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Binding of *Bacillus thuringiensis* Cry1Ac Toxin to Aminopeptidase in Susceptible and Resistant Diamondback Moths (*Plutella xylostella*)

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Bacillus thuringiensis **Cry1Ac toxin bound to a 120-kDa protein isolated from the brush border membranes of both susceptible and resistant larvae of *Plutella xylostella*, the diamondback moth. The 120-kDa protein was purified by Cry1Ac toxin affinity chromatography. Like Cry1Ac-binding aminopeptidase N (EC 3.4.11.2) from other insects, this protein was eluted from the affinity column with 200 mM *N*-acetylgalactosamine. The purified protein had aminopeptidase activity and bound Cry1Ac toxin on ligand blots. Purified aminopeptidase was recognized by antibodies to the cross-reacting determinant found on phosphatidylinositol-specific phospholipase C-solubilized proteins. The results show that the presence of Cry1Ac-binding aminopeptidase in the brush border membrane is not sufficient to confer susceptibility to Cry1Ac. Furthermore, the results do not support the hypothesis that resistance to Cry1Ac was caused by lack of a Cry1Ac-binding aminopeptidase.**

Bacillus thuringiensis Cry1 δ-endotoxins are specifically toxic to insect larvae and uniquely valuable proteins for controlling agricultural pests (2). *B. thuringiensis* δ-endotoxins are applied as biopesticides on vegetables, field crops, and forests. Transgenic corn, cotton, and potatoes expressing *B. thuringiensis* toxins are now part of pest management programs in the United States. Evolution of resistance by pests is a serious threat to the long-term use of these proteins (25). Laboratory selection has produced resistant strains of many insects, including the moths *Heliothis virescens*, *Plodia interpunctella*, and *Spodoptera exigua* (8, 15, 18, 19, 24, 25). The first insect to evolve resistance to *B. thuringiensis* in open field populations is the diamondback moth, *Plutella xylostella* (4, 26).

In *P. xylostella*, the primary resistance mechanism is thought to be reduced binding of Cry1A toxins to the midgut brush border membrane (1, 4, 27, 29). However, some results (3, 17) indicate that Cry1Ac binds to the midgut membranes of resistant larvae of *P. xylostella* derived from the same strain previously investigated by Tabashnik et al. (27). Results with *H. virescens*, *P. interpunctella*, and *S. exigua* suggest that mechanisms other than reduced binding are involved in resistance to *B. thuringiensis* toxins (8, 12, 15, 19, 22).

Cry1Ab, Cry1Ac, and Cry1C toxin-binding proteins have been identified and purified from *Manduca sexta*, *H. virescens*, and *Lymantria dispar* (7, 9, 14, 23, 30, 32). The Cry1Ab receptor was reported to be a cadherin-like glycoprotein (31). Cry1Ac and Cry1C toxin receptors are members of the aminopeptidase N (APN) (EC 3.4.11.2) family (7, 9, 14, 23, 32). The 120-kDa APN has a C-terminal glycosyl-phosphatidylinositol (GPI) anchor (5). This anchor can be cleaved by an endogenous phosphatidylinositol-specific phospholipase C (PIPLC), resulting in conversion of the 120-kDa APN to a soluble form of 115-kDa APN (13). The 115-kDa form binds to Cry1Aa, Cry1Ab, and Cry1Ac toxins but not to Cry1C toxin (16). Functional studies in vitro showed that, when incorporated into phospholipid vesicles, a mixture of the 120-kDa APN and 65-kDa protein could lower by as much as 1,000-fold the concentration of Cry1Ac toxin required to induce ion-conducting channels (23). This was the first evidence that the 120-kDa APN functions as a Cry1Ac receptor in vitro. Knight et al. (10) cloned the 120-kDa-APN gene from *M. sexta*.

In this study we report the purification and identification of a 120-kDa Cry1Ac-binding APN from susceptible and resistant strains of *P. xylostella*. Toxin-binding APN was analyzed for a GPI anchor and evidence of cleavage by a midgut PIPLC.

MATERIALS AND METHODS

Insects. The susceptible strain (LAB-P) was derived from insects collected from Pulehu, Maui, Hawaii (26), and had been reared in the laboratory for >80 generations without exposure to any insecticide. The resistant (NO-QA) strain was derived from insects collected from a watercress farm near Pearl City, Oahu, Hawaii. Repeated exposure to sprays of commercial formulations of *B. thuringiensis* in the field followed by laboratory selection with *B. thuringiensis* had produced extremely high resistance in the NO-QA strain (27, 28).

Purification and iodination of Cry1Ac. Growth of *B. thuringiensis* HD-73, trypsin activation, and fast-performance liquid chromatography (Pharmacia) purification of Cry1Ac toxin were done as previously described (13). Iodination of toxin with iodine-125 using chloramine T was done according to Garczynski et al. (6). Specific activity was 20.5 mCi/mg of input toxin.

Preparation of brush border membrane vesicles (BBMV). Fourth-instar larvae were stored frozen at −70°C. Larvae were thawed, midguts were dissected, and BBMV were prepared by the $MgCl_2$ precipitation method (33) as modified by Ferre et al. (4). The final BBMV pellet was suspended in 0.3 M mannitol–5 mM EGTA–17 mM Tris-Cl, pH 7.5, and stored at −80°C until use.

Affinity purification of Cry1Ac-binding proteins. We performed affinity purification of toxin-binding proteins as described by Lu and Adang (13). BBMV from susceptible and resistant strains were solubilized with 1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulfonate (CHAPS) in buffer A (50 mM Na_2CO_3, pH 9.6, 200 mM NaCl, 5 mM EGTA, 0.1% CHAPS) containing 1 mM phenylmethylsulfonyl fluoride, 4 μM leupeptin, 10 μM aprotinin, and 100 μM pepstatin. The mixture was placed on ice for 30 min. Insoluble material was removed by centrifugation at 27,000 × *g* for 30 min at 4°C. CHAPS-solubilized BBMV (1 mg) were added to 1 ml of Cry1Ac-Sepharose beads equilibrated with buffer A. The gel-BBMV mixture was incubated overnight at 4°C with gentle rotation. After washing with 100 ml of buffer A, bound proteins were eluted from the column with 2 ml of 200 mM *N*-acetylgalactosamine. Eluted proteins were concentrated to 0.2 ml with a Centriprep-30 ultrafiltration device (Amicon, Inc.) and stored at −80°C until analysis.

SDS-PAGE and protein blot analyses. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (11). After SDS-PAGE, proteins were transferred to a nitrocellulose membrane and probed with ^{125}I-labeled Cry1Ac toxin (6).

GPI-anchor detection. The Cry1Ac column eluate was separated by SDS-PAGE (10% polyacrylamide) and blotted onto a nitrocellulose membrane. After

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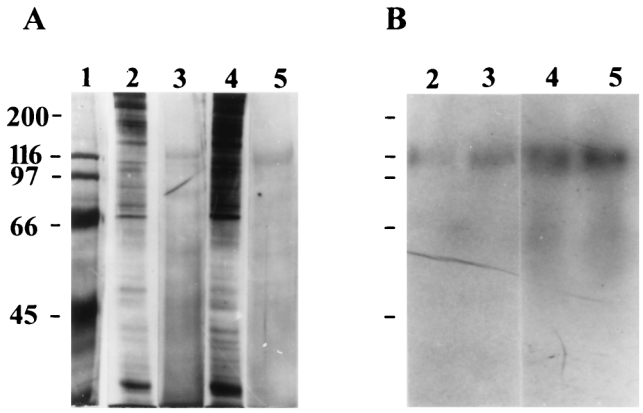

FIG. 1. SDS-PAGE (A) and ligand blot (B) analyses of solubilized BBMV and affinity-purified Cry1Ac-binding proteins from the resistant and susceptible larvae of *P. xylostella*. Lane 1, molecular size markers (in kilodaltons); lane 2, BBMV proteins (3 μg) of the resistant strain; lane 3, affinity-purified Cry1Ac-binding proteins (0.5 μg) from the resistant strain; lane 4, BBMV proteins (3 μg) of the susceptible strain; lane 5, affinity-purified Cry1Ac-binding proteins (0.6 μg) from the susceptible strain.

blocking with 50 mM Tris-HCl (pH 7.4)–150 mM NaCl–1 mM EDTA–0.5% Tween 20 containing 3% bovine serum albumin at room temperature for 2 h, the membrane was incubated either with PIPLC (1.5 U) (Sigma) or without PIPLC in 10 ml of Tris buffer containing 20 mM Tris-HCl (pH 7.4)–0.1% Triton X-100–1 mM dithiothreitol at room temperature overnight. The membrane was then probed with a polyclonal antibody against the cross-reacting determinant (CRD) epitope present on PIPLC-cleaved GPI-anchored proteins (anti-CRD serum was kindly provided by K. Mensa-Wilmot, University of Georgia). The GPI-anchored proteins were visualized with alkaline phosphatase-conjugated secondary antibodies (Sigma) and nitroblue tetrazolium-bromochloroindolyl phosphate as described by Garczynski and Adang (5).

APN activity assays. APN activity was assayed with L-leucine-*p*-nitroanilide as the substrate in a kinetic microplate reader (Molecular Devices) (5).

RESULTS

Affinity purification and ligand blot analysis of Cry1Ac-binding protein. Cry1Ac-affinity selection (Fig. 1A, lanes 3 and 5) and ligand blot analyses of total BBMV proteins (Fig. 1B, lanes 2 and 4) and Cry1Ac affinity-selected protein (Fig. 1B, lanes 3 and 5) identified a 120-kDa protein as the major binding protein in BBMV from both susceptible and resistant *P. xylostella* larvae. No difference was detected in the amount of 120-kDa binding protein between the insect strains. Minor amounts of smaller proteins (<100 kDa) coeluted from the Cry1Ac column. Some toxin binding is visible in a diffuse region near 65 kDa on the ligand blot (Fig. 1B).

Identification of the 120-kDa protein as APN. The 120-kDa protein purified by Cry1Ac-affinity chromatography hydrolyzed leucine-*p*-nitroanilide substrate with a specific activity of 24.3 μmol/min/mg for susceptible larvae and 34.5 μmol/min/mg for resistant NO-QA larvae, representing 19- and 31-fold enrichment compared with *P. xylostella* BBMV, respectively (Table 1). The difference in activity between susceptible and resistant larvae is not significant ($P > 0.05$). Specific activity was greatly reduced by the APN inhibitors amastatin and EDTA (Table 1). Based on these activity data and the protein's similarity in molecular size to *M. sexta* and *L. dispar* APN, we conclude that the 120-kDa protein is APN.

GPI anchor property of the 120-kDa APN. A polyclonal antibody raised to the PIPLC-treated form of *Trypanosoma brucei* variant surface glycoprotein can cross-react with other, unrelated, GPI-anchored proteins at the CRD (5). Figure 2 shows that both untreated 120-kDa APN (Fig. 2A) and PIPLC-treated APN (Fig. 2B) reacted with the anti-CRD antibody, indicating that the toxin-binding APN from *P. xylostella* has a GPI anchor that was cleaved by a midgut PIPLC.

TABLE 1. APN activity in BBMV and affinity-purified binding proteins from susceptible and resistant strains of *P. xylostella*

Treatment	Strain type	Sp act (μmol/min/mg)[a]	
		BBMV	Affinity-purified protein
No inhibitor	Susceptible	1.3 ± 0.1	24.3 ± 0.7
	Resistant	1.1 ± 0.2	34.5 ± 3.2
1 mM amastatin	Susceptible	0.1 ± 0.0	1.0 ± 0.1
	Resistant	0.1 ± 0.0	0.9 ± 0.1
5 mM EDTA	Susceptible	0.2 ± 0.0	10.7 ± 0.0
	Resistant	0.3 ± 0.0	14.7 ± 0.8

[a] APN activity was assayed at 37°C in 10 mM Tris-HCl–150 mM NaCl (pH 7.4)–0.8 mM L-leucine-*p*-nitroanilide–3.3% methanol. The increase in absorbance was measured for 5 min with a kinetic microplate reader. APN activity was calculated with a millimolar extinction coefficient of 9.9 for *p*-nitroanilide at 405 nm. Values are means plus or minus the standard error of triplicate determinations.

DISCUSSION

We found a 120-kDa toxin-binding APN in BBMV from susceptible and resistant *P. xylostella* larvae. The 120-kDa protein was recognized as a toxin-binding protein by Cry1Ac-affinity chromatography and ligand blotting. The 120-kDa protein was identified as APN based on its hydrolysis of leucine-*p*-nitroanilide, molecular size, and inhibition by EDTA and amastatin.

APN is now identified as a major Cry1Ac-binding protein in four insect species (7, 9, 23, 32), including *P. xylostella*. In three of these species, the APN molecule is about 120 kDa. The exception is *H. virescens*, in which Cry1Ac-binding APN molecules are 115, 140, and 170 kDa (7). The APN activity of the partially purified 120-kDa protein from susceptible *P. xylostella* larvae was 24 μmol/min/mg, which is comparable to APN activities reported for *M. sexta* (9, 14). Like the *M. sexta* 115-kDa

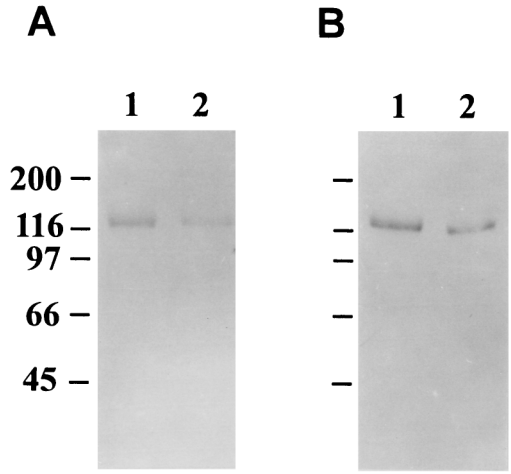

FIG. 2. Immunoblot analysis of affinity-purified Cry1Ac-binding proteins from the resistant and susceptible larvae of *P. xylostella* using anti-CRD antiserum. The affinity-purified toxin-binding proteins were separated by SDS-PAGE (10% polyacrylamide) and blotted onto a nitrocellulose membrane. (A) Membrane not treated with PIPLC; (B) membrane treated with PIPLC. The GPI anchor of toxin-binding protein was then detected with anti-CRD antibodies. Lane 1, affinity-purified Cry1Ac-binding proteins from the susceptible strain; lane 2, affinity-purified Cry1Ac-binding proteins from the resistant strain.

and 106-kDa APNs, the *P. xylostella* 120-kDa APN seems to have a precleaved GPI anchor.

The presence of a Cry1Ac-binding protein in resistant NO-QA larvae is consistent with previous results from histochemical (3) and plasmon resonance techniques (17) and apparently conflicts with data from radiolabel-binding assays (27). Escriche et al. (3) detected binding of Cry1Aa, Cry1Ab, and Cry1Ac to NO-QA midgut sections by a histochemical technique. Masson et al. (17) found only threefold-fewer binding sites in NO-QA larvae versus susceptible LAB-P larvae and no change in toxin-binding site dissociation with an optical biosensor technology based on surface plasmon resonance. Tabashnik et al. (27) observed very low levels of binding of ^{125}I-labeled Cry1Ac to BBMV from NO-QA insects relative to LAB-P insects.

There is a precedent for the apparently incongruous results with *P. xylostella*. In *H. virescens* larvae with >1,000-fold resistance to Cry1A toxins, Cry1Aa toxin did not bind to BBMV from resistant larvae, yet on ligand blots Cry1Aa bound to the protein of the same size from susceptible and resistant larvae (12). Lee et al. (12) also found binding but no toxicity of Cry1Ab and Cry1Ac. They suggested that binding proteins may be masked by associated proteins or other molecules that do not bind toxin (12). Blocking molecules could be dissociated from the toxin-binding protein during solubilization for affinity chromatography (this study), freeze-thaw sonication for the plasmon resonance technique (17), or processing of histological sections (3).

An alternative explanation for the loss of toxin binding to BBMV from resistant larvae is based on the GPI anchorage of APN and the presence of an endogenous PIPLC. The GPI linkage provides a stable yet controllable means of attaching an ectoenzyme to the cell's exterior. For example, in humans, a serum PIPLC selectively releases alkaline phosphatase from hepatocyte membranes (20). Certain GPI-anchored proteins, such as ribosyltransferase, are cleaved by PIPLC in response to external factors such as cross-linking or binding of ligands (21). Altered PIPLC or accessibility of PIPLC to APN may cause the release of APN after toxin binding and prior to membrane insertion and intoxication.

Although the aforementioned explanations are speculative, each is a logical means by which *P. xylostella* could attain resistance by altering toxin binding without eliminating toxin-binding proteins. In any case, our results show that the presence of Cry1Ac-binding APN in the brush border membrane is not sufficient to confer susceptibility to Cry1Ac.

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