolabelled *cryIIIA* toxin in the presence or absence of unlabelled *cryIIIA*, *cryIA(b)* or *cryIVD* toxin. Following three washes with blocking buffer (10 min/wash), membrane strips were exposed to Kodak X-Omat AR x-ray film at -70°C for 1 to 2 days. Bound radioactive toxin was quantitated by counting excised bands in a Beckman γ counter.

Immunodetection of Ligand Binding BBMV proteins were electrophoresed, transferred and blocked as indicated above. Incubations with *cryIIIA* toxin (50 μg), primary antibody (2 hr) and secondary antibody (1 hr) were followed by three washes each with blocking buffer, followed finally by two washes with TBS. Primary antibody was raised in rabbit against purified *cryIIIA* toxin (67 kDa). Secondary antibody consisted of peroxidase-conjugated goat anti-rabbit IgG. Visualization of toxin-binding bands followed treatment with 3,3'-diaminobenzidine (0.2 mg/ml in TBS) and 5 μl 30% H_2O_2.

Immunoprecipitation of *CryIIIA* Toxin-Binding Protein Forty mg of protein A Sepharose beads were incubated for 1 hr at 4°C with 1 ml of *cryIIIA* toxin antiserum in wash buffer consisting of 50 mM Tris, 250 mM NaCl, 6 mM EDTA and 0.5% NP-40 (pH8.0). Following three washes, the beads were incubated with 80 μg of *cryIIIA* toxin for 1 hr at 4°C and washed again three times. BBMV were prepared from 1 gm of midgut tissue and proteins were solubilized for 45 min at 4°C in wash buffer containing protease inhibitors at the concentrations indicated above. Approximately 1 mg of solubilized BBMV protein was diluted in 0.3% NP-40 and incubated with Protein A Sepharose beads for 1 hr at 4°C. Beads were washed extensively with wash buffer containing 0.25% NP-40 and 0.02% SDS. The bound proteins were dissociated from the beads by solubilization in SDS. Proteins were electrophoresed, transferred and visualized by radioligand blotting or immunodetection as indicated above.

Insect Toxicity Assay Insect toxicity assays of purified *cry* toxins for Manduca sexta (tobacco hornworm), Aedes aegypti (mosquito) and Leptinotarsa decemlineata (Colorado potato beetle) were performed according to Vadlamudi et al. (13). Toxicity assays for T. molitor (yellow mealworm) was done precisely as described for M. sexta by Wabiko et al. (29).

RESULTS

Specific Toxicity of *Cry* Toxins

As can be seen in Table I, each of the *cry* toxins from the three different subspecies of B. thuringiensis was specific for only one insect. No toxicity was exhibited by *cryIA(b)* toxin against A. aegypti and L. decemlineata at the highest concentration used in the bioassay (2.5 X 10^4 ng/ml). The *cryIIIA* toxin, active against T. molitor (LC_{50} = 21 μg/cm^2), was not toxic to M. sexta and A. aegypti at the highest concentrations tested (500 ng/cm^2 and 2.5 x 10^4 ng/ml, respectively). *CryIVD* toxin was not effective against L. decemlineata and M. sexta at the highest concentration tested (2.5 x10^4 ng/ml). Because of these extreme specificities, we used the *cryIA(b)* and *cryIVD* toxins as controls in our ligand binding and blotting experiments.

Binding of *CryIIIA* Toxin to BBMV Proteins of T. molitor

Competitive binding of [^{125}I]*cryIIIA* toxin to BBMV of T. molitor was carried out in the presence of increasing concentrations of unlabelled *cryIIIA*, *cryIA(b)* or *cryIVD* toxins. In the absence of competitor, 60% of labelled *cryIIIA* toxin bound to the BBMV proteins. Unlabelled *cryIIIA* toxin at a concentration of 500 nM reduced binding of iodinated toxin to 10%, indicating specificity and saturability of binding (Fig. 1). Scatchard analysis of the binding data revealed a

TABLE I

LC_{50} (LC_{50} = lethal concentration that kills 50% of the insect population; negative values represent no toxicity at the concentrations indicated under "Results") values of B. thuringiensis *cry* toxins

Toxin	T. molitor	M. sexta	A. aegypti
cryIA(b)	--*	7.5 ng/cm^2	--
cryIIIA	21 μg/cm^2	--	--
cryIVD	--*	--	180 ng/ml

* Tested against Leptinotarsa decemlineata (Colorado potato beetle) in lieu of T. molitor.

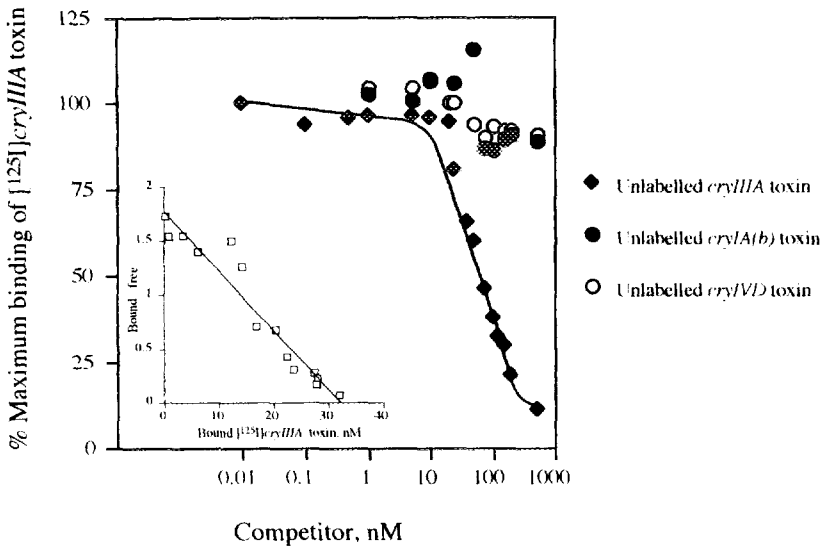

Fig. 1. Binding of [^{125}I]*cryIIIA* toxin to BBMV proteins of T. molitor. BBMV of T. molitor (10 μg protein) were incubated with [^{125}I]*cryIIIA* toxin (0.3 nM) in the presence of unlabelled *cryIIIA* toxin (◆), *cryIA(b)* toxin (●) or *cryIVD* toxin (○). The Scatchard plot of the data is shown in the inset.

single binding site having a K_d of 17.5 nM (mean of four assays, SEM=0.6) and binding site concentration of 304 pmol/mg BBMV protein (SEM=16.5). Neither *cryIA(b)* nor *cryIVD* toxin competed for the *cryIIIA* toxin binding site.

Identification of *CryIIIA* Toxin-Binding Protein by Radioligand Blotting

The pattern of Coomassie blue-stained BBMV proteins appears in Fig. 2, lane 1. Molecular size of the proteins ranged from approximately 30 to greater than 200 kDa. Incubation of [^{125}I]*cryIIIA* toxin with transferred BBMV proteins of T. molitor revealed a toxin binding protein of 144 kDa (Fig. 2, lane 2). In the presence of *cryIIIA* toxin, specific binding was reduced to less than 1% of binding observed in the absence of competitor; neither *cryIA(b)* nor *cryIVD* toxin caused a decrease in binding of [^{125}I]*cryIIIA* toxin to the 144-kDa protein (data not shown). The ligand blot in Fig. 2 was performed at pH 5.0 to simulate the pH conditions of the midgut of T. molitor. However, succeeding ligand blots were carried out at pH 8.0 to approximate the conditions present in the ligand binding assays. Incubation at pH 8.0 produced a pattern similar to that at pH 5.0.

Immunoprecipitation of the *CryIIIA* Toxin Binding Protein

To confirm specificity of binding of *cryIIIA* toxin to the 144-kDa BBMV protein of T. molitor, immunoprecipitation of the protein was performed as indicated in **Materials and Methods**. Radioligand blotting and immunoblotting procedures revealed a band of 144 kDa (Fig. 3). No immunoprecipitate was detected when pre-immune serum or antibody to *cryIA(b)* toxin was substituted for anti-*cryIIIA* toxin antibody. The fainter, lower-molecular-weight bands probably are the result of protein degradation. Protein bands identified as *cryIIIA* toxin and immunoglobulins were detected in immunoblots (Fig. 3, lane 1).

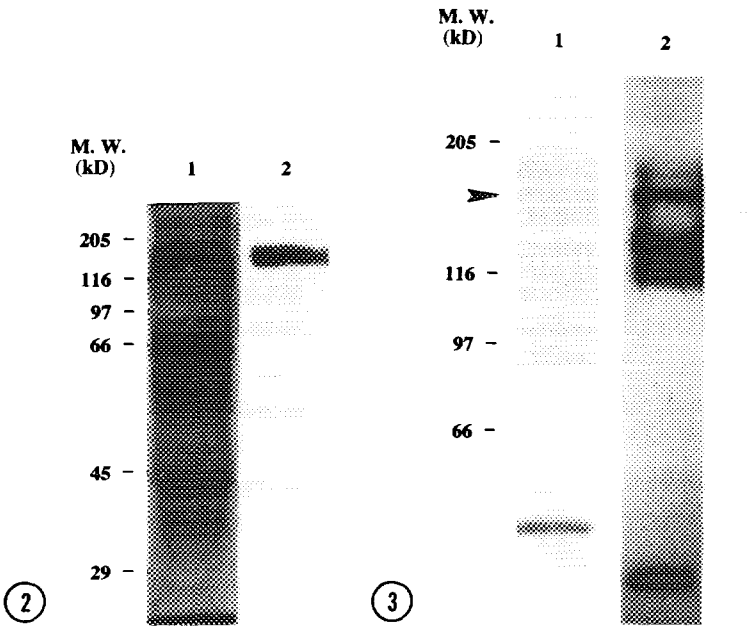

Fig. 2. Binding of [^{125}I]*cryIIIA* toxin to BBMV proteins of T. molitor by radioligand blotting. Solubilized BBMV proteins, separated by SDS-polyacrylamide gel electrophoresis and transferred to Immobilon-P membrane, were incubated with [^{125}I]*cryIIIA* toxin. Membrane strips were subjected to autoradiography. Lane 1, Coomassie blue-stained BBMV proteins. Lane 2, sample blotted with [^{125}I]*cryIIIA* toxin. Position of molecular weight markers is indicated on the left: myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45k Da) and carbonic anhydrase (29 kDa).

Fig. 3. Immunoprecipitation of the *cryIIIA* toxin binding protein. The *cryIIIA* toxin binding protein was precipitated by addition of *cryIIIA* toxin-antitoxin-protein A-Sepharose complex to solubilized BBMV proteins from T. molitor. Precipitated proteins were dissolved in SDS and separated by SDS polyacrylamide gel electrophoresis. The binding protein was detected by blotting with anti-*cryIIIA* toxin antibody (lane 1, indicated by arrow) or [^{125}I]*cryIIIA* toxin (lane 2). Molecular weight markers are as indicated in Fig. 2.

DISCUSSION

There is sufficient evidence to suggest that toxin-binding proteins (receptors) in insect midgut tissue determine target specificity of the *cry* toxins (2). Our data reveal that such a target molecule exists in BBMV of T. molitor. Ligand binding assays showed a single high-affinity binding site with a K_d of 17.5 nM (Fig. 1). This value represents a somewhat lower affinity than those reported previously for the binding of *cryI* toxins to BBMV of susceptible lepidopteran insects (13-16, 18). Such a result may indicate that the susceptibility of T. molitor to the *cryIIIA* toxin is somewhat less than that of M. sexta to *cryIA(b)* toxin (Table I). Recent studies in our laboratory revealed a K_d value of 708 pM for binding of *cryIA(b)* toxin to BBMV of M. sexta (13). Interestingly, we calculated the concentration of binding sites in M. sexta for *cryIA(b)* toxin to be three pmol/mg of BBMV protein, a value 100-fold lower than the binding site concentration for T.

molitor reported in the present study. Such a relationship of reduced affinity with increased binding site concentration has been noted in lepidopteran insects that are variably resistant to *cryI* toxins (17).

Binding specificity was indicated by the inability of either the *cryIA(b)* or *cryIVD* toxin to decrease binding of [^{125}I]*cryIIIA* toxin to BBMV of T. molitor (Fig. 1) and by the lack of competition by these toxins as evidenced in the [^{125}I]*cryIIIA* ligand blotting experiments. Radioligand blotting (Fig.2) revealed a protein of 144 kDa to which the [^{125}I]*cryIIIA* toxin binds. Binding of unlabelled *cryIIIA* toxin to a protein of the same molecular weight was observed following immunoprecipitation with antibody raised against *cryIIIA* toxin (Fig. 3).

To our knowledge, this paper identifies, for the first time, a binding protein in BBMV of a coleopteran insect for the *cryIIIA* toxin of B. thuringiensis subsp. tenebrionis. Further investigation of the biochemical properties of this protein should provide a better understanding of the molecular mechanisms of binding specificity and the molecular basis of insecticidal action of the *cryIIIA* toxin of BTT.

REFERENCES

1. Miller, L. K., Lingg, A. J. and Bulla, L. A., Jr. (1983) Science 219, 715-721.
2. Knowles, B. H. and Dow, J. A. T. (1993) BioEssays 15, 469-476.
3. Lüthy, P. and Ebersold, H. R. (1984) In Pathogenesis of Invertebrate Microbial Diseases, pp. 235-267. Allanheld, Osmun Publishers, Totowa, NJ.
4. Aronson, A. I., Beckman, W. and Dunn, P. (1986) Microbiol. Rev. 50, 1-24.
5. Griego, V. M., Moffett, D. and Spence, K. D. (1979) J. Insect Physiol. 25, 283-288.
6. Harvey, W. R. and Wolfersberger, M. G. (1979) J. Exp. Biol. 83, 293-304.
7. Sacchi, V. F., Parenti, P. et al. (1986) FEBS Lett. 204, 213-218.
8. Nishiitsutsuji-Uwo, J., Endo, Y. and Himeno, M. (1979) J. Invertebr. Pathol. 34, 267-275.
9. Percy, J. and Fast, P. G. (1983) J. Invertebr. Pathol. 41, 86-98.
10. Knowles, B. H. and Ellar, D. J. (1987) Biochim. Biophys. Acta 924, 509-518.
11. Li, J., Carroll, J. and Ellar, D. J. (1991) Nature 353, 815-821.
12. Hofmann, C. and Lüthy, P. (1986) Arch. Microbiol. 146, 7-11.
13. Vadlamudi, R. K., Ji, T. H. and Bulla, L. A., Jr. (1993) J. Biol. Chem. 268, 12334-12340.
14. Garczynski, S. F., Crim, J. W. and Adang, M.J. (1991) Appl. Environ. Microbiol. 57, 2816-2820.
15. Van Rie, J., Jansens, S. et al. (1989) Eur. J. Biochem. 186, 239-247.
16. Van Rie, J., Jansens, S. et al. (1990) Appl. Environ. Microbiol. 56, 1378-1385.
17. MacIntosh, S. C., Stone, T. B. et al. (1991) Proc. Natl. Acad. Sci. USA 88, 8930-8933.
18. Denolf, P., Jansens, S. et al. (1993) Appl. Environ. Microbiol. 59, 1828-1837.
19. Oddou, P., Hartmann, H. and Geiser, M. (1991) Eur. J. Biochem. 202, 673-680.
20. Hofmann, C., Vanderbruggen, H. et al. (1988) Proc. Natl. Acad. Sci. USA 85, 7844-7848.
21. Hurley, J. M., Lee, S. G. et al. (1985) Biochem. Biophys. Res. Commun. 126, 961-965.
22. Tyrell, D. J., Bulla, L. A., Jr. et al. (1981) J. Bacteriol. 145, 1052-1062.
23. Louvard, D., Maroux, S. et al. (1975) Biochim. Biophys. Acta 375, 236-248.
24. Fraker, P. J. and Speck, J. C., Jr. (1978) Biochem. Biophys. Res. Commun. 80, 849-857.
25. English, L. H. and Readdy, T. L. (1989) Insect Biochem. 19, 145-152.
26. Smith, P. K., Krohn, R. I. et al. (1985) Anal. Biochem. 150, 76-85.
27. Scatchard, G. (1949) Ann. N. Y. Acad. Sci. 51, 660-672.
28. Laemmli, U. K. (1970) Nature 227, 680-685.
29. Wabiko, H., Held, G. A. and Bulla, L. A., Jr. (1985) Appl. Environ. Microbiol. 49, 706-708.