Pergamon

0965-1748(95)00102-6

Insect Biochem. Molec. Biol. Vol. 26, No. 4, pp. 365–373, 1996

0965-1748/96 $15.00 + 0.00

Digestion of δ-Endotoxin by Gut Proteases may Explain Reduced Sensitivity of Advanced Instar Larvae of *Spodoptera littoralis* to CryIC

MENACHEM KELLER,* BARUCH SNEH,* NICOLAI STRIZHOV,† EVGENYA PRUDOVSKY,* AVITAL REGEV,* CSABA KONCZ,† JEFF SCHELL,† AVIAH ZILBERSTEIN*‡

Received 9 August 1995; revised and accepted 22 November 1995

The present study describes the correlation between gut protease activity of lepidopteran larvae of different instars, the inactivation of *Bacillus thuringinesis* δ-endotoxins in crystalline and noncrystalline forms, and the reduced susceptibility of advanced larval instars of *Spodoptera littoralis* to the toxin. The original assembly of δ-endotoxins in a crystal structure is essential for causing efficient larval mortality. Denaturation and renaturation (D/R) of δ-ednotoxin crystals increased the vulnerability of the toxin molecules to proteolysis, reduced their capability to kill neonate larvae of *S. littoralis*, but sustained most of their larval growth-inhibition activity. *E. coli*-produced CryIC δ-endotoxin applied as a fraction of inclusion bodies exerted a growth inhibition effect, similar to the molecules released from the crystals by denaturation and subsequent renaturation. Incubation of CryIC with gut juice of 1st or 2nd instar larvae, left part of the CryIC toxin intact, while the toxin was completely degraded when incubated with gut juice of 5th instar larvae. The degradation rate was consistent with the increase of protease specific activity of the gut juice during larval development. This increase in toxin degradation may account for the loss of sensitivity of 5th instar larvae to CryIC. Specific protease inhibitors such as PMSF and Leupeptin were shown to inhibit gut proteases activity in all instar larvae, while, 1,10 phenanthroline, TLCK and TPCK were effective only in young instar larvae. The differential effect of protease inhibitors on proteases obtained from different larval instars indicated that gut juice protease profiles change with larval age. The observed quantitative and qualitative differences in degradation of δ-endotoxin by larval gut proteases that occur during larval maturation may account for the difference in susceptibility to the δ-endotoxin. This finding should be taken into consideration when designing strategies for the development of transgenic crops expressing δ-endotoxins as potent insecticidal proteins.

δ-Endotoxin CryIC Gut endo-proteases Digestion Protease inhibitors *Bacillus thuringiensis* *Spodoptera littoralis*

INTRODUCTION

Delta-endotoxins produced by different strains of *Bacillus thuringiensis* Berliner during sporulation are assembled in parasporal crystalline inclusions. When these crystals are ingested by lepidopteran larvae, the crystals are gradually dissolved, due to the high pH of the larval gut juice, and the δ-endotoxins (crystal proteins) are specifically cleaved at two sites by gut trypsin-like proteases. This proteolysis yields the self-folded N-terminal toxin (60–70 kDa) which lacks the first 29 amino acids. The C-terminal part which is only involved in correct assembly of the crystalline structure during sporulation, is further degraded (Faust *et al.*, 1974; Hofte and Whiteley, 1989; Aronson, 1993). The toxic moiety comprises three conserved structural domains in all δ-endotoxins. The N-terminal domain with its amphipathic α helices exerts the toxic effect and the other two are necessary for correct folding and specific interaction with gut membrane receptors. Due to its specific folding, the processed toxin is considered to be less susceptible to proteolysis (Bai *et al.*, 1993; Li *et al.*, 1991; Schnepf *et al.*, 1985). The different δ-endotoxin types display different specific insecticidal spectra against Lepidoptera, Diptera or Coleoptera species. Furthermore, within these insect orders the different δ-endotoxin proteins have narrower species specificities, which are dictated by the

* Department of Botany, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv, Israel 69978.
† Max Planck Institut für Zuchtungsforschung, Koln, 50829, Germany.
‡ Author for correspondence.

presence of specific membrane receptors in the brush border epithelial cells of the gut and specific receptor binding sequences in domain II of the active toxins (Hofte and Whiteley, 1989; Aronson, 1993; Li *et al.*, 1991). In certain δ-endotoxins, the specificity is also dependent on gut specific endo-proteases required for the primary cleavage of the protoxin to its active form (Haider *et al.*, 1986). Thus, larvicidal specificity of different δ-endotoxins to their respective insects is determined by a combination of species specific membrane receptors, complementary binding sites of the toxin and structural interaction among the three domains (Van Rie *et al.*, 1990; Li *et al.*, 1991; Aronson, 1993).

A wide range of lepidopteran larvae are susceptible to CryIA(a), CryIA(b), or CryIA(c) δ-endotoxins, whereas *Spodoptera littoralis* larvae are almost insensitive to these δ-endotoxins, but susceptible to CryIC and CryIE (Hofte and Whitely, 1989). CryIC toxin is produced by *B. thuringiensis* vars. *entomocidus, aizawai* (Sneh *et al.*, 1981; Visser *et al.*, 1988; Sanchis *et al.*, 1989), or by isolates K26-21 and Mr1-37 (Hamal *et al.*, 1991; Strizhov *et al.*, in preparation). CryIE is produced by *B. thuringiensis* vars. *kenyae* and *dendrolimus* (Van Rice *et al.*, 1990). While the 1st and 2nd instar larvae of *S. littoralis* and other lepidopteran insects were found to be highly susceptible to their specific δ-endotoxins, the 3rd to 6th instar larvae display considerable resistance (Sneh *et al.*, 1981; Bai *et al.*, 1993); whereas, larvae of other insect species such as *Chrysodeixes* (*Plusia*) *chalcites* Esper display high susceptibility to the δ-endotoxin at all instar stages (Broza *et al.*, 1994). The lack of sensitivity may be attributed to the high content of alkaline proteases in the gut juice of the phytophagous lepidopteran larvae (Christeller *et al.*, 1992; Purcell *et al.*, 1992).

Studies of the proteolytic capacity of insect gut proteases have been mainly based on the use of specific substrates and inhibitors. Serine proteases were found to play the major role in proteolytic processes in lepidopteran larval guts (Christeller *et al.*, 1992). Gut proteases obtained from several lepidopteran larvae have shown a significant increase in their activity at pH levels above 8.0. Soybean trypsin inhibitor as well as leupeptin inhibited 60–70% of the gut protease activity (Purcell *et al.*, 1992) indicating that the major gut proteases are trypsin-like serine proteases. Additional proteolytic enzymes such as chymotrypsin, elastase, carboxypeptidase A and B, and leucine amino peptidase were also found at lower amounts in the gut juice of late instar lepidopteran larvae, including *S. litura* (Christeller *et al.*, 1992). As the gut jucie from the advanced larval instars is more experimentally amenable, most of the research related to gut proteases has been carried out using gut juices from advanced larval instars. Less is known about the proteinase profiles of the gut juice of young larvae which were found to be more susceptible to the Cry toxins. Furthermore, diversity in digestive proteinase activities was found among insects (Wolfson and Murdock, 1990). Such a variation could also exist between the early and late instar larvae of the same species. Thus, characterization of δ-endotoxin susceptibility to protease activities in the larval gut of specific insects and larval stages of interest is necessary.

During the last decade, transgenic plants expressing specific full length or C-terminal truncated Cry proteins that exert resistance against insects have been developed (Vaeck *et al.*, 1987; Perlak *et al.*, 1990). The differences in codon usage between *Bacillus thuringiensis* and plants, and the high number of eukaryotic mRNA pretermination signals present in the A+T rich sequence of *cry* genes required drastic modifications in *cry* sequences to efficiently express *cry* genes in plants (Perlak *et al.*, 1990, 1991, 1993). Most of the partially modified or chemically synthesized *cry* genes were introduced into plants as 3′ truncated versions, encoding the toxic part and a limited region of the C-terminal half required for the primary trypsin cleavage that forms the active toxin. As an essential step towards developing transgenic crops resistant to *Spodoptera littoralis*, correlations between the fate of different CryIC forms in the larval gut and their toxicity were investigated in the present study. Differences in the degradation pattern of CryIC by gut juice proteases of young and advanced larval instars of *S. littoralis* were found. These differences may account for more intensive inactivation of CryIC in advanced larval instars.

MATERIALS AND METHODS

Materials

Phenyl methyl sulfonyl fluoride (PMSF), leupeptin, 1.10 phenanthroline, pepstatine, tosyl lysyl chloromethyl ketone (TLCK), tosyl phenylalanyl chloromethyl ketone (TPCK), pre-stained molecular weight markers (SDS-7), bovine serum albumin (BSA), isopropyl D-thiogalactopyranoside (IPTG) and Coomassie Brilliant Blue (R-250) were purchased from Sigma. The other chemicals were purchased from Merck.

Isolation of crystals

Isolate K26-21 of *B. thuringiensis*, highly effective against *Spodoptera littoralis* (Hamal *et al.*, 1991) was grown on solid LB plates for 3 days at 28°C, until reaching the sporulation stage. Spores and crystals from each plate were suspended in 1 ml H_2O and transferred to an Eppendorf tube. After centrifugation at 350 ***g*** for 20 min at room temperature, the supernatant containing the floating crystals was collected and stored at −20°C.

Expression of 3′ truncated cryIC in E. coli *and isolation of inclusion bodies*

The 5′ region of *cry*IC encoding 756 amino acids was synthesized by polymerase chain reaction (PCR) on total DNA template purified from isolate K26-21. The PCR product was cloned and completely sequenced. The sequence of the PCR synthesized gene is similar to the

sequence of the native B15 gene described by Van Rie *et al.* (1990) and differs in one amino acid residue from the CryIC described by Honee *et al.* (1988). Thus, despite using a PCR approach to synthesize the 3′ truncated gene, no amino acid substitutions were introduced. The synthesized *cry*IC was cloned in the expression vector pET11a (Studier *et al.*, 1990) and the resulting plasmid was designated as pC_2.

To isolate inclusion bodies enriched in CryIC, 400 ml log phase culture (A_{600}=0.4) of *E. coli* BL21 (DE3) harboring pC_2 were induced to over-produce CryIC by adding IPTG to a final concentration of 0.4 mM. After 3 h induction, the cells were collected by centrifugation at 5000 ***g*** and the pellet was frozen at −20°C for at least 30 min. The cells were suspended in 8 ml Buffer A (25% sucrose, 50 mM Tris–HCl pH 8, 1 mM PMSF, 1 μg/ml lysozyme) at room temperature. After 60 min lysis at 4°C, 3.2 ml buffer B (0.4% Triton X-100, 50 mM Tris–HC1 pH 8.0) were added and the incubation continued for additional 60 min at 4°C. The lysate was passed 4 times through a syringe needle (25G) to shear the DNA, then centrifuged at 20,000 ***g*** for 25 min at 4°C. The pellet was re-suspended in 4 ml buffer A (without lysozyme) and recentrifuged as above. The pellet, mainly containing inclusion bodies, was resuspended in 1 ml buffer A (without lysozyme) and the protein concentration was determined.

Denaturation and renaturation of CryIC inclusion bodies and crystals

CryIC containing inclusion bodies isolated from *E. coli* or crystals isolated from Bt isolate K26-21 were dissolved in denaturation buffer (6M Guanidinium–HC1, 10 mM DTT) at a final protein concentration of 3 μg/μl. After 60 min at room temperature, the insoluble material was removed by centrifugation at 10,000 ***g*** for 10 min. The supernatant with the denatured CryIC molecules was subjected to gradual renaturation by stepwise addition of 1/10 vol of H_2O every 30 min, until reaching a final concentration of 0.5 M Guanidinium-HC1. The solution was then dialyzed at 25°C against 300 ml solution A (10 mM EDTA, 50 mM NH_4HCO_3, pH 9.5) for 30 min, 300 ml solution B (10 mM EDTA pH 8.0, 50 mM Tris–HC1 pH 8.0) for 30 min and against solution B overnight (Wu and Aronson, 1992).

Bioassays with S. littoralis *larvae*

S. littoralis larvae were reared as previously described (Sneh *et al.*, 1981). For assaying larvicidal activity of CryIC, neonate larvae (100/treatment) were fed on an artificial diet (Yawetz *et al.*, 1983) mixed with the required concentrations of inclusions bodies or crystals. Five neonate larvae were placed in each of 45 × 35 mm (dia.) plastic vials (covered with perforated covers for aeration) containing 0.5 ml diet. After 6 days' incubation at 25°C, 65% relative humidity and 12 h photoperiod, larval weight values were separately recorded for each vial. Data were statistically analyzed by ANOVA program.

Extraction of larval gut juice

To perform CryIC digestion experiments with pure gut juice of the early instar larvae, a method to obtain non-diluted, pure juice samples was developed. Larvae of each instar were placed in 0.65 ml conical tubes (Sorenson), with a small hole at the bottom, covered with fiber glass. The tubes were inserted into 1.6 ml Eppendorf tubes and centrifuged at room temperature until the Eppendorf centrifuge reached its maximal speed. After 5 min rest, to let the larvae regain their motility in the tube, they were spun again under the same conditions. Following the second centrifugation, the gut juice was collected from the outer tube. To obtain gut juice from the 1st and 2nd instar larvae, several hundred individuals were placed in the inner tube, whereas, only one or two 4th to 6th instar larvae were spun to yield the required amount of gut juice. Protein concentrations of each juice preparation was determined.

Protease assays

The truncated CryIC (first 756 a.a) produced in *E. coli* and packed in inclusion bodies, or isolated crystals from the isolate K26-21 were incubated with gut juice of different larval instars in a final volume of 20 μl 50 mM Tris–HC1 pH 8.0, at 25°C. Specific proteinase inhibitors were added to the reactions at the following concentrations: 0.001 to 8 mM leupeptin — a trypsin-like serine protease inhibitor, 50 mM 1,10 phenanthroline — a metallo-protease inhibitor, 0.001 to 10 mM PMSF — a general serine protease inhibitor, 200 μM TLCK — a trypsin-like serine protease inhibitor, 200 μM TPCK — a chymotrypsin-like serine protease inhibitor. All inhibitors were applied at concentrations higher than usually recommended for use in inhibition assays (Beynon and Bond, 1990). Assays with or without proteinase inhibitors were carried out in parallel. Reactions were terminated by adding equal volume of 2 × SDS–PAGE loading buffer (Laemmli, 1970), boiling for 3 min and then loading onto 12% SDS-polyacrylamide gel (Laemmli, 1970). The Coomassie Brilliant Blue stained gels were scanned and the percent inhibition of CryIC degradation caused by the specific inhibitors was calculated.

Extraction of larval hemolymph fluid

To extract larval hemolymph fluid from the 4th and 5th instar larvae, one of the larval pseudopodia was excised. The hemolymph fluid which was leaking from the open wound was collected into an Eppendorf tube and stored at −20°C until used.

Determination of total protein concentration

Protein concentration was determined by applying 5 μl protein samples dissolved in × 2 loading buffer (0.1 M Tris–HC1 pH 7.0, 4% SDS, 20% glycerol, 10% 2-Mercaptoethanol) on Whatman 3 MM filter paper in parallel to 5 μl aliquots of 0.1–5 μg/μl BSA samples dissolved in the same buffer. After staining with "staining" solution (50% methanol, 10% acetic acid, 0.1% Coomas-

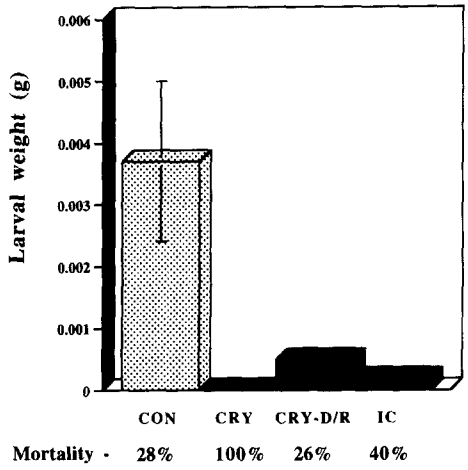

FIGURE 1. The toxic effect of different forms of CryIC on neonate larvae of *Spodoptera littoralis*. The effect of three CryIC preparations (30 μg protein/ml diet) on the mortality and weight of *S. littoralis* neonature larvae was assessed after 6 days of feeding on artificial diet. CRY–crystals from isolate K26-21; CRY-D/R—crystals subjected to denaturation and renaturation; IC—CryIC containing inclusion bodies produced by *E. coli* BL21 (DE3) harboring pC2. The percentage of larval mortality is indicated in each treatment. The histogram depicts weight of the larvae which survived in each of the toxic treatments.

sie Brilliant Blue) for 10 min and de-staining twice with 50% methanol, 10% acetic acid for 10 min, the stained spots were excised, extracted in 1% SDS for at least 2 h at room temperature and their absorbance at 600 nm was measured. Protein concentrations were estimated according to a BSA standard curve (Marder *et al.*, 1986).

RESULTS

Insecticidal activity of CryIC

Preliminary bioassay experiments with *E. coli*-produced CryIC indicated that increasing CryIC concentrations in the artificial diet of neonate *S. littoralis* larvae reduced the larval weight as recorded after 6 days incubation. The recorded percentage of larval mortality did not exceed 40% even at a CryIC concentration of 30 μg/ml. However, when neonate larvae were fed on diet containing isolated crystals, 100% mortality was recorded at a concentration of 30 μg Cry/ml (Fig. 1). However, when the same crystal preparation was subjected to successive denaturation and renaturation steps, yielding spontaneously folded Cry molecules and then added to the diet, the over-all larvicidal effect expressed in mortality and reduction in larval weight was close to that obtained by the *E. coli*-produced CryIC (Fig. 1). There was no significant difference between the larval weight of those which survived the 135 kD natural Cry protein and the 86 kDa C-terminal truncated CryIC treatments. The differences in larval mortality caused by the two treatments may be attributed to the presence of other Cry proteins which are not toxic to *S. littoralis* in the native Bt crystals, thus reducing the proportion of CryIC in the applied 135 kDa mixture.

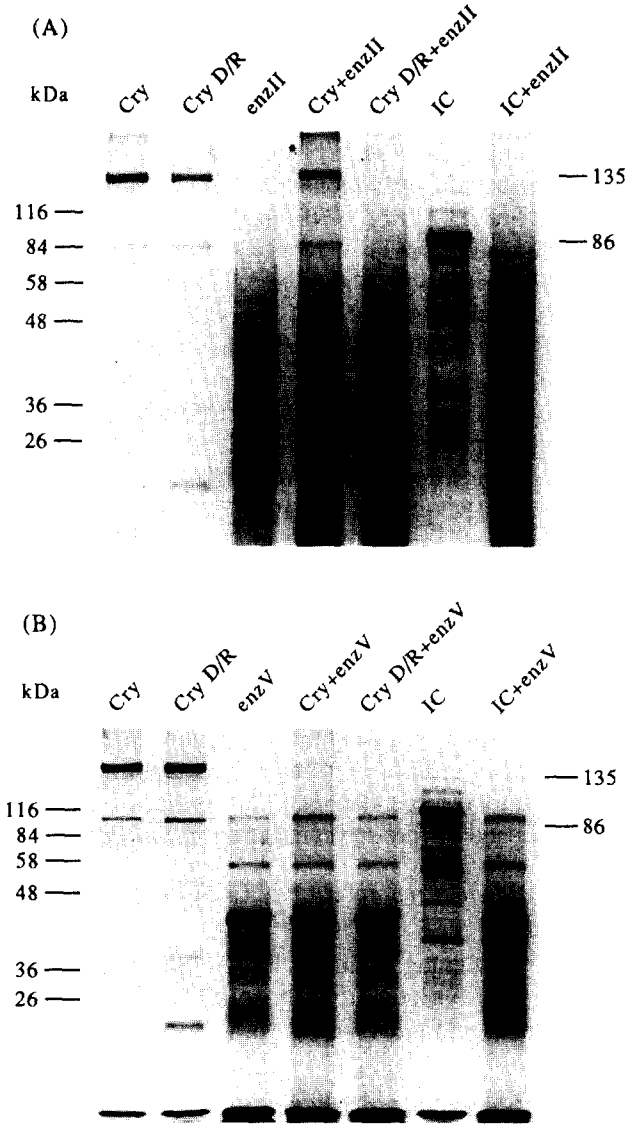

FIGURE 2. Digestion of different forms of δ-endotoxin by gut enzymes from the 2nd and 5th instar larvae of *Spodoptera littoralis*: SDS–PAGE separation of proteolyzed products obtained as follows: gut juices extracted from the 2nd (A) or 5th (B) instar *S. littoralis* larvae were incubated in 50 mM Tris–HCl, pH 8.0, in a final volume of 20 μl, at room temperature for 20 min (for 2nd instar), or 3 min (for 5th instar) with CryIC proteins. Cry–2 μg crystal isolated from *Bacillus thuringiensis* isolate K26-21; IC–5 μg *E. coli*-produced CryIC; D/R — denaturation–renaturation treatment; enz II and V— 40 μg and 5 μg total gut juice proteins from the 2nd (II) and 5th (V) instars, respectively. Molecular weight values are indicated on the left. The size of Cry molecules released from crystals or inclusion bodies by boiling in loading buffer, are indicated on the right.

Susceptibility of crystals and E. coli*-produced CryIC to gut proteases*

Natural CryIC-containing crystals, or those subjected to denaturation and renaturation (D/R) (2 μg), were incubated with gut juice (40 μg total protein) of 2nd instar larvae for 20 min. Most of the 135 kDa Cry proteins assembled in crystalline structure remained intact after the incubation (Fig. 2A). However, the crystal-free 135 kDa CryIC proteins resulting from crystal denaturation, as well as the *E. coli*-produced 86 kDa CryIC were

completely digested under the same incubation conditions (Fig 2A). When native crystals were incubated for only 3 min with gut juice extracted from 5th instar larvae containing only 5 μg total proteins, even the assembled crystalline CryIC was degraded, leaving only a small fraction of 135 kDa molecules undigested. Furthermore, disruption of the crystalline structure by successive denaturation and renaturation of crystals prior to the incubation with the 5th instar gut enzymes strongly increased their susceptibility, leading to their complete proteolysis. As was the case for the proteolysis of *E. coli*-produced CryIC, it was completely digested (Fig. 2B).

CryIC proteolysis by gut juices from different larval instars

CryIC proteolysis by gut juices obtained from different larval instars was determined by estimating the amount of degraded CryIC after 5 min incubation with gut proteases from different instar larvae (5 μg CryIC with 2 μg juice proteins). Under these conditions, gut proteases from 1st and 2nd instar larvae digested the CryIC protein very mildly, and therefore most of the CryIC (86 kDA) molecules remained intact. Proteases from the 3rd and the 4th instar larvae degraded 54 and 90% of the CryIC molecules respectively, within 5 min incubation (Fig. 3A). The proteolytic capability of gut proteases gradually increased during larval development, while concomitantly, the protein concentration of the gut juice decreased (Fig. 3B). Thus, during larval development, the capability of the gut juice to degrade CryIC gradually increased and was about 4-fold higher at the 4th instar stage (Fig. 3B).

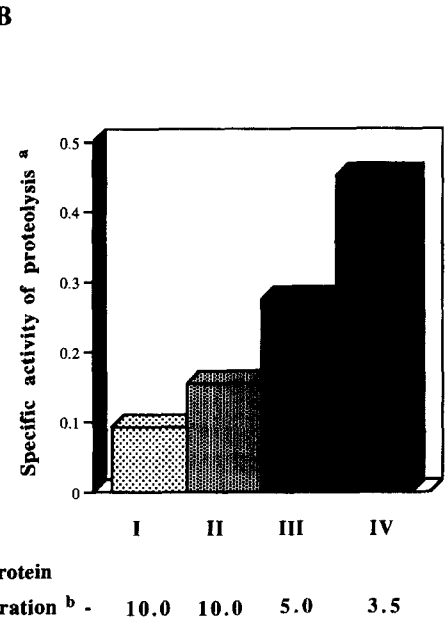

FIGURE 3. (B) Specific activity of gut juice proteases extracted from 1st to 4th larval instars evaluated by measurements of CryIC protein degradation displayed in A. [a]CryIC proteolysis expressed in μg CryIC degraded per μg gut juice protein per min. [b]Total protein concentration (μg/μl) of gut juice for each indicated instar.

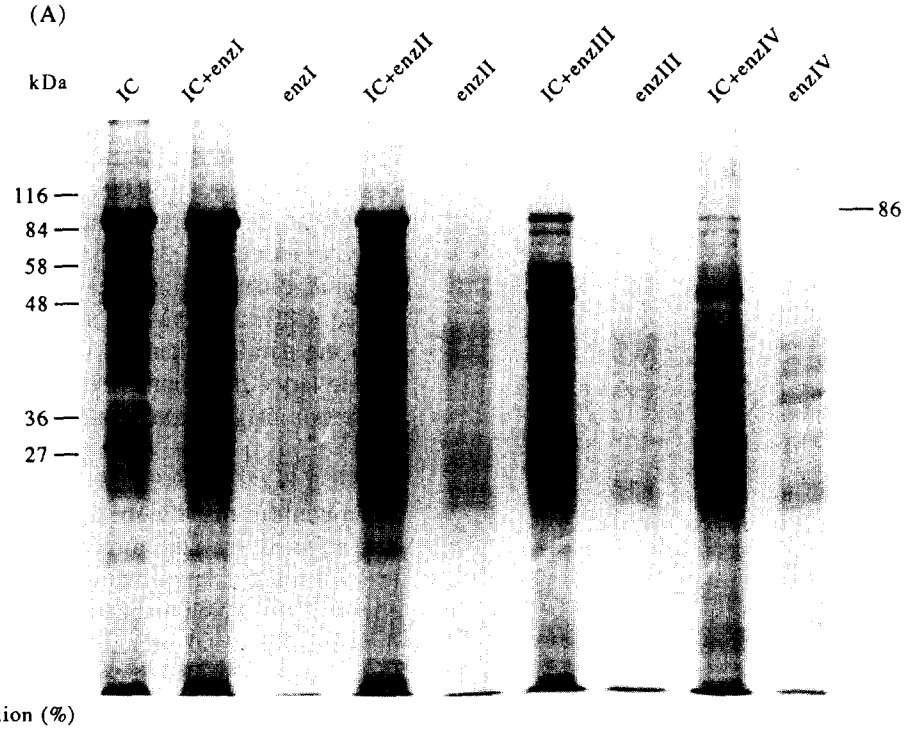

FIGURE 3. CryIC proteolysis by gut juice of different larval instars. (A) Increasing CryIC-protease activities in *Spodoptera littoralis* gut juices extracted from 1st to 4th instar larvae. Incubation with 5 μg *E. coli*-produced CryIC (86 kDa) was carried out in 50 mM Tris–HCl, pH 8.0, in a final volume of 20 μl, at room temperature for 5 min. Molecular mass markers are indicated on the left; IC-5 μg *E. coli*-produced CryIC; Enz I to IV — 2 μg gut juice total proteins extracted from 1st to 4th instar larvae. The percentage of CryIC degradation is indicated below each treatment, and was evaluated by densitometer scanning.

Qualitative differences in gut proteases

To evaluate the role of different gut proteases of early and late instar larvae in digesting CryIC toxin, specific proteinase inhibitors were included in the proteolysis assays. Previous studies with lepidopteran midgut enzymes showed that partially purified proteases from late instar larvae of *S. litura* were strongly inhibited by the serine proteinase inhibitor PMSF. Moreover, the trypsin-like proteinase inhibitor TLCK, showed 96% inhibition of the three identified proteases, while the chymotrypsin-specific inhibitor TPCK and the metallo-protease inhibitor EDTA had no inhibitory effect (Ahmad *et al.*, 1980). Christeller *et al.* (1992) as well as Terra (1988) in his review confirmed that the gut juice of the phytophagous species of the Noctuidae showed serine endopeptidase activity with alkaline pH optimum, while lacking activity of cysteine proteases when evaluated either at their optimal pH (pH 5.9) or at alkaline pH. Results summarized in Fig. 4 show that when *S. littoralis* gut juice samples of 2nd and 5th instar larvae, containing equal amount of total proteins, were incubated with CryIC in the presence of PMSF, leupeptin (trypsin-like serine protease inhibitor) as well as 1,10 phenanthroline (metallo-proteinase inhibitor), similar inhibitory effects of the different inhibitors were recorded with the 2nd instar larvae (Fig. 4A). However, the latter two inhibitors were less effective in inhibiting extensive proteolysis of CryIC by the 5th instar gut juice (Fig. 4B). In assays with gut juice proteases of the 5th instar larvae, PMSF considerably inhibited CryIC degradation, while leupeptin and 1,10 phenanthroline caused only a minor inhibition (Fig. 4B). To compensate for the considerable difference in gut juice protease specific activity between the early and late instar larvae (Fig 3B), another series of reactions was performed using 2nd and 5th instar larval gut juices with almost similar proteolytic capacity and more specific serine protease inhibitors (see legend to Fig. 5). Results summarized in Fig. 5 show that while in the 2nd instar larval gut juice TLCK (trypsin inhibitor) and TPCK (chymotryspin inhibitor) partially inhibited CryIC proteolysis, they had no effect on the 5th instar larval gut juice. PMSF appeared to be the most effective inhibitor in both assays, and co-application of PMSF and leupeptin inhibited most of CryIC proteolysis in both 2nd and 5th instar larval juices.

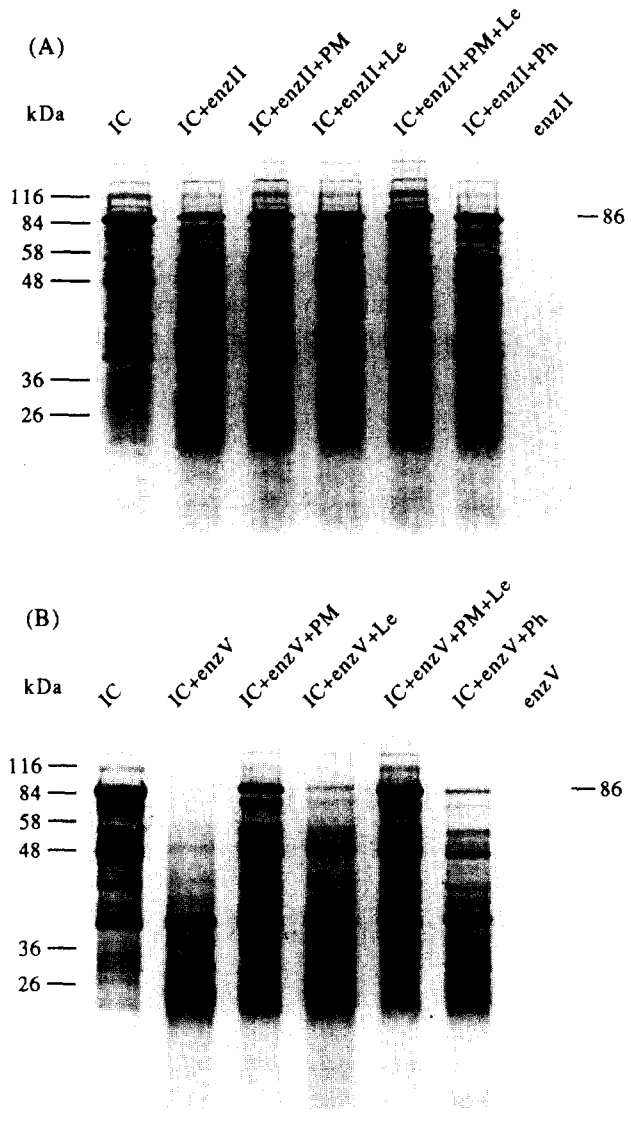

FIGURE 4. Effect of proteinase inhibitors on CryIC proteolysis by gut juice samples of equal protein content from the 2nd (A) and 5th (B) instar larvae. *E. coli*-produced CryIC (5 μg, depicted as IC) incubated with gut juice (2 μg total proteins) of the 2nd (enzII) and 5th (enzV) larval instars for 60 and 3 min, respectively, in the presence or absence of the following proteinase inhibitors: 200 μM leupeptin (Le), PH-50 mM 1,10-phenanthroline, PM-10 mM PMSF.

Since PMSF and leupeptin were found to play the major role in avoiding proteolysis in all larval stages, increasing concentrations of two inhibitors were used to titrate proteolytic activity and thereby quantify their differential effect in young and advanced instar larvae. PMSF, was found to be effective in inhibiting the proteloytic activity in 5th instar larval gut proteases at a concentration range of 0.001–4 mM (Fig. 6). Leupeptin applied at similar concentration range inhibited the proteolytic activity of 2nd instar gut proteases (40% inhibition at 4 mM) to a greater extent than that of 5th instar larvae (28% at 4 mM) (Fig. 6). These results together with the data summarized in Figs 3–5 demonstrate that during larval development both the profile and total concentration of gut proteases are gradually changed.

Absence of protease activity in the larval hemolymph

To confirm that the measured proteolytic activity was not affected by additional accidentally extracted proteases from the larval hemolymph, 5 μg of *E. coli*-produced CryIC were incubated for 30 min with hemolymph fluid (1–2 μg total protein) extracted from 4th and 5th instar larvae. Results summarized in Fig. 7A and B indicate that the hemolymph fluid of both instars did not display any proteolytic activity and the CryIC remained intact at the end of the incubation period. Therefore, the proteolytic activities of the gut juice samples demonstrated in Figs 2–6 can be attributed solely to the gut juice.

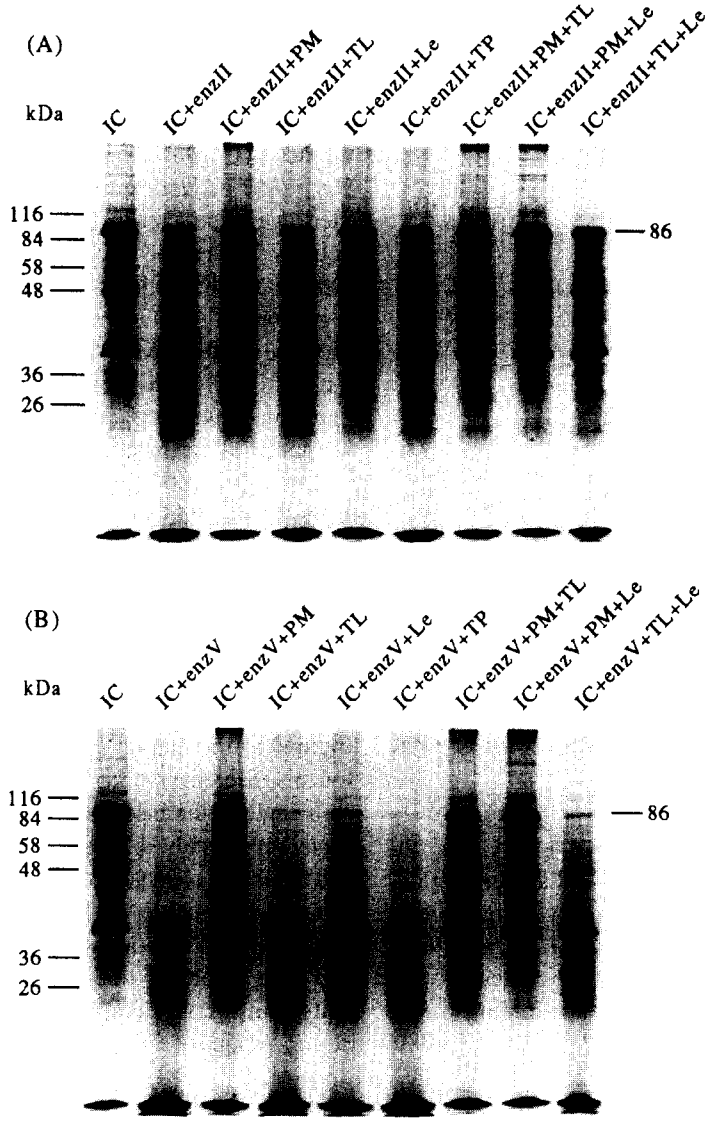

FIGURE 5. Effect of specific serine protease inhibitors on proteolysis of CryIC in gut juice of early and late larval instars. CryIC (5 μg) was incubated with gut juice of 2nd (enzII) larval instar (10 μg) or 5th (enzV) larval instar (0.35 μg) for 20 and 15 min, respectively. Proteinase inhibitors were added as follows: 200 μM leupeptin (Le), 5 mM PMSF (PM), 200 μM TLCK, and 200 μM TPCK. The reactions were stopped and their proteolytic products were analyzed as described in Fig. 4.

DISCUSSION

The first and second instar larvae of *S. littoralis*, as well as those of certain other lepidopteran insects, were found to be more susceptible to δ-endotoxins of *B. thuringiensis* than the advanced instar larvae (Sneh *et al.*, 1981; Bai *et al.*, 1993). The present work demonstrates that gut juice of advanced *S. littoralis* larval instars (3rd–5th) exhibits very high proteolytic activities which lead to a complete degradation of Cry proteins either supplied as a recombinant CryIC protein produced in *E. coli* or derived from denaturation and renaturation of Bt crystal proteins. The proteolysis of CryIC is considerably lower during incubation with gut juice of 1st and the 2nd instar larvae, but gradually increased during larval maturation.

The observed quantitative increase of larval gut protease activity during larval instar development is also accompained by qualitative differences in the protease profiles. Most of the lepidopteran larval gut proteases belong to the group of serine proteases (Purcell *et al.*, 1992; Christeller *et al.*, 1992). Thus, by using specific protease inhibitors such as PMSF and leupeptin it was possible to demonstrate that most of the proteases responsible for inactivation of CryIC in the advanced instar larvae of *S. littoralis* can be inhibited. In early instar larval juice metallo-proteases as well as chymotrypsin, also play a certain role in CryIC proteolysis, as demonstrated by inhibition exerted by 1,10 phenanthroline and TPCK, respectively. Quantitative analysis with increased concentrations of leupeptin and PMSF showed that protease activity, which can be inhibited by leupeptin decreases in advanced larval instars whereas the level of other serine proteases inhibited by PMSF increases during larval aging. PMSF-sensitive proteases are thus responsible for inactivation of most of CryIC at the 5th larval instar (Fig 6). Coapplication of leupeptin and PMSF resulted in more than 80% inhibition of CryIC degradation in both young and advanced larval instars (Figs 4 and 5).

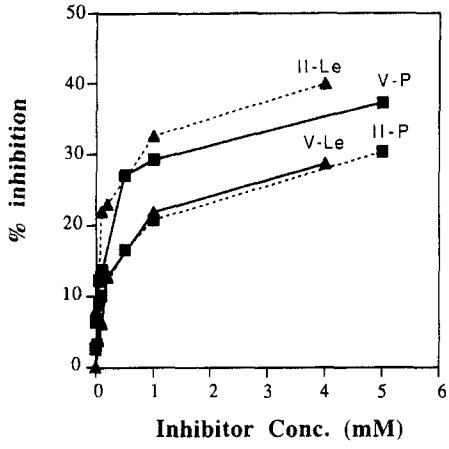

FIGURE 6. Quantitative evaluation of PMSF and leupeptin inhibition of CryIC proteolysis by gut proteases from the 2nd (II) and 5th (V) instar larvae of *Spodoptera littoralis*. Gut juices extracted from the 2nd and 5th instar larvae (10 and 3.5 μg gut juice proteins, respectively) were incubated with 5 μg CryIC at room temperature for 10 min with increasing concentrations of PMSF (P) and leupeptin (Le). The reaction was terminated by addition of equal volume of ×2 SDS–PAGE loading buffer followed by 3 min boiling. Following separation on 12% SDS–polyacrylamide gels and Coomassie staining, the amount of integrated CryIC was determined using an imaging densitometer (BioRad, GS-670) and expressed as percent inhibition of CryIC proteolysis.

Specific cleavage of CryIA(c) protoxin yields the mature δ-endotoxin when exposed to low concentrations of trypsin-like proteases isolated from larval gut juice of *Choristoneura fumiferana* (Milne and Kaplan, 1993). Similar δ-endotoxin processing by gut proteases was detected in other lepidopteran species, including *S. littoralis* (Faust *et al.*, 1974; Hofte and Whiteley, 1989; Strizhov *et al.*, in preparation). The results presented in this study show that the combination of relatively low protease content and the presence of trypsin-like protease (TLCK inhibited, Fig. 5) in the gut of 1st or 2nd instar larvae of *S. littoralis* provides a more suitable environment for proper activation of the toxic part of CryIC by tryptic cleavage. The presence of considerably higher protease activity in the gut juice of the 3rd and 5th instar larvae reduces CryIC toxicity by promoting a fast and complete digestion of the ingested Cry proteins. The observed increase in the PMSF-sensitive serine proteases and the lack of TLCK-sensitive proteases in the 5th instar larvae agree well with the apparent reduction in sensitivity of advanced larval instars to the CryIC toxin.

The absence of similar protease activities in the larval hemolymph fluid shows that the observed CryIC proteolysis is attributed only to the gut juice and not to possible leakage of hemolymph fluid into the gut juice extract.

It was puzzling at first that relatively high concentrations (30 μg/ml) of *E. coli*-produced CryIC applied in the artificial diet of neonate *S. littoralis* larvae did not cause 100% mortality within 6 days, while similar concentrations of the natrual Bt crystals did (Fig. 1). The reason for this discrepancy has been clarified by the present work showing that the insecticidal activity of assembled *B. thuringiensis* crystals differs from crystal preparations which had undergone denaturation and subsequent renaturation. Whereas the native CryIC-containing crystals are capable of causing 100% mortality of neonate larvae, the denatured and then renatured toxin does not cause significant mortality, but a considerable reduction in larval weight. The *E. coli*-produced CryIC included in the diet causes a simi-

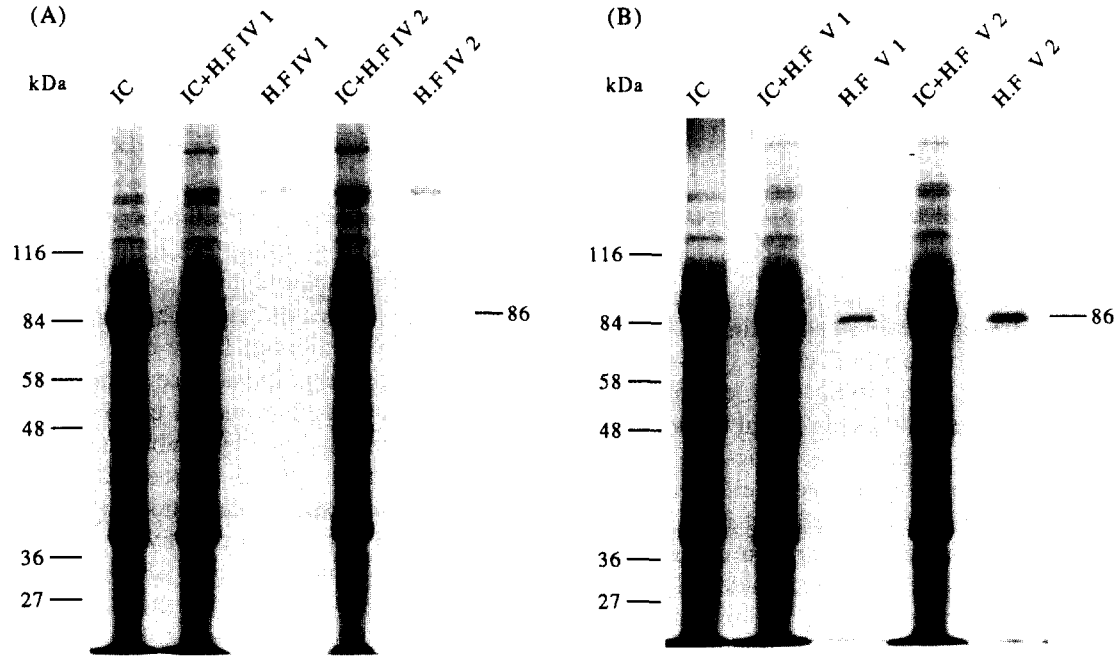

FIGURE 7. Proteolytic activity of hemolymph fluid in *Spodoptera littoralis* larvae. Hemolymph fluid extracted from the 4th (A) and 5th (B) instar *S. littoralis* larvae was incubated with *E. coli*-produced CryIC at room temperature for 30 min in 10 μl final reaction volume. IC 5 μg *E. coli*-produced CryIC. H.F-1 or 2-1 or 2 μg total proteins respectively, from hemolymph fluid extracted from the 4th (IV) or 5th (V) instar larvae. Molecular mass standards are shown on the left.

lar weight reduction effect. Thus, it is apparent that the assembly of the δ-endotoxin molecules in the crystalline structure is crucial to avoid efficient proteolysis by gut proteases and obtain high larvicidal performance. When the crystal structure is disrupted by denaturation and the released Cry molecules acquire a self-folded structure upon renaturation, they become more vulnerable to the digestion by increasing gut protease activity throughout larval maturation. Since δ-endotoxin crystals are incapable of directly interacting with the receptors located on the epithelial membrane, free Cry molecules must be first released from the crystals. At this stage the free Cry proteins are vulnerable to inactivation by gut proteases, similar to the ingested recombinant Cry protein. In this respect there is no difference between 135 kDa Cry proteins or the C-terminal truncated forms. This information may be of significant importance for understanding the larvicidal potential of specific δ-endotoxins expressed in transgenic plants. In this setting they would not be packed in the natural protective crystalline structures and could therefore be more exposed to proteolysis, which might lead to reduced larvicidal activity.

REFERENCES

Ahmad Z., Saleemuddin M. and Siddi M. (1980) Purification and characterization of three alkaline proteases from the gut of larvae of the armyworm. *Spodoptera litura*. *Insect Biochem.* **10**, 667–673.

Aronson A. I. (1993) The two faces of *Bacillus thuringiensis* insecticidal proteins and post-exponential survival. *Microbiol. Rev.* **7**, 489–496.

Bai C., Degheele D., Jansens S. and Lambert B. (1993) Activity of insecticidal proteins and strains of *Bacillus thruingiensis* against *Spodoptera exempta* (Walker). *J. Invertebr. Pathol.* **62**, 211–215.

Beynon R. J. and Bond J. S. (1990) *Proteolytic Enzymes, a Practical Approach*, p. 259, IRL Press, Oxford University Press, Oxford, UK.

Broza M. and Sneh B. (1994) *Bacillus thuringiensis* var. *kurstaki* as an effective control agent of lepidopteran pests in tomato fields in Israel. *J. Econ. Entomol.* **87**, 923–928.

Christeller J. T., Laing W. A., Markwick N. P. and Burgess E. P. J. (1992) Midgut protease activities in twelve phytophagous lepidopteran larvae: dietary and protease inhibitor interactions. *Insect Biochem. Molec. Biol.* **22**, 735–746.

Faust R. M., Hallam G. M. and Travers R. S. (1974) Degradation of the parasporal crystal produced by *Bacillus thuringiensis* var. *kurstaki*. *J. Invertebr. Pathol.* **2**, 265–272.

Haider M. Z., Knowles B. H. and Ellar D. J. (1986) Specificity of *Bacillus thuringiensis* var. *colmeri* insecticidal δ-endotoxin as determined by differential proteolytic processing of the protoxin with larval gut proteases. *J. Biochem.* **156**, 531–540.

Hamal M., Brownbridge M,. Broza M. and Sneh B. (1991) Screening for highly effective isolates of *Bacillus thuringiensis* against *Spodoptera exempta* and *S. littoralis*. *Phytoparasitica* **19**, 9–21.

Hofte G. and Whiteley H. R. (1989) Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiol. Rev.* **53**, 212–255.

Honee G., Van der Salm T. and Visser B. (1988) Sequence of crystal protein gene isolated from *Bacillus thuringiensis* subsp. *entomocidus* 60.5 coding for a toxin highly active against *Spodoptera* species. *Nucl. Acids Res.* **16**, 6240.

Laemmli U. K. (1970) Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Li J., Carroll J. and Ellar D. (1991) Crystal structure of insecticidal δ-endotoxin from *Bacillus thuringiensis* at 2.5 A resolution. *Nature* **353**, 815–821.

Marder J. B. J., Mattoo A. K. and Edelman M. (1986) Identification and characterization of *psb*A gene product: the 32 kDa chloroplast membrane protein. *Methods Enzymol.* **118**, 384–396.

Milne R. and Kaplan H. (1993) Purification and characterization of a trypsin-like digestive enzyme from spruce budworm (*Choristoneura fumiferana*) responsible for the activation of δ-endotoxin from *Bacillus thuringiensis*. *Insect Biochem. Molec. Biol.* **23**, 663–673.

Perlak F. J., Deaton R. W., Armstrong T. A., Fuchs R. L., Sims S. R., Greenplate J. T. and Fischhoff D. A. (1990) Insect resistant plants. *Bio Tech.* **8**, 939–943.

Perlak F. J., Fuchs R. L., Dean D. A., McPherson S. L. and Fischhoff D. A. (1991) Modification of the coding sequence enhances plant expression of insect control protein genes. *Proc. Natn Acad. Sci. U.S.A.* **88**, 3324–3328.

Perlak F. J., Stone T. B., Muskopf Y. M., Petersen L. J., Parker G. B., McPherson S. L., Wyman J., Love S., Reed G., Biever D. and Fischhoff D. A. (1993) Genetically improved potatoes: protection from damage by Colorado potato beetles. *Plant Mol. Biol.* **22**, 313–321.

Purcell J. P., Greenplate J. T. and Sammons R. D. (1992) Examination of midgut luminal proteinase activities in six economically important insects. *Insect Biochem. Molec. Biol.* **22**, 41–47.

Sanchis V., Lereclus D., Menou G., Chaufaux J., Guo S. and Lecadet M. M. (1989) Nucleotide sequence and analysis of the N-terminal coding region of the *Spodoptera*-active δ-endotoxin gene of *Bacillus thuringiensis* var. *aizawai* 7.29, *Mol. Microbiol.* **3**, 229–238.

Schnepf H. E., Wong H. C. and Whiteley H. R. (1985) The amino acid sequence of a crystal protein from *Bacillus thuringiensis* deduced from the DNA base sequence. *J. Biol. Chem.* **260**, 6261–6272.

Sneh B., Schuster S. and Broza M. (1981) Insecticidal activity of *Bacillus thuringiensis* strains against the Egyptian cotton leafworm, *Spodoptera littoralis* Boisd. (Lep. Noctuidae). *Entomophaga* **26**, 179–190.

Studier W. F., Rosenberg A. H., Dan J. J. and Dubendorf J. W. (1990) Use of the T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* **185**, 60–89.

Terra W. R. (1988) Physiology and biochemistry of insect digestion: an evolutionary perspective. *Physiol. J. Med. Biol. Res.* **21**, 675–739.

Vaeck M., Reynaerts A., Hofte H., Jansens S., De Beuckeleer M., Dean C., Zabeau M., Van Montague M. and Leemans J. (1987) Transgenic plants protected from insect attack. *Nature* **328**, 33–37.

Van Rie J., Jansens S., Hofte H., Degheele D. and Van Mellaert H. (1990) Receptors on the brush border membrane of the insect midgut as determinants of the specificity of *Bacillus thuringiensis* δ-endotoxins. *Appl. Env. Microbiol.* **56**, 1378–1385.

Visser B., Van der Salm T., Brink W. and Polkers G. (1988) Gene from *Bacillus thuringiensis* var. *entomocidus* 60.5. *Mol. Gen. Genet.* **212**, 219–224.

Wolfson J. L. and Murdock L. L. (1990) Diversity in digestive proteinase activity among insects. *J. Chem. Ecol.* **16**, 1089–1102.

Wu D. and Aronson A. I. (1992) Localized mutagenesis defines regions of the Bt δ-endotoxin involved in toxicity and specificity. *J. Biol. Chem.* **267**, 2311–2317.

Yawetz A., Sneh B. and Oron U. (1983) Purification and hydrophobic properties of the δ-endotoxin in the parasporal crystal produced by *Bacillus thuringiensis* var. *entomocidus*. *J. Invertebr. Pathol.* **42**, 106–112.

Acknowledgements—The research was supported by the German–Israeli Foundation (GIF) grant No. 0223-146.12/91.