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Synergistic Effect of the *Bacillus thuringiensis* Toxins CryIAa and CryIAc on the Gypsy Moth, *Lymantria dispar*

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The insecticidal activity of toxins CryIAa, CryIAb, and CryIAc against *Lymantria dispar* (gypsy moth) and *Bombyx mori* (silkworm) was examined by force-feeding bioassays. Toxin CryIAa exhibited higher toxicity than toxins CryIAb and CryIAc for *L. dispar* and *B. mori*. To evaluate possible synergism among these toxins, bioassays were performed with mixtures of CryIAa and CryIAb, CryIAb and CryIAc, and CryIAa and CryIAc. Expected toxicity was calculated from the activity of each individual toxin and its proportion in the mixture by using the equation described by Tabashnik (B. E. Tabashnik, Appl. Environ. Microbiol. 58:3343–3346, 1992). Observed 50% growth-inhibitory doses were calculated from mixing experiments by probit analysis. In *L. dispar* bioassays, synergism was observed with a mixture of CryIAa and CryIAc while a mixture of CryIAa and CryIAb exhibited an antagonistic effect. No synergistic effect on *B. mori* was observed with any toxin combination. Voltage clamping assays of isolated *L. dispar* midguts also demonstrated that the mixture of CryIAa and CryIAc induced a greater slope of inhibition of short circuit current than did other toxin combinations.

Many strains of *Bacillus thuringiensis* produce more than one type of insecticidal crystal protein, called δ-endotoxins, each of which has its own insecticidal specificity. The HD-1 strain of *B. thuringiensis* subsp. *kurstaki* contains genes for different insecticidal crystal proteins: CryIAa, CryIAb, CryIAc, CryIIA, and CryIIB. Although the CryIA-type proteins share about 90% amino acid sequence identity, different insecticidal specificities have been reported for several lepidopteran insects (3, 4, 13).

It has been suggested that a mixture of different toxins could be more effective than a single toxin and might delay rapid onset of resistance (5, 18, 22). In an earlier report, van Frankenhuyzen et al. (20) assessed possible interactions between toxins CryIAa, CryIAb, and CryIAc by comparing the observed toxicity (50% frass failure dose) of native *B. thuringiensis* subsp. *kurstaki* HD-1 crystals with the expected toxicity. The expected 50% frass failure dose was calculated from a weighted arithmetic mean of the 50% frass failure doses of the individual toxins, that is, multiplication of the toxicity of each individual component and the proportion of each toxin in the HD-1 crystal. They reported synergistic interaction of the HD-1 toxins with three insect species, *Lymantria dispar*, *Choristoneura pinus*, and *Orygia leucostigma* (28, 2, and 2 times, respectively). Tabashnik (17) reevaluated the previously published data on interactions of *B. thuringiensis* toxins, including the data of van Frankenhuyzen et al. (20). He demonstrated that an inappropriate test for synergism can cause a misleading conclusion. The reevaluation of synergism of CryIA toxins against two insect species, *O. leucostigma* and *L. dispar*, revealed no evidence of synergism. The reason for this contradiction between van Frankenhuyzen et al. and Tabashnik was the use of different methods to calculate the expected toxicity. Moar et al. (14) also demonstrated that CryIA toxins from the HD-1 or NRD-12 strain of *B. thuringiensis* subsp. *kurstaki* did not show synergistic effects against *Spodoptera exigua*. Therefore, absence of positive synergism among CryIA toxins seemed to be the general observation.

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In the present study, we investigated possible synergistic effects among the CryIAa, CryIAb, and CryIAc toxins on *L. dispar* and *B. mori* by force feeding combinations of toxins CryIAa and CryIAb, CryIAb and CryIAc, CryIAa and CryIAc. We observed about four to seven times higher toxicity than expected with a combination of toxins CryIAa and CryIAc for *L. dispar*, while a mixture of toxins CryIAa and CryIAb showed an antagonistic effect. In assays with *B. mori*, no such synergism was observed.

MATERIALS AND METHODS

Purification and activation of recombinant *B. thuringiensis* δ-endotoxins. The *cryIAa*, *cryIAb*, and *cryIAc* toxin genes were overexpressed in *Escherichia coli* JM103 by using the expression vector pKK223-3 (2). Crystal proteins were purified and solubilized as described previously (10). The solubilized protoxin was digested with trypsin in a trypsin-protoxin ratio of 1:25 (by mass) for 2 h at 37°C. Activated toxins were dialyzed against 50 mM sodium carbonate buffer, pH 9.5. The purity of the protoxins and activated toxins was examined by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (9).

Force-feeding bioassays. Fourth-instar *L. dispar* larvae were kindly supplied by Normand Dubois (U.S. Forestry Service, Hamden, Conn.). Activity of toxins was determined by force feeding fourth-instar larvae of *L. dispar* and *B. mori*. Toxins were serially diluted with 50 mM sodium carbonate buffer, pH 9.5. A 2-μl drop of each dilution was fed to the larvae with a 0.25-ml syringe (polished tip; Hamilton Inc.) fitted with a 30-gauge blunt needle by using a Houston-Atlas (Houston, Tex.) 1003 Micro-jector syringe drive and a 1010 Micro-doser. Dilution buffer was fed to the control larvae. Fifteen to twenty insects were used for each toxin dose, and bioassays were repeated more than three times. Toxin-fed *L. dispar* larvae were placed into 30-ml plastic cups with a Bio-serv diet (U.S. Department of Agriculture Formula, product F9630B), and 50% growth inhibition dosages (ID_{50}s) were measured after 5 days. The definition of growth inhibition is decreased or unchanged weight after dose administration. Toxin-fed *B. mori* larvae were placed in a cage containing mulberry leaves and monitored for 24 to 30 h.

ID_{50}s were obtained from mixing experiments with the toxin combinations (1:1 ratio) CryIAa-CryIAb, CryIAb-CryIAc, and CryIAa-CryIAc and individual-toxin bioassays by using the PROBIT.SAS program (16). Observed values represent the ID_{50}s obtained from force-feeding bioassays with each of the toxins and toxin mixtures (1:1 ratio). Expected ID_{50}s were calculated from ID_{50}s of individual toxins by using the following equation described by Tabashnik (17): $LD_{50(m)} = \{[r_a/LD_{50(a)}] + [r_b/LD_{50(b)}]\}^{-1}$ where $LD_{50(m)}$ represents the expected 50% lethal dose (LD_{50}) of the mixture, $LD_{50(a)}$ and $LD_{50(b)}$ represent the LD_{50} of toxins *a* and *b*, and r_a and r_b represent the relative proportions of toxins *a* and *b* in the mixture.

Voltage clamping assays. Voltage clamping assays were performed as described by Chen et al. (1). Fifth-instar *L. dispar* larvae were dissected, and midguts were mounted on a disk 3.9 mm in diameter. A standard chamber buffer (32 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 240 mM sucrose in 5 mM Tris, pH 8.3)

TABLE 1. Force-feeding bioassay of each individual toxin and toxin mixtures (1:1) with *L. dispar* larvae

Toxin(s)	Observed ID_{50} (ng)[a]	Avg slope ± SE	Expected ID_{50} (ng)[b]	Expected/observed ID_{50} ratio
CryIAa	106 (69.2–146.6)	2.34 ± 0.41		
CryIAb	214 (136–301)	1.68 ± 0.32		
CryIAc	871 (651–1147)	1.78 ± 0.23		
CryIAa-CryIAb	431 (281–922)	1.58 ± 0.46	141.5	0.33
CryIAb-CryIAc	226 (151–350)	1.62 ± 0.34	343.5	1.5
CryIAa-CryIAc	49.9 (34.4–67.7)	2.44 ± 0.49	188.5	3.8

[a] 95% confidence limits are given in parentheses.
[b] Expected ID_{50}s were calculated as described in Materials and Methods.

(6) was used. After 15 to 30 min of stabilization of the midgut, trypsin-activated toxins (0.4 or 0.8 nM in 20 μl of chamber buffer) were injected into the lumen side of the chamber and the changes in short circuit current (Isc) over time (minutes) were recorded. The total volume of the chamber was 4 ml. During the experiment, the chamber solution was bubbled continuously with O_2. The Isc was tracked with a Kipp and Zonen recorder, and data were collected with the MacLab data acquisition system on a Macintosh computer.

RESULTS

Force-feeding bioassays. Force-feeding bioassay results are summarized in Table 1. Toward *L. dispar*, toxin CryIAa was about two times more toxic than CryIAb and CryIAb was about four times more toxic than CryIAc toxin. In the bioassays with 1:1 toxin mixtures, only the CryIAa-CryIAc combination showed a synergistic effect, about four times. Mixed toxins CryIAb and CryIAc showed about 1.5-fold higher toxicity for *L. dispar* than expected, but this difference was not statistically significant. On the other hand, the CryIAa-CryIAb toxin mixture exhibited an antagonistic effect on *L. dispar* (Table 1). The observed toxicity was only one-third of the expected toxicity. More-detailed bioassays with a series of different ratios of toxins CryIAa and CryIAc are summarized in Table 2. At a CryIAa-CryIAc ratio of 1:2, an about sevenfold synergistic effect was observed. Even at a 1:12 ratio, about 2.7 times higher toxicity was still observed.

Against *B. mori*, CryIAa was much more toxic than CryIAb or CryIAc. A 25-ng dose of toxin CryIAa produced 70% mortality, while toxins CryIAb and CryIAc showed no measurable toxicity. CryIAb and CryIAc did not kill *B. mori* larvae at 100 and 2,700 ng, respectively. Mixing experiments did not show evidence of synergism against *B. mori* for any of these combinations.

Voltage clamping analysis. Data from voltage clamping experiments performed with *L. dispar* larval midguts are summarized in Table 3. We measured the slope of inhibition of Isc in response to the addition of individual toxins and toxin mixtures to the lumen side of the midgut. Isc is proportional to the flux of the ion being actively transported from the hemolymph side of the midgut membrane to the lumen side (6). High negative values of the slopes mean better ion channel-forming activity and represent higher toxicity. The Isc inhibition slopes for 0.4 nM CryIAa, CryIAb, and CryIAc were −9.8, −5.9, and −4.2 μA/min, respectively. A 0.8 nM concentration of CryIAa, CryIAb, and CryIAc gave Isc inhibition slopes of −20.7, −10.8, and −8.1 μA/min, respectively. The Isc inhibition slopes for CryIAa-CryIAb, CryIAb-CryIAc, and CryIAa-CryIAc (0.4 nM each toxin; total, 0.8 nM) were −15.2, −11.3, and −23.5 μA/min, respectively.

TABLE 2. Toxicity of different ratios of CryIAa-CryIAc toxin mixtures for *L. dispar*

Ratio of CryIAa to CryIAc	Observed ID_{50} (ng)[a]	Avg slope ± SE	Expected ID_{50} (ng)	Expected/observed ID_{50} ratio
1:0	106 (69.2–146.6)	2.34 ± 0.41		
0:1	871 (651–1147)	1.78 ± 0.23		
1:1	49.9 (34.4–67.7)	2.44 ± 0.49	188.5	3.8
1:2	34.9 (6.1–55.9)	2.32 ± 0.76	255.2	7.3
1:4	73.3 (48.9–96.0)	1.26 ± 0.59	355.8	4.9
1:6	103.9 (73.1–133.4)	1.65 ± 0.71	428.2	4.1
1:8	106.0 (75.2–135.6)	1.86 ± 0.43	482.7	4.6
1:12	210.7 (167–352)	1.75 ± 0.32	559.4	2.7

[a] ID_{50}s were calculated from the bioassays with toxin mixtures as described in Materials and Methods. The values in parentheses are 95% confidence limits.

TABLE 3. Slopes of Isc inhibition from voltage clamping experiments with individual toxins, toxin mixtures (1:1), and *L. dispar* larvae

Toxin(s)	Mean Isc inhibition slope (μA/min) ± SEM	
	0.4 nM[a]	0.8 nM[a]
CryIAa	−9.8 ± 1.7	−20.7 ± 2.7
CryIAb	−5.9 ± 1.6	−10.8 ± 2.3
CryIAc	−4.2 ± 1.4	−8.1 ± 1.9
CryIAa-CryIAb	—[b]	−15.2 ± 2.3[c]
CryIAb-CryIAc	—	−11.3 ± 2.1[c]
CryIAa-CryIAc	—	−23.5 ± 2.5[c]

[a] Concentration of toxin added to the lumen side of the voltage clamp chamber.
[b] —, not tested.
[c] Value obtained from a mixing experiment with a 0.4 nM concentration of toxin (total, 0.8 nM). Each value is the mean of three independent experiments.

DISCUSSION

In this study, we investigated the interactive effect of purified CryIA toxins (CryIAa, CryIAb, and CryIAc) on *L. dispar* and *B. mori* larvae. We performed force-feeding bioassays with different toxin mixtures and obtained ID_{50}s by probit analysis (observed toxicity). The observed toxicity values were compared to the expected toxicity calculated on the basis of the toxicity of each toxin and the proportion of each toxin in the mixture by using the equation described by Tabashnik (17). Pairs of toxins (CryIAa-CryIAb, CryIAb-CryIAc, and CryIAa-CryIAc) were mixed in a 1:1 ratio and force fed to insects. When we compared the effects of three combinations of mixtures on *L. dispar*, the CryIAa-CryIAc mixture was about eight times more effective than CryIAa-CryIAb and four times more effective than the CryIAb-CryIAc mixture (Table 1). The observed toxicity of the CryIAa-CryIAc mixture was about 3.8 times higher than expected toxicity, suggesting a possible synergistic effect. In more-detailed assays, the ratio of toxins CryIAa and CryIAc was varied from 1:1 to 1:12 to limit the amount of CryIAa toxin. We still observed about four to seven times higher toxicity than expected up to a 1:8 ratio (Table 2). We observed the highest synergy at a 1:2 ratio, and this result was consistent throughout the repetitions. It should be noted that toxin CryIAc was much less toxic than CryIAa; however, only a small amount of toxin CryIAa mixed with toxin CryIAc was enough for high toxicity. Indeed, synergism was still observed in the 1:12 mixture. A CryIAb-CryIAc mixture showed about 1.5 times higher toxicity than expected. However, the confidence limits overlapped, suggesting an additive effect or weak synergism. On the contrary, a CryIAa-CryIAb mixture was

about three times less toxic than expected, suggesting possible antagonism (Table 1). From these results, we may explain the observations of no positive synergism among three CryIA toxins in the reevaluation by Tabashnik (17) of the work of van Frankenhuyzen et al. (20). The synergistic effect of the CryIAa-CryIAc toxin mixture might be masked by the antagonistic effect of the CryIAa-CryIAb mixture. When we compared our data on toxicity against *L. dispar* to that of van Frankenhuyzen et al. (20), we observed an eightfold difference between the ID_{50}s of toxins CryIAa and CryIAc, compared with an about 44-fold difference between 50% frass failure doses in the earlier work (20). This difference could be due to different assay systems. Recently, we observed that *L. dispar* insects reared on an iron-sufficient diet were somewhat more susceptible to toxin CryIAc (1a). We used these larvae for our bioassays; therefore, the difference between our data and those from the earlier study (20) might be due to this fact.

We further confirmed the synergistic effect of the CryIAa-CryIAc toxin mixture by performing voltage clamping experiments. Voltage clamping data appeared to be correlated with in vivo bioassay data obtained with different insects (12, 15). Toxin CryIAa showed a better response than did toxins CryIAb and CryIAc. The CryIAa-CryIAc toxin mixture (0.4 nM each; total, 0.8 nM) showed a greater slope of Isc inhibition than did the CryIAa-CryIAb and CryIAb-CryIAc mixtures (Table 3). Furthermore, the slope of Isc inhibition of the CryIAa-CryIAc toxin mixture (-23.5 μA/min) was similar to that of 0.8 nM toxin CryIAa (-20.7 μA/min). No such synergistic effects were observed with the CryIAa-CryIAb and CryIAb-CryIAc toxin mixtures. These results are consistent with the bioassay data. However, no antagonistic effect of the CryIAa-CryIAb toxin mixture was observed in the voltage clamping experiment. In contrast to the bioassay data, a greater slope of Isc inhibition was observed for CryIAa-CryIAb than for the CryIAb-CryIAc mixture, suggesting the possibility that voltage clamping data are not always correlated with bioassay data.

We have no definitive explanations for the synergistic effect of the CryIAa-CryIAc mixture. However, we do suggest a possible way to interpret our data. The mechanism of action of *B. thuringiensis* toxin consists of solubilization of *B. thuringiensis* crystal, activation of protoxin to toxin by midgut proteases, binding of the toxin to receptors, insertion into the membrane, and pore formation (5, 7, 8, 21). It has been suggested that the toxin might oligomerize before or after binding to the receptor (5). Possibly, a hetero-oligomer (CryIAa and CryIAc combined in one oligomer) could be formed in a toxin mixture. It has been observed that the toxins are inserted in the order CryIAa > CryIAb > CryIAc, suggesting that irreversible binding is directly related to toxicity in *L. dispar* (11). A possible model to explain our results is that a hetero-oligomer forms between CryIAa and CryIAc and has better insertion ability than the CryIAc homocomplex; this hetero-oligomer may bind to the CryIAc receptor with better insertion ability, resulting in higher toxicity. Binding and insertion of this heterocomplex to the CryIAa receptor may not be reduced by inclusion of CryIAc if a small number of CryIAa units in a hetero-oligomer is sufficient to form a pore. Indeed, a synergistic effect (2.7 times) was still observed with a 1:12 mixture of CryIAa and CryIAc.

Our brush border membrane vesicle ligand blot experiments (19) showed that CryIAa recognizes a 210-kDa brush border membrane vesicle protein, while CryIAc recognizes a 120-kDa brush border membrane vesicle protein, which is aminopeptidase N and different from the CryIAa-binding protein. The mixture of toxins CryIAa and CryIAc might bind to their own receptors, insert, and make pores. Another possibility for synergism between these toxins is that the individual pores made by different toxins may act together and show higher toxicity than the individual pores. We have no physical evidence to prove any of these hypotheses.

As an explanation for the lack of synergy of the CryIAa-CryIAb mixture, we have observed that toxins CryIAa and CryIAb bind to the same-size brush border membrane vesicle protein, 210 kDa (9a), and we have observed that these two toxins compete with each other for the same binding sites. This competition might reduce any potential synergism. With the CryIAb-CryIAc mixture, less synergistic effect is expected than with the CryIAa-CryIAc mixture, since the pore-forming activity of CryIAb measured by voltage clamping is not as high as that of toxin CryIAa (Table 3).

Interestingly, no synergistic effect against *B. mori* was observed with the CryIAa-CryIAc mixture. In earlier studies, we identified a high-affinity binding site for toxin CryIAa and a very low-affinity binding site for toxin CryIAc in *B. mori* (10). Therefore, the lack of synergism with the CryIAa-CryIAc mixture might be due to the lack of a high-affinity binding site for toxin CryIAc. The CryIAa-CryIAc hetero-oligomer might bind to the CryIAc receptor with very low affinity which is not sufficient to show toxicity. Understanding the mechanisms for synergism between toxins could improve the potency of *B. thuringiensis* toxin and may contribute to resistance management.

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