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Synergistic Activity of a *Bacillus thuringiensis* δ-Endotoxin and a Bacterial Endochitinase against *Spodoptera littoralis* Larvae

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In an attempt to increase the insecticidal effect of the δ-endotoxin crystal protein CryIC on the relatively Cry-insensitive larvae of *Spodoptera littoralis*, a combination of CryIC and endochitinase was used. CryIC comprising the first 756 amino acids from *Bacillus thuringiensis* K26-21 and endochitinase ChiAII encoded by *Serratia marcescens* were separately produced in *Escherichia coli* carrying the genes in overexpression vectors. The endochitinase on its own, even at very low concentrations (0.1 μg/ml), perforated the larval midgut peritrophic membrane. When applied together with low concentrations of CryIC, a synergistic toxic effect was obtained. In the absence of chitinase, about 20 μg of CryIC per ml was required to obtain maximal reduction in larval weight, while only 3.0 μg of CryIC per ml caused a similar toxic effect in the presence of endochitinase. Thus, a combination of the Cry protein and an endochitinase could result in effective insect control in transgenic systems in which the Cry protein is not expressed in a crystalline form.

Crystal (Cry) proteins of different *Bacillus thuringiensis* strains exhibit a high insecticidal activity against lepidopteran larvae (2, 17). The protoxins of 130 to 140 kDa are synthesized during sporulation and assembled as bipyramidal crystals. In lepidopteran larval midguts, the protoxin molecules undergo specific trypsin cleavage, yielding the N-terminal 60- to 65-kDa toxin, which lacks the first 27 to 28 amino acid residues and the carboxy-terminal half (2, 24). The resulting toxin comprises three structural domains. The N-terminal domain with its seven to eight amphipathic α-helices disrupts the midgut epithelial cell membrane by forming ion pores (14, 21, 22). The second and third domains are involved in specific folding and interaction with membrane receptors. This toxin-receptor interaction is a prerequisite for pore formation and thus dictates toxin specificity (3, 7, 13, 14, 33, 36, 49–51). It has been shown that C-terminally truncated versions of different Cry proteins and even 5′ translational fusions with *nptII* are capable of exerting the insecticidal effect as long as the specific trypsin cleavage sites which delimit the toxin are maintained (16, 32, 46, 48). The strict specificity of the receptor-toxin interaction has promoted the use of *cry* genes as very promising tools to construct insecticidal bacteria and transgenic plants with specific insect resistance (6, 12, 25, 28). While in *cry*-carrying bacteria and plant chloroplasts (25) high levels of Cry proteins have been recorded, the native *cry* genes were poorly expressed as plant nuclear transgenes, and consequently their larvicidal activity was limited (12). The relatively low level of *cry* gene expression in transgenic plants is presumably due to a high A+T content, the presence of transcription pretermination signals, and differences in codon usage (12, 27). Thus, achieving high levels of transgenic δ-endotoxin expression in plants was considered imperative for complete insect tolerance. Modifications of *cry* coding sequences to remove the AT-rich transcription-termination elements and improve codon usage elevated the expression of C-terminally truncated Cry proteins in transgenic plants up to 500-fold and greatly increased their tolerance to specific insects (12, 29). The successful use of C-terminally truncated versions is currently being extended to monocotyledonous transgenic plants (20, 52), circumventing the need to synthesize full-length *cry* genes.

On the other hand, exposure to high levels of Cry proteins produced in transgenic hosts may promote development of resistant lepidopteran larval populations (26, 42, 44). Resistance has been attributed to a loss of specific midgut receptors or their binding capacity (42). Reversal of such resistance was reported to occur when exposure to *B. thuringiensis* was stopped for many generations (43). Regained sensitivity was linked to restored Cry-binding capacity and higher biotic fitness, indicating that the Cry receptors might also play a role in normal physiological processes of the epithelial cells. Although the molecular basis of this phenomenon is still unclear, it may indicate a delicate equilibrium between larval biotic fitness and the antagonizing selectable pressure exerted by exposure to specific Cry toxins. Consequently, it has led to the strategy that in fields of transgenic plants expressing Cry toxins, it is essential to maintain a certain proportion of wild-type refuge plants or rotation with crops that do not express Cry proteins (12, 43).

An additional conceptual strategy may increase the efficacy and potential future of *B. thuringiensis* Cry toxins in insect control. Smirnoff (35) observed that coapplication of *B. thuringiensis* (Sandoz-Wander preparation 26B or Dipel 36B) and chitinase significantly increased the insecticidal effect of *B. thuringiensis* on *Choristoneura fumiferana* larvae. Similarly, application of low concentrations of a mixture of spore crystal suspension from *B. thuringiensis* subsp. *entomocidus* and chitinolytic bacteria resulted in a significant synergistic insecticidal effect against *Spodoptera littoralis* larvae (39). It was suggested that the chitinolytic bacteria affect the larval peritrophic membrane, which normally forms a cylindrical sheet separating the gut epithelium from the lumen. The peritrophic membrane consists of a network of chitin embedded in a protein-carbo-

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hydrate matrix and provides a physical barrier against mechanical damage and invasion of microorganisms (4, 10, 45). Isolated peritrophic membranes were shown to limit penetration of dissolved δ-endotoxin in vitro (53). Thus, increasing the level of endochitinases in the larval midgut may elevate the larvicidal effect as a result of peritrophic membrane perforation and increase accessibility of the δ-endotoxin molecules to the epithelial membranes.

In the present work, the combined effect of *Escherichia coli*-produced CryIC and chitinase on young larvae of the polyphagous insect *S. littoralis* was examined. Larvae of *Spodoptera* spp. are relatively tolerant of most of the Cry proteins and are sensitive only to CryIC, CryIE, and CryIH. However, a relatively high dosage of CryIC is required to effectively control them (5). The results indicate that while CryIC-containing crystals are highly insecticidal, carboxy-terminally truncated or full-length CryIC, applied as self-folded molecules, exerts low mortality but high growth retardation. This limited effect might be synergized by the addition of endochitinase, which perforates the peritrophic membrane lining the larval midgut.

MATERIALS AND METHODS

Bacterial strains. DNA of *B. thuringiensis* K26-21 (15) was used to synthesize the 3′-truncated *cryIC* gene by PCR. *E. coli* BL21(DE3) (42) was used to overexpress the cloned *cryIC*, and *E. coli* A5187 harboring pLCHIA served to overproduce *Serratia marcescens* endochitinase encoded by *chiAII* (33). The acrystalliferous, plasmid-cured derivative of *B. thuringiensis* subsp. *kurstaki* HD-1, strain CryB$^-$ (40), carrying plasmid pHT3101-*cry*IC (kindly provided by A. I. Aronson) was used for isolating homogenous CryIC crystals.

Construction of plasmids. Recombinant DNA techniques were carried out essentially as described by Sambrook et al. (30). The 3′-truncated *cryIC* gene was obtained from isolate K26-21 of *B. thuringiensis*, which was found to be highly insecticidal for the larvae of *S. littoralis* (15). Synthesis of the 2.2-kb *cryIC* coding region, starting from the translation start site and containing the sequence of the first 756 amino acids, was obtained by PCR amplification (40a). The PCR mixture in a total volume of 100 μl contained 1 μg of total DNA of isolate K26-21, 150 pmol of each primer, 0.2 mM deoxynucleoside triphosphates, 5 U of *Taq* polymerase (Promega), 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, and 2.5 mM $MgCl_2$. The amplification reaction was carried out using 30 cycles of 95°C (20 s), 42°C (45 s), and 72°C (60 s) and then a 10-min termination at 72°C. The forward primer with the sequence 5′-ACGGAGGATCCAT**ATG**G AGGAAAATAATCAAAATC-3′ contained the translation start site (boldfaced) and additional *Bam*HI and *Nde*I sites suitable for cloning in pBluescript (Stratagene) and pET11a (41) vectors, respectively. The reverse primer with the sequence 5′-CTCTTGGATCC**TAA**CGGGTATAAGCTTTTAATTTC-3′ contained a translation stop codon (boldfaced) and a *Bam*HI site and is complementary to the *cryIC* sequence located 2,247 bp downstream of the translation start site. The synthesized 2.2-kb fragment was cloned in pBluescript SK+, and its two strands were completely sequenced. The sequence was found to be identical to that of *cryIC* which was isolated from the *B. thuringiensis* subsp. *entomocidus* HD-110 and designated B15 (47), except for a single nucleotide difference, i.e., codon 370 is GCA instead of GAA, leading to a single amino acid difference (A124E). The presence of an A residue at the position 124 was also confirmed by sequencing of the *cryIC* gene from a cosmid clone obtained from the isolate K26-21 and is conserved in CryI proteins (40a). The 3′-truncated *cryIC* was recloned into the *Nde*I and *Bam*HI sites of the expression vector pET11a (41). The resulting plasmid, pC2, was transformed into *E. coli* BL21(DE3). A set of *cryIC* 3′ deletion mutants which were generated and bioassayed on *S. littoralis* neonate larvae showed that an N-terminal toxic part of 627 amino acid residues is capable of exerting toxicity (40a).

To overexpress the endochitinase encoded by *chiAII* (hereafter referred to as ChiA), *E. coli* A5187 harboring plasmid pLCHIA was used. This plasmid carries the *chiAII* gene of *Serratia marcescens* driven by the λpL promoter (34). ChiA was shown to maintain its activity at basic pH values (8), contrary to plant chitinases.

Protein production and estimation. Overproduction of the CryIC protein (86 kDa) was obtained by adding 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to 200 ml of log-phase culture (A_{600} = 0.5) of BL21(DE3) harboring the pC2 plasmid. After 3 h of induction, the cells were harvested, kept at −20°C for at least 2 h, and then lysed in 4 ml of 50 mM Tris-HCl (pH 8.0)–25% sucrose–1 mM phenylmethylsulfonyl fluoride–60 μg of lysozyme per ml for 45 min at 4°C. Following addition of 0.5 volume of 50 mM Tris-HCl (pH 8.0)–0.4% Triton X-100, the incubation on ice continued for an additional 45 min. The lysate was then passed several times through a syringe needle (25 gauge) and stored at −15°C. Inclusion bodies were isolated as described by Keller et al. (19).

Induction of *chiAII* expression in *E. coli* A5187 harboring plasmid pLCHTA to produce ChiA (58 kDa) was obtained by growing a log-phase culture (A_{600} = 0.5) at 42°C for 3 h (34). After the cells were harvested, a lysate with high ChiA content was obtained by the procedure described for CryIC. To estimate the amount of CryIC or ChiA in the total protein lysate, equal amounts of lysate proteins were separated on sodium dodecyl sulfate (SDS)–7.5% polyacrylamide gels in parallel to increasing concentrations of bovine serum albumin as standards. The concentrations of CryIC and ChiA were evaluated after scanning the gel stained with Coomassie brilliant blue R-250 with a laser scanner (LKB Ultra scan XL 2222-020). The total protein concentration of the bacterial lysate was measured by Coomassie staining (19, 23).

To obtain protein lysates devoid of CryIC, cultures of BL21(DE3) harboring plasmid pET11a were induced by IPTG for 3 h, harvested, and lysed as described above. Protein extracts lacking ChiAII were prepared from *E. coli* A5187(pLCHIA) grown at 30°C. Aliquots of control lysates were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (see Fig. 1) to confirm the lack of CryIC or ChiAII.

Isolation of CryIC crystal-spore mixture. The acrystalliferous HD-1 strain harboring plasmid pHT3101-*cry*IC [kindly supplied by A. Aronson and designated *B. thuringiensis* CryB$^-$ (pHT3101-*cry*IC)] was used to isolate pure CryIC crystals. pHT3101-*cry*IC carries a 7-kb *Eco*RI fragment from *B. thuringiensis* subsp. *aizawai* 7.29 which includes the entire coding region of *cryIC* and its native promoter and terminator regions (31, 33). pHT3101 is a stable multicopy plasmid developed to obtain defined crystals in originally acrystalliferous *B. thuringiensis* strains (1). An overnight culture of *B. thuringiensis* CryB$^-$-(pHT3101-*cry*IC) grown in 500 ml of Luria broth with 25 μg of erythromycin per ml at 30°C was centrifuged for 5 min at 6,000 rpm (GSA rotor) and resuspended in an equal volume of sterilized tap water. After 3 days of incubation at 30°C, crystals were separated by centrifugation at 12,000 rpm (SLA 1,500 rotor) for 10 min. The pellet containing the crystals was resuspended in 5 ml of H_2O and used for protein evaluation by SDS-PAGE and larval bioassays. A single 135-kDa band specifically recognized by anti-CryIC polyclonal antibodies in a Western blot (immunoblot) was identified (data not presented). Denaturation and renaturation of crystals to obtain self-folded 135-kDa CryIC molecules were performed as described by Keller et al. (19).

Insect bioassays. The insecticidal effect of CryIC alone or in combination with ChiA on neonate *S. littoralis* larvae was determined by feeding the larvae an artificial diet as previously described (15), with few modifications. Aliquots (170 μl) of artificial diet (50°C) containing the required concentrations of CryIC and ChiA were poured into Eppendorf tubes with punctured caps (for aeration). One neonate larva was placed in each tube, which was then covered with a piece of Miracloth (Calbiochem). A total of 100 tubes were used for each treatment, arranged in replicates of 10 tubes each. All treatments contained equal amounts of bacterial proteins. Corresponding protein lysates devoid of CryIC or ChiA were used to compensate the protein content in each treatment and in the control treatments of every assay. After 6 feeding days at 25°C and 65% relative humidity with a 12-h photoperiod, larval weight values were separately recorded for each of the 10 replicates of 10 larvae. CryIC concentrations required to cause a 50% reduction in larval weight compared with that of larvae in the control treatment and standard errors of the mean were determined by log probit analysis. Data were statistically analyzed by Duncan's multiple-range test (11).

Effect of ChiA on the peritrophic membrane of *S. littoralis*. The pH values of the midgut lumen content of *S. littoralis* larvae ranged between 8.5 and 9.5 in our measurements. Similar values were reported by Ishaaya et al. (18). Peritrophic membranes were isolated from dissected midguts of fifth-instar larvae of *S. littoralis*. After rinsing with saline (0.85% NaCl), the membranes were placed in Eppendorf tubes with increasing concentrations (0.1, 1, 10, and 100 μg/ml) of *E. coli*-produced ChiA in 0.1 M Tris-HCl, pH 8.5. In the control treatments, the membranes were incubated in equal amounts of *E. coli* A5187(pLCHIA) lysate proteins without endochitinase. After 30 min of incubation at 25°C with gentle shaking (100 rpm), the treated membranes were rinsed and then gradually dehydrated through increasing concentrations of ethanol (25 to 100%), 10 min in each concentration. The critical-point-dried specimens were mounted on stubs. The specimens were sputter coated with gold and viewed in an AMR-900 scanning electron microscope, as described by Sneh et al. (38). In parallel, fifth-instar larvae were fed with 2-cm^2 pieces of castor bean leaf, overlaid with *E. coli* A5187(pLCHIA) lysate containing 2 μg of ChiA or an equal concentration of lysate proteins (control), and then the peritrophic membranes were isolated and subjected to the same scanning electron microscopy preparation procedure.

RESULTS

Production of CryIC and ChiA in *E. coli*. The 86-kDa CryIC protein was produced in *E. coli* harboring plasmid pC2 upon IPTG induction (Fig. 1). Most of the protein was packed in inclusion bodies (40a). The endochitinase ChiA was produced in *E. coli* expressing the *chiAII* gene from a λpL promoter by induction at 42°C (Fig. 1). ChiA was both secreted extracellularly (data not shown) and stored as a soluble cytosolic or

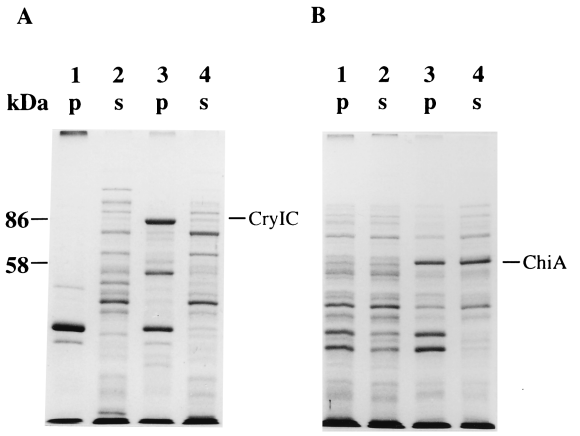


FIG. 1. SDS-PAGE analysis of CryIC (A) and ChiA (B) proteins expressed in *E. coli*. Lanes 1 and 2, inclusion bodies (p) and the supernatants (s) of the noninduced control, respectively; lanes 3 and 4, similar fractions of the induced cultures producing CryIC (A) or ChiA (B), respectively.

inclusion body protein (Fig. 1). *E. coli* lysates overproducing either CryIC or ChiA were used in all assays described below.

Effect of ChiA on the peritrophic membrane. Peritrophic membranes isolated from fifth-instar larvae were subjected to increasing concentrations of *E. coli*-produced ChiA. All peritrophic membrane samples which were incubated with 0.1 to 10 μg of chitinase per ml underwent perforation (Fig. 2). Even a ChiA concentration as low as 0.1 μg/ml was still effective (Fig. 2d), while peritrophic membranes of control treatments remained intact. Moreover, perforation of the peritrophic membrane by chitinase also occurred in vivo, upon feeding the fifth-instar larvae with 2 μg of *E. coli*-produced ChiA per larva (Fig. 2b).

CryIC toxicity. The anti-insect activity of the 86-kDa CryIC was evaluated in larval bioassays with increasing CryIC concentrations (0.01 to 35 μg/ml) (Fig. 3). An ineffective CryIC concentration (0.01 μg/ml) was used as a control in all larval bioassays. Each treatment included an equal amount of *E. coli* total proteins (2.1 μg/ml). Larval weights were recorded after 6 days of feeding. The effective concentration of CryIC causing a 50% reduction in larval weight was determined as 0.5 μg/ml. According to the dose-response curve (Fig. 3A), the highest toxic activity of CryIC was obtained at a concentration of ca. 20 μg/ml. Higher toxin concentrations did not cause any further reduction in larval weight. Larval mortality recorded at 20 and 30 μg of CryIC per ml did not exceed the level of 30 to 40% of the control treatments. Although 6 days of feeding on CryIC toxin did not lead to a high level of larval mortality, the inhibited larvae could not survive the toxic effect when they continued to feed on CryIC-containing medium.

The toxic effect of the 86-kDa CryIC was compared with that of the native full-length Cry protein (135 kDa) produced as a sole crystalline component of *B. thuringiensis* subsp. *kurstaki* CryB$^-$ harboring pHT3101-*cry*IC. Figure 3B shows that when the two CryIC types were applied in the larval diet at a concentration of 30 μg/ml, only the crystalline form of CryIC applied as crystal-spore mixtures caused 88% mortality. However, when the CryIC crystals were disrupted by sequential

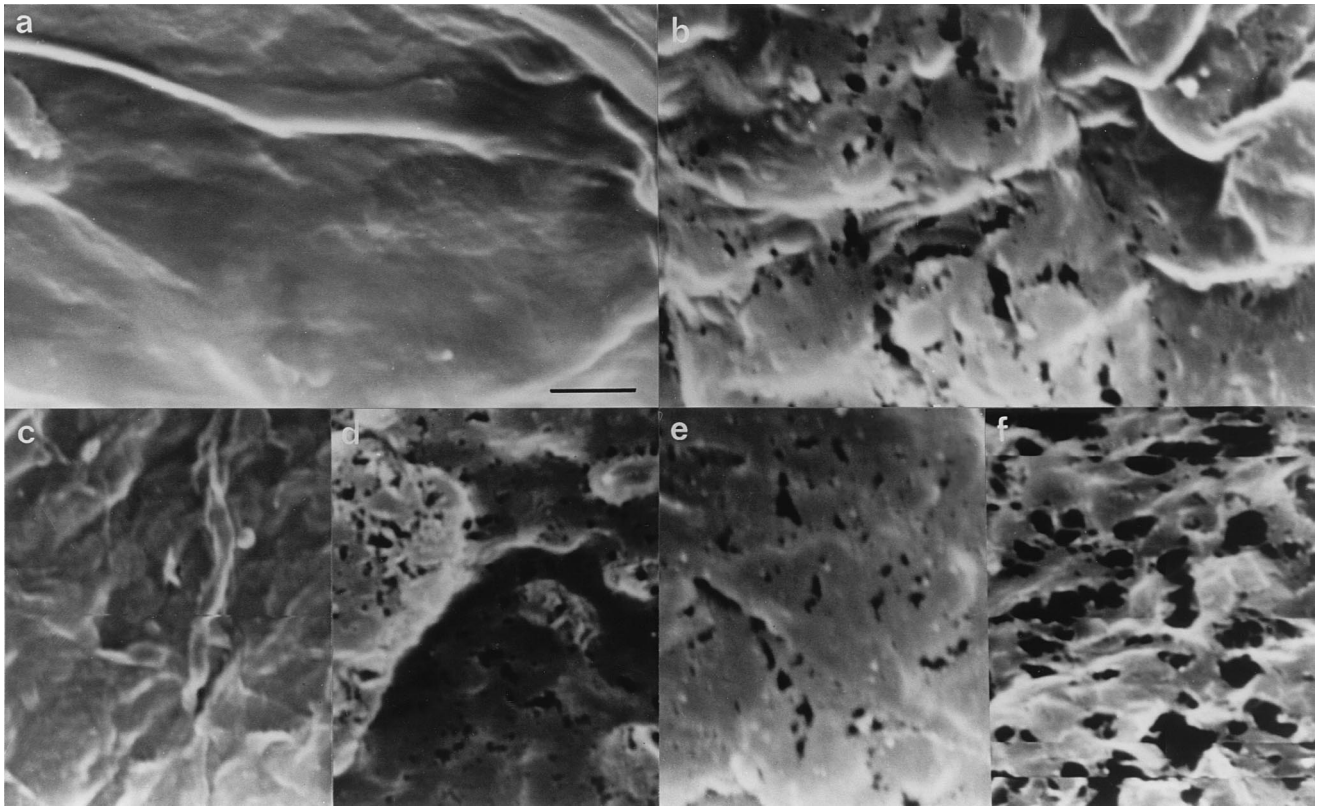
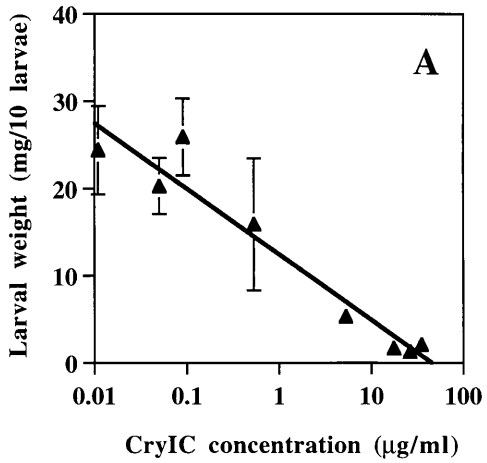


FIG. 2. Effect of ChiA on the peritrophic membrane of fifth-instar *S. littoralis* larvae displayed by scanning electron micrographs. (a and b) Feeding of the fifth-instar larvae on a 2-cm^2 leaf piece with 0 (a) and 2 (b) μg of ChiA; (c to f) incubation of isolated periotrophic membranes for 30 min with 0 (c), 0.1 (d), 1 (e), and 10 (d) μg of ChiA per ml applied as an *E. coli* lysate in Tris-HCl, pH 8.5. Bar = 1 μM. Compared treatments contained equal concentrations of lysate proteins.



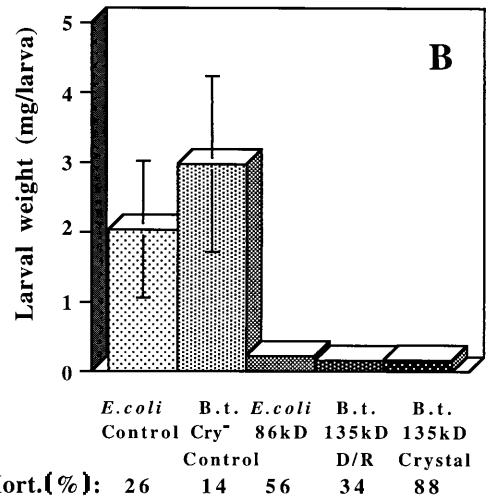


FIG. 3. Toxic effects of the 68-kDa C-terminally truncated CryIC produced in *E. coli* and the native 135-kDa CryIC produced in *B. thuringiensis*. (A) Determination of the concentration of *E. coli*-produced 86-kDa CryIC causing a 50% reduction in larval weight. (B) Comparison of the toxic effect exerted by CryIC crystal-spore mixture with that obtained by free CryIC released from the same crystals by denaturation and subsequent renaturation and with that of the 86-kDa CryIC produced in *E. coli*. Neonate larvae (100 per treatment) were fed on artificial diet containing 30 μg of the CryIC form per ml and an equal amount of proteins. Larval weights and mortality (Mort.) were recorded after 6 days. Control treatments contained similar amounts of proteins extracted from the same *B. thuringiensis* (B.t.) strain devoid of the CryIC-carrying plasmid or from *E. coli* harboring pET11a. Vertical bars signify the standard errors of the mean.

denaturation and renaturation, resulting in self-folded 135-kDa molecules, the overall insecticidal effect (mortality and growth retardation) did not exceed the effect obtained by the *E. coli*-produced 86-kDa CryIC. These results indicate that free self-folded CryIC molecules, produced from transgenes in different organisms, are less toxic than the native crystalline form.

Combined insecticidal effect of CryIC and ChiA. It has previously been demonstrated that coapplication of spore-crystal suspensions of *B. thuringiensis* subsp. *entomocidus* (Bt_{24}) and culture suspensions of several different chitinase-producing bacteria resulted in a synergistic insecticidal effect (39). To directly evaluate the combined toxic effect, neonate larvae were fed on mixtures of 86-kDa CryIC and ChiA. The CryIC concentrations used in this experiment were close to the concentration causing a 50% reduction in larval weight (1.0 or 3.0 μg/ml), and ChiA was used at a concentration range of 0.1 to 110 μg/ml. In the control treatments, the larval diet contained only one of the proteins, either CryIC or ChiA at equivalent concentrations. The lowest CryIC (1.0 μg/ml) and ChiA (0.1 μg/ml) concentrations used were within the range of expected expression levels of single-copy transgenes in plants. Coapplication of CryIC and increasing ChiA concentrations (starting from 1 μg/ml) always caused a synergistic reduction in larval weight (62.0 to 98.2%), while 1 μg of CryIC per ml alone caused only a 37% reduction in larval weight (Fig. 4).

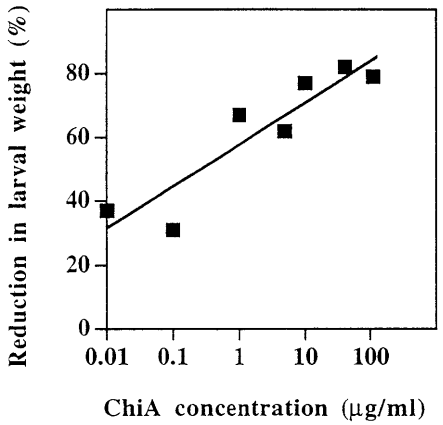


FIG. 4. Combined toxic effect of 1 μg of CryIC per ml and various concentrations of ChiA on neonate larvae of *S. littoralis*. Weights after *E. coli*-produced CryIC and ChiA combined treatments were compared with those recorded after CryIC treatment alone and expressed as percent reduction. Experimental conditions were as described in the legend to Fig. 3.

When a higher concentration of ChiA (110 μg/ml) was applied, it was possible to show that endochitinase alone was capable of causing an increase in larval weight (Fig. 5, control without toxin). But when endochitinase was coapplied with CryIC, the presence of CryIC abolished the growth effect of endochitinase, leading to a synergistic increase of CryIC toxicity (Fig. 5) even at relatively low concentrations of CryIC. Thus, in the presence of ChiA, a CryIC concentration as low as 3 μg/ml caused a toxic effect equivalent to that of 20 μg of CryIC per ml used alone (Fig. 3). CryIC did not kill the majority of larvae but caused an extreme retardation of growth. The probability of such underdeveloped larvae (Fig. 5B) surviving in the natural environment is extremely low.

DISCUSSION

A considerable weight reduction in neonate *S. littoralis* larvae, but not high larval mortality, was caused by the C-terminally truncated version of CryIC (756 amino acids) overexpressed in *E. coli*. This growth retardation was irreversible, and the larvae could not recover from the toxic effect. On the other hand, high-percentage (Fig. 3B) or complete (19) mortality was obtained when the larvae were fed with intact CryIC crystal-spore mixture produced by acrystalliferous *B. thuringiensis* harboring pHT3101-*cry*IC or in *B. thuringiensis* K26-21, respectively. But when these crystals were denatured and renatured prior to their application to the diet, their toxicity decreased to the same level obtained by the 86-kDa C-terminally truncated CryIC. Most of the self-folded 135-kDa CryIC molecules, similarly to the C-terminally truncated versions, were shown to be accessible to proteolysis by the midgut proteases (19, 37). Perhaps these self-folded CryIC molecules are more vulnerable to

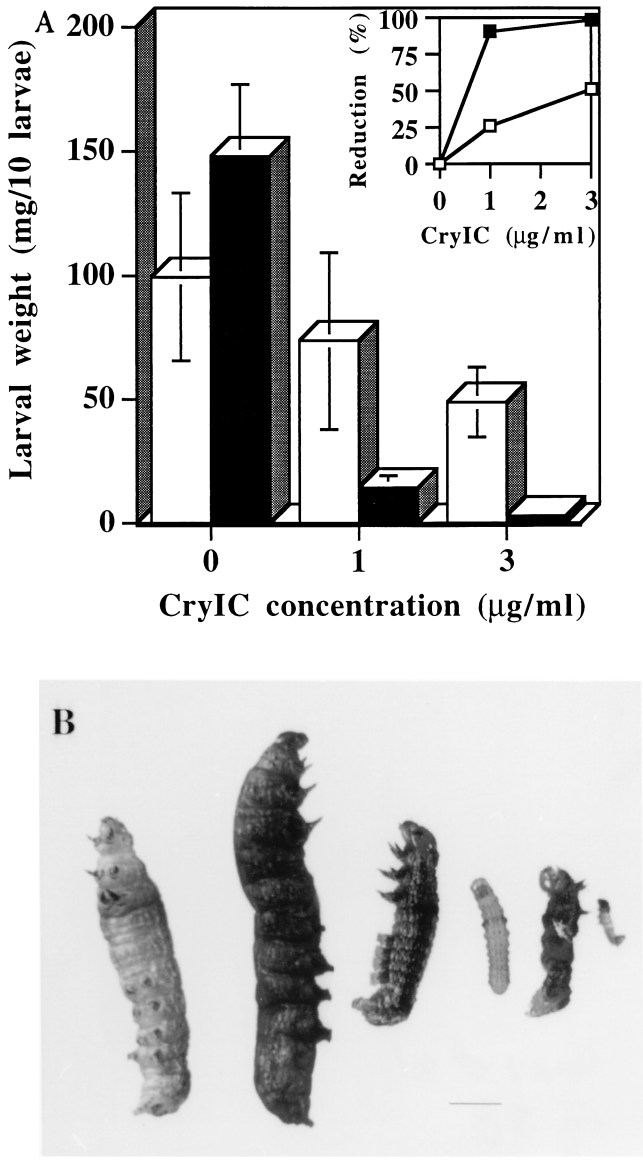


FIG. 5. Combined toxic effect of 1 and 3 μg of CryIC per ml and 110 μg of ChiA per ml on neonate larvae of *S. littoralis*. (A) Larval weight recorded for each CryIC and ChiA combination. The vertical bars indicate standard errors of the mean. (Inset) The reduction in larval weight obtained by addition of ChiA extract to defined concentrations of CryIC calculated from panel A. ■, with chitinase; □, without chitinase. (B) A photograph of corresponding larvae arranged according to the treatment order demonstrated in panel A. Bar, 1 mm.

the larval midgut proteases, as demonstrated by Keller et al. (19) for both CryIC forms or by Smith et al. (37) for the C-terminally truncated 92-kDa CryIC. Consequently, it appears that only a small proportion of ingested Cry molecules can actually reach the vicinity of the midgut epithelium membrane and interact with the epithelial specific receptors.

Feeding the larvae with an *E. coli* lysate containing recombinant ChiA resulted in perforation of the chitin-containing peritrophic membrane which separates the midgut lumen content from the brush border epithelium membrane throughout larval development. Pore formation in the peritrophic membrane was observed even at a chitinase concentration as low as 0.1 μg/ml (Fig. 2). This perforation caused an increase in CryIC toxicity, most probably by increasing the availability of the limited number of functional toxin molecules to the epithelial membrane receptors. An extreme reduction in larval weight was achieved at a CryIC concentration as low as 3 μg/ml, in the presence of only 1 μg of ChiA per ml. Thus, a clear synergistic toxic effect on neonate larvae was obtained when *E. coli*-produced CryIC and ChiA were cointroduced to the larval diet. This confirmed previous results obtained in the laboratory and in the field (39). Another alteration of the lepidopteran peritrophic membrane by a baculovirus similarly enhanced viral infections by exposing the underlying epithelial cells to more virus particles (9).

The effective concentrations of CryIC and ChiA mixtures obtained in the present study are within the expression range of a single transgene in a transgenic plant (12). Therefore, it is very likely that cointroduction of *cry* and *chiA* genes into transgenic bacteria or plants could increase the insecticidal efficacy of low concentrations of transgenic, self-folded δ-endotoxins which lack the protection against the larval midgut proteases provided by the natural folding in the crystalline structure.

Development of Cry-resistant larval populations has been shown to correlate with the intensive and consistent application of *B. thuringiensis* preparations. Diverse effects, including cross-resistance responses (26, 43, 44) and reversal of resistance to certain δ-endotoxins linked to increasing biotic fitness, have been reported previously (43). The approach examined in the present study, based on coapplication of low concentrations of endochitinase and Cry proteins, still requires further biological verification in terms of acquired Cry resistance that antagonizes biotic fitness (43). Experiments are currently under way to verify this approach in transgenic plants expressing CryIC and ChiA.

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