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Antagonistic effect of Cry1Ac and Cry1Jb on cotton bollworm (*Helicoverpa armigera*)*

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The insecticidal bacterium *Bacillus thuringiensis* has been widely used in agriculture for the control of insect pests in crops (Federici 2005). The main *Bt* toxic factor, cry δ-endotoxins, is produced as crystal parasporal bodies that form a large family of proteins with a remarkable structure similarity but with a wide target diversity. Cry1 class comprises several cry toxins that are highly specific for the lepidopteran pests, which kill them following a unique mode of action. These proteins are based on solubilization and partial proteolysis in the insect intestine, in which the activated toxins interact with the member of columnar cells of the intestinal epithelium and damage the integrity of gut lining; this is followed by paralysis of the host and death. Although the cry1 type proteins share about 90% amino acid sequence identity, different insecticidal specificities have been reported for several lepidopteran insects (Milne *et al.* 1990). It has been suggested that mixture of functionally diverse toxins might be more effective than single toxins and might also delay evolution of resistance in target insects. Few workers have attempted to evaluate the combined action of toxins that naturally occur together or experimental mixtures of different toxins. Synergistic interactions between different δ-endotoxins have been described earlier. Other studies of interactions between δ-endotoxins have revealed additive effects and there has been only very few reports concerning unambiguous antagonism between closely related toxins. This is the first report where Cry1Ac and Cry1Jb toxins are showing antagonistic effect on cotton bollworm (*Helicoverpa armigera*).

The recombinant plasmids pET29a-*cry1Jb* and pKK233-3-*cry1Ac* were overexpressed in BL21(DE3) and JM103 to produce their respective proteins. Crystal proteins were isolated and solubilized as described previously. The solubilized protoxin was digested with trypsin for 2 hr at 37ºC. The purity of the protoxins and the activated toxins was examined by 12% SDS-PAGE. Laboratory culture of *H. armigera* was maintained in environmental simulation walk-in chamber at temperature 27±1ºC, relative humidity 65±5%, 16:8 h scoot-photophase regime on the artificial diet (Gupta *et al.* 2004). To determine the LC_{50} of Cry1Ac and Cry1Jb eight concentrations were tested against 30 neonate larvae in each concentration. The bioassays in which toxin combinations were used were performed in a similar manner as that of individual toxins. Mortality was recorded after incubation for 6 days under laboratory conditions. The LC_{50} were obtained from mixing experiments with the toxin combinations Cry1Ac and Cry1Jb (1:1, 1:2, 2:1) and individual toxin bioassay by using the probit analysis Indostat® statistical software. Expected LC_{50}s were calculated from LC_{50}s of individual toxins by using the following equations described by Tabashnik 1992.

$$LD_{50}(m) = \left(\frac{r_a}{LD_{50}(a)} + \frac{r_b}{LC_{50}(b)} \right)^{-1}$$

H. armigera BBMVs were dissolved in concentrated SDS-polyacrylamide gel electrophoresis sample buffer and heated to 100ºC for 5 min. before being loaded on a 12% acrylamide gel. After electrophoretic separation, BBMV proteins (25 µg per lane) were transferred to nitrocellulose by electroblotting. Strips were cut from the filter and washed in deionized water and subsequently in Tris-buffered saline (TBS; 10 mM Tris HCl [*p*H 8], 150 mM NaCl) containing 0.5% Tween 20. Next, the strips were incubated for 2 hr in blocking solution (TBS containing 1% dried nonfat milk,

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Table 1 Toxicity of different ratios of Cry1Ac-Cry1Jb toxin mixtures for *Helicoverpa armigera*

Ratio Cry1Ac:Cry1Jb	Regression equation Y=	Observed LC_{50} (ng/ml)	Fiducial Limit	Expected LC_{50} (ng/ml)	Expected/ Observed
1:0	4.61+1.07x	2 .29	1.31 – 4.00		
0:1	2.98+2.11x	6039.55	4785.0-7622.9		
1:1	3.91+1.11x	9.44	6.51-13.69	4.58	0.485
1:2	2.36+2.43x	12.09	9.32 – 15.69	6.93	0.573
2:1	3.68+1.57x	6.84	5.14 -9.11	3.41	0.499

0.1% bovine serum albumin, and 0.5% Tween 20). They were then incubated for 3 hr in 1 to 2 mg of purified Cry protein/ml in blocking solution, washed three times for 10 min each in TBS containing 0.5% Tween 20, and incubated for 1.5 hr in rabbit anti-Cry1Ac serum diluted 1:70,000 in blocking solution. After three more washes for 10 min each in TBS–0.5% Tween 20, bound toxin was detected by incubation for 45 min. in goat anti-rabbit–horseradish peroxidase conjugate diluted 1:20,000 in blocking buffer, followed by three washes for 10 min. each in TBS–0.5% Tween 20 and visualized.

Bioassay results are summarized in Table 1. Cry1Ac is 3000 times more toxic than Cry1Jb towards *H. armigera*. Mixtures of Cry1Ac and Cry1Jb toxin (1:1) showed antagonistic effect in which the LC_{50} of Cry1Ac was lowered 4.58 times due to the presence of Cry1Jb. This was even increased to 6.9 times in the case of 1:2 ratio of Cry1Ac and Cry1Jb. Ligand blotting experiment performed with *H. armigera* larval midguts are shown in Fig 1. Both Cry1Ac and Cry1Jb recognize an 110kDa BBMV protein, which is a class 4 amino peptidase (APN).

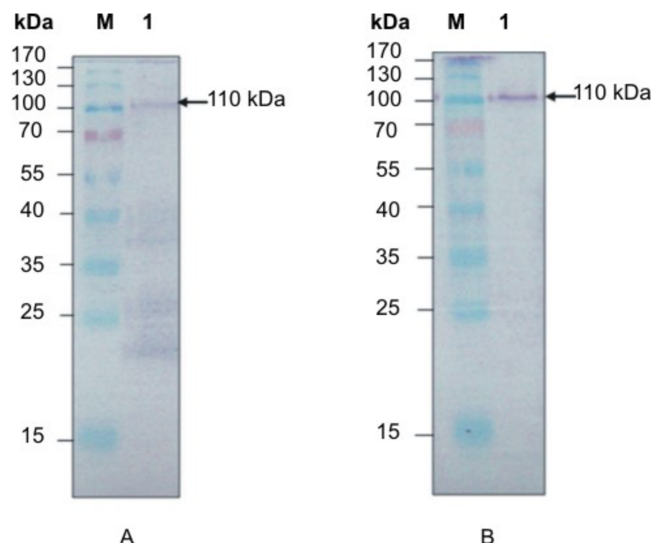

Fig 1 Binding of the Cry1Ac and Cry1Jb to *Helicoverpa armigera* BBMV. Ligand blotting reactions were performed with 25 μg of BBMVs. Both the toxins bound to110 kDa APN. Lane M = PageRuler™ Prestained Protein Ladder (#SM0671), Lane A1 = Cry1Ac bound to 110 kDa APN. Lane B1 = Cry1Jb bound to 110 kDa APN.

Both the study in this paper revealed that there is some antagonism between these two toxins. The Cry1Ac toxin was very toxic to the *H. armigera* larvae (LC_{50}= 2.29 ng/ml), while Cry1Jb exhibited LC_{50} at 6039 ng/ml. Both the toxins caused cell lysis *in vivo*, which was expected. The mechanism behind the antagonism may be that the toxins may interact physically to form a complex, thus blocking one or more of the active sites on one or more molecules (Castro *et al.* 1999). Alternatively, antagonism may be a result of competition for space on the cell surface. But our BBMV Ligand blot showed that both Cry1Ac and Cry1Jb toxins binds to the same 110kDa APN receptor on BBMV and these two toxins compete with each other for the same binding sites. This competition might reduce any potent synergism (Gao *et al.* 2010). Cry1Ac and Cry1Jb toxins bind to common sites explains, in an elegant form, the biochemical basis of multiple resistances and cross resistance among these toxins observed in some insect species. Insects that lack additional sites for Cry1Jb could become resistant simultaneously to the other toxins. However, from resistance management stand point neither transgenic plants expressing pairwise combinations of Cry1Ac and Cry1Jb nor rotations of *Bt* crops containing single genes of these toxins will offer a good strategy for controlling those insects susceptible to more than one of these toxins. Evolution of resistance to more than one toxin is associated with the alteration of a common binding site in several insect species. Thus, knowledge of which toxins share binding sites can help in choosing the appropriate sets of toxins for delaying resistance. Because Cry1Jb and Cry1Ac toxins share common binding sites in *H. armigera*, it is not advisable to use the combination of Cry1Ac toxin with Cry1Jb for pest control.

SUMMARY

The interaction of two crystal proteins, Cry1Ac and Cry1Jb was evaluated against cotton bollworm (*Helicoverpa armigera*). The 50% lethal concentrations (LC_{50}s) were estimated to be 2.29 ng/ml and 6 039 ng/ml for Cry1Ac and Cry1Jb, respectively. When mixtures of these toxins in different proportion were assayed, the expected LC_{50}s were lower than the observed LC_{50}s, indicating a clear antagonism

between the two toxins. Ligand blotting showed that both the toxins bind to 110kDa APN corroborating the results. This is the first report of antagonism of Cry1Jb with Cry1Ac toxin against cotton bollworm. This trait may prove useful for combating insecticide resistance and for improving the activity of microbial insecticides.

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