Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 8930–8933, October 1991
Biochemistry

Binding of *Bacillus thuringiensis* proteins to a laboratory-selected line of *Heliothis virescens*

(mode of action/receptor binding/insecticidal activity)

SUSAN C. MACINTOSH*, TERRY B. STONE, R. SCOTT JOKERST, AND ROY L. FUCHS†

Monsanto Agricultural Company, A unit of Monsanto Company, 700 Chesterfield Village Parkway, Saint Louis, MO 63198

Communicated by Clarence A. Ryan, July 12, 1991 (received for review January 31, 1991)

ABSTRACT **A laboratory-selected colony of *Heliothis virescens* displaying a 20- to 70-fold level of resistance to *Bacillus thuringiensis* proteins was evaluated to identify mechanism(s) of resistance. Brush-border membrane vesicles were isolated from larval midgut epithelium from the susceptible and resistant strains of *H. virescens*. Two *B. thuringiensis* proteins, CryIA(b) and CryIA(c), were iodinated and shown to specifically bind to brush-border membrane vesicles of both insect strains. Multiple changes in the receptor-binding parameters were seen in the resistant strain as compared with the susceptible strain. A 2- to 4-fold reduction in binding affinity was accompanied by a 4- to 6-fold increase in binding-site concentration for both proteins. Although these two *B. thuringiensis* proteins competed for the same high-affinity binding site, competition experiments revealed different receptor specificity toward these proteins in the resistant *H. virescens* line. The *H. virescens* strains were not sensitive to a coleopteran-active protein, CryIIIA, nor did these proteins compete with the CryIA proteins for binding. Complexity of the mechanism of resistance is consistent with the complex mode of action of *B. thuringiensis* proteins.**

Insect-control proteins from *Bacillus thuringiensis* ssp. *kurstaki* are active against a wide range of agronomically important lepidopteran larvae (1, 2). Use of these proteins in optimized microbial strains and genetically improved plants will become increasingly important for insect control (3), particularly as the popularity of many commercial chemical insecticides is declining due to the onset of resistance by target pests (4). Although commercial preparations of *B. thuringiensis* strains have been used for >25 yr, only recently have insects with reduced susceptibility been identified (5) and obtained in laboratory-selection experiments (6–8). Management strategies are being developed to prevent or delay the onset of insect resistance to assure the long-term efficacy of *B. thuringiensis* proteins. Biochemical characterization of *B. thuringiensis* proteins, their mode of action, and mechanisms of increased resistance are critical for the development of appropriate management strategies.

The mode of action of *B. thuringiensis* protein insecticides is complex, as evidenced in a number of reports over the last few years (9–20). Upon ingestion by the insect, the proteins are proteolytically processed (9–14), cross the peritrophic membrane, and bind to high-affinity receptors on the midgut epithelium (15–20). The membrane-bound *B. thuringiensis* protein disrupts the membrane integrity by forming a pore, causing an electrolyte imbalance that ultimately kills the insect (21, 22). Receptor binding has been analyzed by using midgut brush-border vesicles from the gut epithelium from various lepidopteran larvae and for several *B. thuringiensis* proteins (16, 18–20, 23). High-affinity binding sites were initially identified that directly correlated with the observed toxicity in these insects (16, 18–20). More recently, Wolfersberger (23) reported that, although he observed a qualitative correlation of insecticidal activity and binding of two *B. thuringiensis* proteins, CryIA(c) and CryIA(b), he found an inverse quantitative relationship between insecticidal activity of these two proteins and receptor binding for *Lymantria dispar*.

Insects that have evolved to tolerate *B. thuringiensis* protein have developed a mechanism(s) that interferes with one or a combination of steps involved in the mode of action. One recent study with a laboratory-selected colony of *Plodia interpunctella* that had significantly reduced susceptibility to *B. thuringiensis* protein showed a correlation between reduction of *B. thuringiensis* toxicity and decreased binding affinity (24).

Selection for resistance to *B. thuringiensis* in the laboratory has been documented with several different lepidopteran species (6–8). Laboratory-selected resistance in a field-crop insect, *Heliothis virescens*, was described by Stone *et al.* (8) in 1989. To evaluate mechanism(s) of resistance in the laboratory-selected *H. virescens* strain, the binding parameters of purified *B. thuringiensis* proteins to midgut brush-border membrane vesicles (BBMV) of the susceptible and resistant insect lines were determined.

METHODS AND MATERIALS

Reagents. All chemicals were reagent grade and purchased from Sigma, unless otherwise indicated.

Protein Purification. The *cryIA(b)* and *cryIIIA* genes were isolated from *B. thuringiensis* ssp. *kurstaki* HD-1 and *B. thuringiensis* ssp. *tenebrionis* strains, respectively. Construction of the genes (25, 26) and purification of the *Escherichia coli*-expressed gene products (27, 28) have been described. The *Pseudomonas fluorescens* strain expressing the *cryIA(b)* gene, Ps112-12a, was constructed (29) and fermented (8), as described. CryIA(c) protein was purified from *B. thuringiensis* ssp. *kurstaki* HD-73 strain, as described (28, 30). The trypsin-resistant fragments of CryIA(b) and CryIA(c) proteins were further purified on a fast protein liquid chromatography Superose 12 column (Pharmacia). All proteins were, at least, 95% pure, as judged by SDS/PAGE analysis (31).

Iodination of *B. thuringiensis* Proteins. CryIA(b) and CryIA(c) proteins were iodinated using the Iodo-Bead technique (Pierce technical bulletin 28666). One hundred micrograms of each protein was mixed with two Iodo-Beads in a final volume of 0.5 ml of 100 mM sodium carbonate buffer, pH 10. The free iodine was separated from the protein-bound fraction by using prepacked PD-10 G-25M desalting columns

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Abbreviation: BBMV, brush-border membrane vesicles.

*Permanent address: Entotech, Inc., 1497 Drew Avenue, Davis, CA 95616.
†To whom reprint requests should be addressed.

(Pharmacia). Column fractions of 0.5 ml were collected, and the three fractions with the highest specific activity were pooled. Radioactivity was measured in a gamma counter. Specific activity was typically 200–480 Ci/mmol (1 Ci = 37 GBq).

Isolation of BBMV. BBMV were prepared from the midgut tissue of third-instar *H. virescens* larvae by the method of Biber *et al.* (32), as described and modified by Wolfersberger *et al.* (33). Vesicle preparations were quickly frozen in liquid nitrogen and stored at −80°C for up to 3 mo.

Protein Determination. Protein content of the BBMV preparations and purified *B. thuringiensis* proteins was assessed by the method of Bradford (34), using bovine serum albumin as the standard.

Receptor-Binding Assay. Binding assays were done, as described (18), with minor modifications. All experimental data represent the average of three replicates from three separate experiments. Two different analyses were used to evaluate binding: (*i*) saturated binding assays to determine affinity (K_d) and concentration of binding sites (B_{max}) and (*ii*) competition experiments to estimate the concentration of a competing ligand that displaced 50% of the bound radiolabeled protein (IC_{50}). In saturated binding experiments, the vesicles (20 μg of protein) were combined with eight concentrations (serially diluted, 1000-fold range) of radiolabeled CryIA(b) or CryIA(c) protein. For each point, total bound radioactivity was determined as well as nonspecific bound radioactivity by adding 100-fold excess of unlabeled ligand. Data were analyzed by Scatchard analysis (35).

In competition binding assays, a constant tracer dose (0.1 times the K_d, as calculated from saturated binding experiments) of ^{125}I-labeled *B. thuringiensis* protein was mixed with 10–12 concentrations of unlabeled *B. thuringiensis* protein.

A strain of *H. virescens* was subcultured from a colony maintained at the Tobacco Research Laboratory, U.S. Department of Agriculture, Oxford, NC. Larvae of the strain were further subcultured on a pinto bean-based diet (36) containing *P. fluorescens* (Ps112-12a) genetically modified to contain the 130-kDa protoxin of *B. thuringiensis* ssp. *kurstaki* [CryIA(b)]. The line was selected on this diet for 13 generations (8). From F14 to F18 generations the line was selected on Dipel, a commercial *B. thuringiensis* formulation. Dipel contains the CryIA(a), CryIA(b), CryIA(c), and CryIIA insecticidal crystal proteins (2), as well as spores and other formulation products. Selection of the F18 to F22 generations was on Ps112-12a.

Insect Bioassays. *B. thuringiensis* insecticidal activity was assayed with neonate *H. virescens* larvae, as described (8). Toxicity of the ^{125}I-labeled *B. thuringiensis* CryIA(c) to *Trichoplusia ni* was tested by using a modified droplet assay (37). *T. ni* neonates were exposed to droplets of a discriminating dose (5 μl, 0.33-μg dose) of *B. thuringiensis* proteins spiked with glucose and FD&C blue food coloring to stimulate feeding and indicate consumption. Larvae that ingested the toxin were placed on an artificial diet. Mortality was determined on day 7 after treatment.

Table 1. Activity of insecticidal proteins to resistant and susceptible strains of *H. virescens*

Protein	LC_{50}*		RF†
	Susceptible	Resistant	
Dipel	8.7	500	57
CryIA(b)‡	0.21	15	71
CryIA(c)§	0.11	1.8	16
CryIIIA§	>500	>500	—

*Fifty percent lethal concentrations (LC_{50}) were calculated from probit analysis and expressed in μg/ml, except where noted.
†Resistance factor, ratio of resistant LC_{50}/sensitive LC_{50}.
‡CryIA(b) protein expressed in *P. fluorescens* (mg of dry weight per ml).
§Purified *B. thuringiensis* protein.

Table 2. Sensitivity of *T. ni* to radiolabeled CryIA(c) protein

Sample	Alive/total, no.
CryIA(c)	3/15
^{125}I-CryIA(c)	1/15, 4/15
Buffer control	14/15

Dose of CryIA(c) protein was 0.33 μg for each insect. Mortality was assessed at 7 days.

RESULTS

The activity of three *B. thuringiensis* proteins toward the resistant and susceptible *H. virescens* strains is listed in Table 1. Approximately a 70-fold level of resistance was observed for the CryIA(b) protein expressed and contained in *Pseudomonas* Ps112-12a, 20-fold for the purified CryIA(c) protein, and 57-fold for the commercial preparation Dipel. As expected, no activity was seen for the coleopteran-active protein CryIIIA.

CryIA(b) and CryIA(c) proteins were radiolabeled to investigate binding. Both radiolabeled CryIA(b) and CryIA(c) proteins maintained the integrity of the original protein, as evidenced by unaltered migration on SDS/PAGE with radiography. Insect toxicity was also measured for CryIA(c) (Table 2). Integrity of the BBMV preparations was established by demonstrating a 6- to 10-fold enrichment for the BBMV marker enzyme alkaline phosphatase and a 20-fold reduction of the mitochondrial marker cytochrome *c*, relative to the concentration from the initial insect-midgut homogenate (data not shown).

High-affinity, specific, and saturable binding of radiolabeled CryIA(b) and CryIA(c) proteins to isolated BBMV was observed for both resistant and susceptible *H. virescens* strains. Binding of radiolabeled CryIA(b) and CryIA(c) proteins to the BBMV was not completely reversible but was complete in 15 min and remained stable for up to 2 hr (data not shown). The dissociation constant calculated from the Scatchard plot for the CryIA(c) protein was 2- to 5-fold lower than for the CryIA(b) protein in both the resistant and susceptible *H. virescens* strains (Table 3). This result corresponds with the insect bioassay data reported for the sensitive line, which showed that the LC_{50} value for the purified active fragment of the CryIA(c) protein was ≈2-fold lower than the LC_{50} for the purified active fragment of the CryIA(b) protein (28). The concentration of high-affinity binding sites (B_{max}) was the same for both *B. thuringiensis* proteins for the susceptible insects and differed only negligibly between the two insecticidal proteins in the resistant *H. virescens* strain.

The dissociation constants for CryIA(b) and CryIA(c) proteins were 2-fold to 4-fold higher for the resistant strain than for the susceptible strain (Table 3). Compared to the susceptible strain, a 6-fold and 4-fold increase in the binding-site concentration for the CryIA(b) and CryIA(c) proteins, respectively, was also observed for the resistant insects. Although the binding parameter differences were statistically significant, the magnitude of the changes does not reflect the 20- to 70-fold increase in the LC_{50} values seen for the resistant *H. virescens* strain.

Table 3. Saturated binding data for resistant and susceptible strains of *H. virescens*

Protein	Strain	K_d, nM	B_{max}, pM/mg
CryIA(b)	Susceptible	5.92 ± 1.34	0.37 ± 0.11
CryIA(b)	Resistant	9.48 ± 1.84	2.14 ± 0.11
CryIA(c)	Susceptible	1.29 ± 0.11	0.37 ± 0.06
CryIA(c)	Resistant	4.93 ± 1.03	1.40 ± 0.36

Table 4. Competitive binding data for resistant and susceptible strains of *H. virescens*

Protein			
^{125}I-labeled tracer	Unlabeled competitor	Strain	IC_{50}, nM
CryIA(c)	CryIA(c)	Susceptible	1.17 ± 0.05
CryIA(c)	CryIA(c)	Resistant	2.50 ± 0.29
CryIA(c)	CryIA(b)	Susceptible	1.74 ± 0.38
CryIA(c)	CryIA(b)	Resistant	15.33 ± 2.91
CryIA(b)	CryIA(c)	Susceptible	1.96 ± 0.95

In competition assays, IC_{50} values for various combinations of CryIA(b) and CryIA(c) proteins for the susceptible *H. virescens* strain did not differ significantly, 1.17–1.96 nM (Table 4, Fig. 1), suggesting that both ligands compete for the same high-affinity site. The IC_{50} value for the resistant insects was only 2-fold higher than the susceptible strain with unlabeled CryIA(c) protein as the competing ligand for radiolabeled CryIA(c). However, a 9-fold higher concentration of unlabeled CryIA(b) protein was required to displace the radiolabeled CryIA(c) protein for the resistant line (Table 4, Fig. 2). The CryIIIA protein, even at 100-fold higher concentrations, was unable to displace the CryIA(b) protein (Fig. 1).

DISCUSSION

The receptor-binding characteristics of a resistant strain of *H. virescens* were determined and compared to a susceptible strain for three *B. thuringiensis* proteins, CryIA(b), CryIA(c), and CryIIIA. High-affinity, saturable, and specific binding was observed for the lepidopteran active proteins CryIA(b) and CryIA(c) (Table 3). The disassociation constants (K_d) and binding-site concentrations (B_{max}) determined for the susceptible insects were consistent with the reported insect bioassay data (28). Unlabeled CryIA(b) protein successfully competed for iodinated CryIA(c) protein and *vice versa*. These two proteins compete for a common receptor, as evidenced by the competition experiments (Table 4, Figs. 1 and 2). CryIIIA protein, a coleopteran-active *B. thuringiensis* protein, did not bind to the BBMV of the sensitive *H. virescens* (Fig. 1). These data support the results of Hofmann *et al.* (16, 18) and illustrate both the link of insecticidal specificity to receptor binding and the identification of a common receptor for CryIA(c) and CryIA(b) proteins.

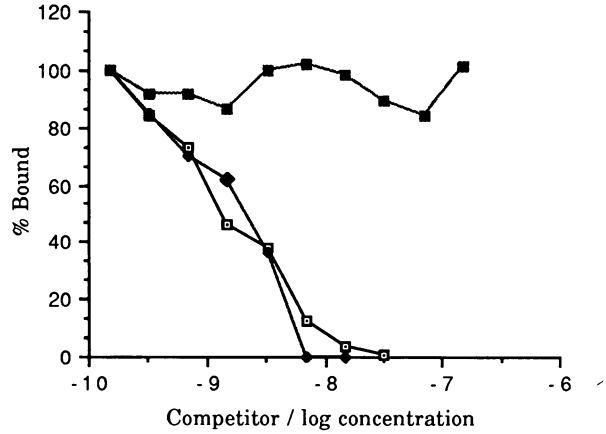

FIG. 1. Competitive binding of ^{125}I-labeled *B. thuringiensis* proteins to BBMV from the susceptible strain of *H. virescens*. Vesicles (20 μg of protein) were incubated with ^{125}I-labeled CryIA(b) protein (0.5 nM) or ^{125}I-labeled CryIA(c) protein (0.1 nM) in the presence of an unlabeled competitor at indicated concentrations in the following combinations: □, ^{125}I-labeled CryIA(c)/unlabeled CryIA(c); ◆, ^{125}I-labeled CryIA(c)/unlabeled CryIA(b); and ■, ^{125}I-labeled CryIA(c)/unlabeled CryIIIA. Binding is expressed as percentage of labeled protein bound in the absence of unlabeled protein.

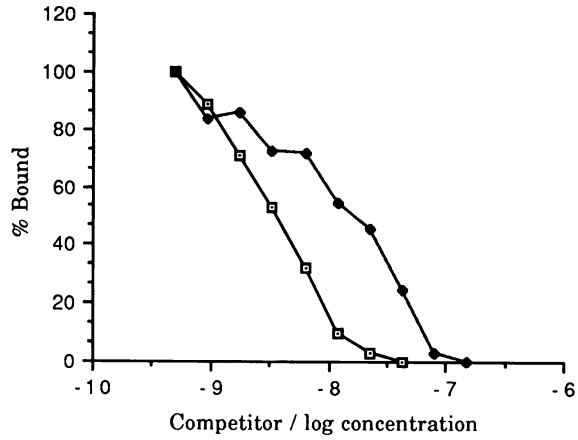

FIG. 2. Competitive binding of ^{125}I-labeled *B. thuringiensis* proteins to BBMV from the resistant strain of *H. virescens*. Vesicles (20 μg of protein) were incubated with ^{125}I-labeled CryIA(c) protein (0.5 nM) in the presence of an unlabeled competitor at indicated concentrations in the following combinations: □, ^{125}I-labeled CryIA(c)/unlabeled CryIA(c); ◆, ^{125}I-labeled CryIA(c)/unlabeled CryIA(b). Binding is expressed as percentage of labeled protein bound in the absence of unlabeled protein.

Both the dissociation constant (K_d) and the binding-site concentration (B_{max}) were altered in the resistant strain. The alterations seen were compensatory in nature. As binding affinity decreased, binding-site concentration increased. Empirically these differences would appear to offset each other; yet, a significant reduction in susceptibility to *B. thuringiensis* proteins is evident from insect bioassays. One striking difference was observed in the competition experiments. Although CryIA(b) and CryIA(c) proteins competed equally in competition experiments using susceptible insects, significant differences between these two proteins was seen with the resistant strain. Unlabeled CryIA(b) protein was far less effective in displacing radiolabeled CryIA(c) than was unlabeled CryIA(c) protein (Table 4, Fig. 2). These data suggest that the receptor specificity, in the resistant insects, has been differentially modified relative to these two proteins. This observation plus the apparent offsetting changes in binding affinity and binding-site concentration suggests that the mechanism of resistance is complicated and results from multiple genetic changes. This conclusion is supported by genetic analysis (38) that revealed a multigenic, partially recessive mode of inheritance for resistance in the selected *H. virescens* population. The putative insect receptor for *B. thuringiensis* proteins almost certainly possesses other physiological functions vital to the insect. If the altered binding site in the resistant insects also affects the normal biological function of this site, it is not surprising that the resistant insect strain increases the number of these receptors to offset their reduced activity.

This study focused only on the initial binding step. Although binding is no doubt critical in the entire scheme of the mode of action of *B. thuringiensis* proteins, postbinding events may be even more important. Postbinding events, such as integration of *B. thuringiensis* proteins into the membrane, formation of a membrane pore, and, therefore, alteration of amino acid transport by means of the ion gradient also may have been altered in the resistant insects. The fact that the magnitude of the observed changes in the receptor affinities does not account for the magnitude of the changes in insect susceptibilities for the resistant line suggests that one or more of these subsequent events may also

be altered. Results of genetic studies are consistent with this hypothesis.

It must be emphasized that the resistant colony of *H. virescens* used for this study originated from laboratory-selection experiments that cannot predict development of insect resistance in the field. Laboratory-produced resistance neglects some major biological influences—such as insect migration, refugia, and the vast numbers of insects present in a field as compared to the laboratory. Nonetheless, laboratory-selected insect colonies are valuable for a wide range of research topics. The mechanism by which an insect evolves resistance to a particular substance, such as *B. thuringiensis* protein, is unavoidably tied to the mode of action of that substance. Exploring this mechanism of resistance will lead to a better understanding of the mode of action. Likewise, strategies for delaying or overcoming decreased susceptibility to *B. thuringiensis* proteins require a fundamental understanding of the potential mechanisms of resistance.

For example, Van Rie *et al.* (24) showed that a significant decrease in binding affinity of *B. thuringiensis* protein to a resistant line of *Plodia interpunctella* accounted for the decrease in toxicity observed. Knowing this, Van Rie *et al.* illustrated that at least one unrelated *B. thuringiensis* protein, CryIC type, was effective in overcoming the resistance of this *P. interpunctella* colony. Insect sensitivity to the CryIC protein and the number of CryIC receptors increased in the insects, which showed a decreased sensitivity to CryIA(b). This decrease suggested that the changes in CryIA(b) may have led to compensational changes in the CryIC receptor. These data imply that multiple *B. thuringiensis* proteins with specific binding-site properties could be combined, rotated, or otherwise used together in an integrated pest-management system to delay or prevent the occurrence of insect resistance to *B. thuringiensis* proteins.

In contrast to the results reported by Van Rie *et al.* (24), in which altered receptor binding alone appears to explain resistance development, the results presented here for laboratory-selected *H. virescens* suggest that: (*i*) multiple mutations occur in insects developing resistance to *B. thuringiensis* products; (*ii*) postbinding events are also modified; and (*iii*) mutations in receptor-binding affinity are deleterious to the insect. Whereas Van Rie *et al.* (23) observed a concurrent reduction in binding affinity to the CryIA(b) receptor and an increase in sensitivity to and number of receptors for the CryIC protein in *P. interpunctella*, our results show a change both in receptor affinity and in the number of receptors for the same protein to which resistance was selected. This response of compensatory mutations to achieve resistance implies that mutations in the receptors for binding *B. thuringiensis* proteins confer a selective disadvantage. The information in this study will be coupled with the variety of management strategies available (e.g., use of integrated pest-management systems, use of refugia, use of other insect-control agents with independent modes of action, including multiple genes with specific receptor-binding activities) to assure the long and effective use of *B. thuringiensis* proteins.

We thank David Fischhoff and Pam Marrone for support of this project and critical review of this manuscript. We are also grateful to Mike Wolfersberger for thoughtful discussions and support of this research.

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