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Mapping of the entomocidal fragment of *Spodoptera*-specific *Bacillus thuringiensis* toxin CryIC

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Abstract Insecticidal CryI protoxins of *Bacillus thuringiensis* are activated by proteolysis in the midgut of insects. A conservation of proteolytic cleavage sites in the CryI proteins facilitates the expression of active toxins in transgenic plants to obtain protection from various insects. However, the engineering of CryIC toxins has, thus far, failed to yield applicable resistance to armyworms of *Spodoptera* species representing common insect pests worldwide. To improve the production of recombinant CryIC toxins, we established a CryIC consensus sequence by comparative analysis of three *cryIC* genes and tested the stability and protease sensitivity of truncated CryIC toxins in *Escherichia coli* and in vitro. In contrast to previous data, the boundaries of trypsin-resistant CryIC core toxin were mapped to amino acid residues I28 and R627. Proteolysis of the truncated CryIC proteins showed that *Spodoptera* midgut proteases may further shorten the C-terminus of CryIC toxin to residue A615. However, C-terminal truncation of CryIC to residue L614, and a mutation causing amino acid replacement I610T, abolished the insecticidal activity of CryIC toxin to *S. littoralis* larvae, as well as its resistance to trypsin and *Spodoptera* midgut proteases. Because no CryIC toxin carrying a proteolytically processed N-terminus could be stably expressed in bacteria, our data indicate that, in contrast to other CryI poteins, an entomocidal fragment located between amino acid positions 1 and 627 is required for stable production of recombinant CryIC toxins.

Key words CryIC · *Spodoptera littoralis* · δ-Endotoxin · Proteolytic processing · Site-specific mutagenesis

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Introduction

Insecticidal crystal proteins of *Bacillus thuringiensis* (Cry ICPs) are widely used as environmentally friendly alternatives to chemical pesticides. Thus far, over 100 Cry proteins have been identified and classified according to their sequence homology and insect specificity (for review see Höfte and Whiteley 1989; Aronson 1993; Schnepf 1995). The Lepidoptera-specific CryI proteins are synthesized as protoxins of 130–140 kDa in parasporal crystals of bacteria. Once ingested, the ICP crystals are dissolved in the reducing alkaline environment of insect midgut and processed to active toxins of 60–65 kDa by trypsin-like serine proteases (for review see Knowles 1994). The N-terminal protease cleavage sites appear to be conserved in all CryI protoxins and located at the amino acid residue R27/28 (Bietlot et al. 1989). By contrast, the positions of C-terminal protease cleavage sites determined in the different CryI proteins vary between amino acid positions 609 and 630 (Höfte et al. 1986; Wabiko et al, 1986; Haider and Ellar 1989; Martens et al. 1995). X-ray crystallography studies of CryIA(a) and CryIIIA (Li et al. 1991; Grochulski et al. 1995) show that the Cry toxins fold into three structurally distinct domains. Protein sequences conserved among the Cry toxins are located in domains I and III, and referred to as boxes 1–5 (Höfte and Whiteley 1989). Domain I, extending from the N-terminal proteolytic cleavage site to residue 253 in CryIA(a), consists of amphipatic α-helices that play a role in ion channel formation during interaction with the outer membrane of insect midgut brush-border columnar cells (Walters et al. 1993; Gazit et al. 1994). Domain III, located at the C-terminus of CryIA(a) between positions 463 and 609, forms a sandwich of antiparallel β-sheets that are

required for conformational stabilization of the processed toxin (Chen et al. 1993; Nishimoto et al 1994; Aronson et al. 1995). Domain II carries the variable regions of Cry toxins with three β-sheets in a Greek-key structure. Surface-exposed loops of domain II are important determinants of toxin specificity (Lu et al. 1994; Smith and Ellar 1994; Rajamohan et al. 1995) and involved in binding to cadherin and aminopeptidase N membrane receptors of insects in the case of CryIA(b) and CryIA(c), respectively (Knight et al. 1995; Masson et al. 1995; Vadlamudi et al. 1995).

Genes encoding the processed forms of CryIA(a), (b), (c), and CryIIIA have been expressed in plant-associated bacteria and transgenic plants to control major insect pests of maize, rice, cotton, tomato, potato, and tobacco (for review see Perlak et al. 1990; Salm van der et al. 1994; Schnepf 1995). Nonetheless, *Spodoptera* species causing severe agricultural losses have thus far escaped efficient control because of problems preventing a high level expression of CryIC toxins in transgenic plants (van der Salm et al. 1994). To improve the engineering of CryIC toxins, we established a consensus CryIC sequence and determined the boundaries of the entomocidal fragment of CryIC toxin that confers resistance to midgut proteases and toxicity to the larvae of *Spodoptera littoralis*.

Materials and methods

Comparative DNA sequence analysis of *cryIC* genes

Total DNAs from *B. thuringiensis* isolates K26-21 and MR1-37 (Hamal et al. 1991), and subsp. *aizawai* 7.29 (Sanchis et al. 1988) were prepared as described (Dhaese et al. 1979). DNAs from strains K26-21 and MR1-37 were partially digested with *Hin*d III and used for preparation of pHC79 cosmid libraries (Hohn and Collins 1980). The libraries were screened by colony hybridization (Sambrook et al. 1991) with a PCR-amplified DNA fragment of 900 bp carrying the 5′ end of the *cryIC* coding region from *B. thuringiensis* subsp. *aizawai* 7.29. PCR primers used for amplification of *cryIC* and *cryIE* sequences are listed in Table 1. Primers for *cryIC* (1 and 2) and *cryIE* (7 and 8) were designed to amplify a *cryIC* fragment of 2294 bp and a *cryIE* fragment of 2249 bp, coding for the N-terminal δ-endotoxin domains with translational start codons preceded by *Nde*I and *Bam*HI cleavage sites. The 3′ reverse primers carried a *Bam*HI site following a stop codon, which replaced codon 757 in *cryIC* and codon 735 in *cryIE*. The PCR reactions with 100 pM of each *cry* primer, 500 ng DNA template, 250 µM dNTP, and 2.5 U *Taq* polymerase (Perkin-Elmer) were performed in 100 µl of *Taq* buffer (10 mM TRIS.HCl pH 9.0, 50 mM KCl, and 0.1% Triton X-100) using 30 cycles of 1 min at 92° C, 45 s at 55° C, and 3 min at 72° C after denaturation of the template at 95° C for 5 min. The PCR-amplified fragments were gel-purified, digested by *Bam*HI, and cloned in pBluescript SK$^+$ (Stratagene) for DNA sequencing using a T7 DNA polymerase sequencing kit (Pharmacia) and double-stranded DNA templates. For each *cryIC* isolate cloned by PCR amplification, as well as for a *cryIC* gene cloned in cosmid 1-37H19 from *B. thuringiensis* MR1-37, a series of deletions was generated by subcloning and both DNA strands were sequenced using a set of *cryIC*-specific sequencing primers. The DNA sequence data were analyzed using GCG and BLAST computer program packages (Deveraux et al. 1984; Altschul et al. 1990). The sequence of *cryIC* coding domains carrying the N-terminal 756 amino acid codons and found to be identical in three *B. thuringinesis* isolates K26-21, MR1-37, and subsp. *aizawai* 7.29 is deposited in the EMBL database under the accession number X96682.

Expression, purification, proteolysis, and sequencing of CryIC proteins

*Nde*I- *Bam*HI fragments carrying the first 756 codons of *cryIC* genes from *B. thuringiensis* strains K26-21, MR1-37, and *aizawai* 7.29 were subcloned from pBluescript clones p21, p35, and p76 in pET-11a, derived (Studier et al. 1990) plasmids pA2, pB4, and pC2 to express wild-type and mutated CryIC proteins in *Escherichia coli*. The *Bam*HI fragment of clone p76, carrying the *cryIC* gene from *B. thuringiensis* subsp. *aizawai* 7.29, was used as template (0.5 ng) to generate 5′ and 3′ deletions by PCR amplification as described above, using 42° C for primer annealing. Primer 3 (Table 1) was designed to add methionine and alanine upstream of the N-terminal I28 residue of CryIC core toxin. Primer 3 was combined with primer 4, 5 or 6 to PCR- amplify and clone in pBluescript three *Bam*HI fragments encoding truncated CryIC toxins, carrying a deletion of 27 N-terminal amino acids and stop codons following amino acid positions 609, 614, and 630. *Nco*I-*Bam*HI fragments from these plasmids were cloned in pET-11d (Studier et al. 1990). After verifying the sequence of these truncated *cryIC* constructs, their *Xba*I fragments were replaced by a corresponding *Xba*I fragment of pA2 to reconstruct the native 5′ end of *cryIC* coding regions. In CryIC expression plasmids pΔK, pΔL, and pΔR, the truncated *cryIC* coding regions start from amino acid position 28 and, respectively, extend to positions 609, 614, and 630, whereas plasmids pKE1, pLE1, and pRN2 contain *cryIC* coding sequences extending from amino acid position 1 to positions 609, 614, and 630.

The *cryIC* expression plasmids were transformed into *E. coli* host BL21(DE3) (Studier et al. 1990) and the production of CryIC proteins in bacterial cultures was induced by 0.8 mM IPTG (isopropylD-β-thiogalactopyranoside) for 4–6 h. Proteins from soluble and inclusion body fractions of total cell extracts were resolved in 7.5 or 10% SDS-polyacrylamide gels and stained with Coomassie

Table 1 PCR primers used for amplification of *cryIC* and *cryIE* sequences. Cleavage sites for restriction endonucleases *Bam*HI, *Nco*I, and *Nde*I are printed in bold, ATG codons and trinucleotides complementary to stop codons are underlined. The A nucleotide of ATG start codons corresponds to position 1

Primer number		Sequence (5′ → 3′)	*cry* positions
cryIC	1	ACGGA**GGATCCATATG**GAGGAAAATAATCAAAATC	(− 13)–22
	2	CTCTT**GGATCC**TAACGGGTATAAGCTTTTAATTTC	2281–2247
	3	CTTTT**GGATCCCATGG**CGATATCAACTGGTAATTCA	64–99
	4	AGAAT**GGATCC**TCATTTATCTATATAAAGTTCAC	1841–1808
	5	ATGTT**GGATCC**TATAGAATAATTTCAATTTTATC	1855–1822
	6	GGGCA**GGATCC**TACTTTTGTGCTCTTTCTAAATC	1903–1870
cryIE	7	ATGGA**GGATCCATATG**GAGATAGTGAATAATCAGA	(− 13)–22
	8	CTCTT**GGATCC**TAGCGGGTATAAGCTTTTAACTTC	2236–2202

Brilliant Blue (Laemmli 1970). Protein concentrations were determined according to Bradford (1976) and by laser-scanning of SDS-polyacrylamide gels using BSA as standard. Inclusion bodies were isolated and purified as described (Schmidt et al. 1986), suspended at a concentration of 2 mg/ml in 0.5 ml of 25 mM TRIS.HCl pH 8.8 buffer, and preincubated for 5 min at 37° C. For tryptic digestion, trypsin (Boehringer, sequencing grade, dissolved at 1 mg/ml concentration in 1 mM HCl) was added at an enzyme : substrate ratio of 1 : 200 (w/w) and aliquots were removed at different time intervals of incubation at 37° C. To terminate the proteolysis, proteins were precipitated by trichloracetic acid (TCA) at 12% final concentration at 4° C, then dissolved in sample buffer and analyzed by SDS-PAGE. To prepare midgut protease extracts, the midgut content from ten 5th instar larvae of *S. littoralis* was mixed with 1 ml of carbonate buffer (50 mM Na_2CO_3 pH 9.0 and 10 mM dithiotreithol) and sonicated on ice. After removing the cell debris by centrifugation (15 000 *g*, 10 min at 4° C), aliquots of protein extract (5 mg/ml) were stored at −20° C. Proteolysis with midgut extract was carried out as described for tryptic digestion, using an approximate enzyme : substrate ratio of 1 : 15 (w/w). CryIC proteins processed by proteases were separated from contaminating peptides by SDS-PAGE and blotted to PVDF membrane (Millipore) to determine their N-terminal sequence by Edman degradation, using an Applied Biosystem protein sequencer 477A.

Insecticide bioassays

Inclusion bodies containing known amounts of CryIC proteins were added at different concentrations to an artificial diet of *S. littoralis* neonate larvae. As control, soluble protein extract from IPTG-induced *E. coli* BL21(DE3) cultures carrying pET-11a was used at concentrations identical to that of the CryIC inclusion body proteins assayed. The insect diet contained 25 g sucrose, 10 g yeast extract, 30 g ground wheat germ, 0.4 g cholesterol, 1 g sorbic acid, 1 g methyl paraben, 0.1 g chloramphenicol, and 18 g agar per l, (Hamal et al. 1991). The larvae were separately placed in Eppendorf tubes containing 0.3 ml of diet and reared at 25° C and 65% humidity. Each treatment was performed with 100 larvae in ten replicates with 10 larvae and repeated 3 times. Larval weights were recorded after 6 days and the results were evaluated by standard statistical procedures.

Results

Spodoptera-specific *B. thuringiensis* strains share identical *cryIC* sequences

To identify new *cryIC* genes, two *B. thuringiensis* isolates, K26-21 and MR1-37 selected for high insecticidal activity against *Spodoptera* (Hamal et al. 1991), were characterized. *Hin*dIII fingerprints of total and plasmid DNAs from the new isolates were hybridized with a *cryIA*(*c*) probe, using DNAs from *B. thuringiensis* strains subsp. *berliner* 1715, HD1, and HD73 as controls. The Southern hybridizations revealed the presence of *Hin*dIII fragments of 4.5 and 5.3 kb diagnostic for the *cryIA*(*a*) and (*b*) genes (Kronstad and Whiteley 1986), but indicated the absence of a *Hin*dIII fragment of 6.6 kb corresponding to the *cryIA*(*c*) gene (Adang et al. 1985) in the new isolates (data not shown). PCR amplification and Southern DNA hybridization, using *cryIC*- and *cryIE*-specific oligonucleotides and probes, respectively (Table 1), also showed that strains K26-21 and MR1-37 carried *cryIC*, but no *cryIE* sequences. A probe of 900 bp carrying the 5′ end of the PCR-amplified *cryIC* coding region from *B. thuringiensis* subsp. *aizawai* 7.29 detected a *Hin*dIII fragment of 2.5 kb in plasmid DNAs of strains K26-21 and MR1-37, as described earlier for *B. thuringiensis* strains *aizawai* 7.29, HD229, and *entomocidus* 60.1 (data not shown; Sanchis et al. 1988; Smith et al. 1994). The presence of *cryIC* sequences carried by plasmids in the new isolates thus correlated with their high activity against *Spodoptera* because members of the CryIA toxin family confer no or only low entomocidal activity against these insects (Höfte and Whiteley 1989; Visser et al. 1990).

The *cryIC* genes were cloned from strains K26-21, MR1-37, and *aizawai* 7.29 by generating pHC79 cosmid libraries using total DNA partially digested with *Hin*dIII, as well as by PCR amplification of their 5′ segments coding for an N-terminal toxin fragment extending to amino acid position 756. The PCR products were inserted as *Bam*HI fragments into pBluescript, and three clones derived from three independent PCR reactions with template DNAs from each *B. thuringiensis* strain were sequenced. Using a set of *cryIC*-specific sequencing primers, a total of 13 466 bp DNA sequence information was obtained from the PCR clones and compared to a *cryIC* sequence carried by a cosmid clone (1-37H19) from strain MR1-37. In the PCR clones, no deletion or insertion but seven substitutions (six transitions and one transversion) were identified at different positions, corresponding to an error frequency of 5.2×10^{-4} (1/1924 bp) and a misincorporation rate of 3.4×10^{-5} per nucleotide per PCR cycle. Except for these random nucleotide exchanges, all *cryIC* sequences isolated by PCR were identical to that of the *cryIC* gene isolated by cosmid cloning. These redundant data resulted in a consensus CryIC sequence (EMBL X96682) that differed by amino acid replacements A124E, A294R, and H453D from the CryIC sequence of *B. thuringiensis* subsp. *entomocidus* 60.5 (Honée et al. 1988) and by a T405Q exchange from the corresponding sequence of subsp. *aizawai* PS81I (Genbank m73251). Similarly, multiple sequence shifts resulting in N366I, V386G, and 376WPAPPFN382 to 376CQRHHFN382 amino acid replacements were found in the previously published *cryIC* sequence from subsp. *aizawai* 7.29 (Sanchis et al. 1989). The occurrence of glutamate in position 124 and glutamine in position 405 was clearly due to previous errors, since A124 and T405 were found to be conserved in all CryI proteins. Similarly, sequence variations detected between positions 366 and 386 of the CryIC sequence from subsp. *aizawai* 7.29 could safely be excluded because they would either create a new tryptic cleavage site, such as the R378 residue, or affect the activity and insect specificity of the toxin, such as the W376C replacement and the 374QPWP377 motif that are located in

a surface-exposed loop of the variable toxin domain II (Convents et al. 1991; Smith and Ellar 1994; Grochulski et al. 1995).

Proteolysis of the N-terminal entomocidal fragment of wild-type and mutant CryIC toxins expressed in *E. coli*

*Nde*I-*Bam*HI fragments of PCR-amplified *cryIC* sequences were cloned in pET-11a to express wild-type and mutant derivatives of the N-terminal CryIC fragment of 756 amino acids in *E. coli* . Expression plasmid pC2 carried wild-type *cryIC* sequences, pA2 contained a GAT to GGT mutation replacing Asp by Gly at amino acid position 735 (D735G replacement), whereas a *cryIC* sequence with amino acid replacement I610T (ATT to ACT mutation at amino acid codon 610) was cloned in pB4 (Fig. 1). *E. coli* cultures harboring these plasmids accumulated proteins of 86 and 52 kDa in inclusion bodies, following an induction by IPTG for 4–6 h (Fig. 2). N-terminal sequencing of these proteins showed that the 86-kDa protein corresponded to the expected CryIC toxin fragment of 756 amino acids starting at amino acid position 1. The N-terminus of the 52-kDa protein carried a methionine located in CryIC at position 290, indicating that translation from a second in-frame ATG codon was initiated in *E. coli* (data not shown).

Both 86- and 52-kDa proteins expressed from the pB4 construct showed a minor, but reproducible mobility shift in comparison to wild-type and A2 mutant CryIC proteins in SDS-PAGE (Fig. 2). This suggested that a conformational alteration maintained under denaturing conditions was caused by the Ile to Thr (hydrophobic to polar) replacement at amino acid position 610. In fact, hydrolysis by trypsin (Fig. 3a) and complete degradation by *S. littoralis* midgut proteases (Fig. 3b) of the pB4-encoded CryIC protein of 86 kDa showed that the C-terminal I610T mutation resulted in a structural change, rendering the B4 protein sensitive to proteases. By contrast, trypsin digestion of the A2 (D735G) mutant and wild-type (C2) CryIC proteins resulted in similar hydrolysis patterns, leading to the formation of a protease-resistant toxin fragment of 64 kDa.

During tryptic digestion, a rapid hydrolysis of the 86-kDa protein to an intermediate of 68 kDa occurred within 10 min at an approximate enzyme : substrate ratio of 1 : 200 (w/w), but a further 4 h of incubation was required to complete the conversion of this intermediate to a core toxin of 64 kDa (Fig. 3c). The N-terminal

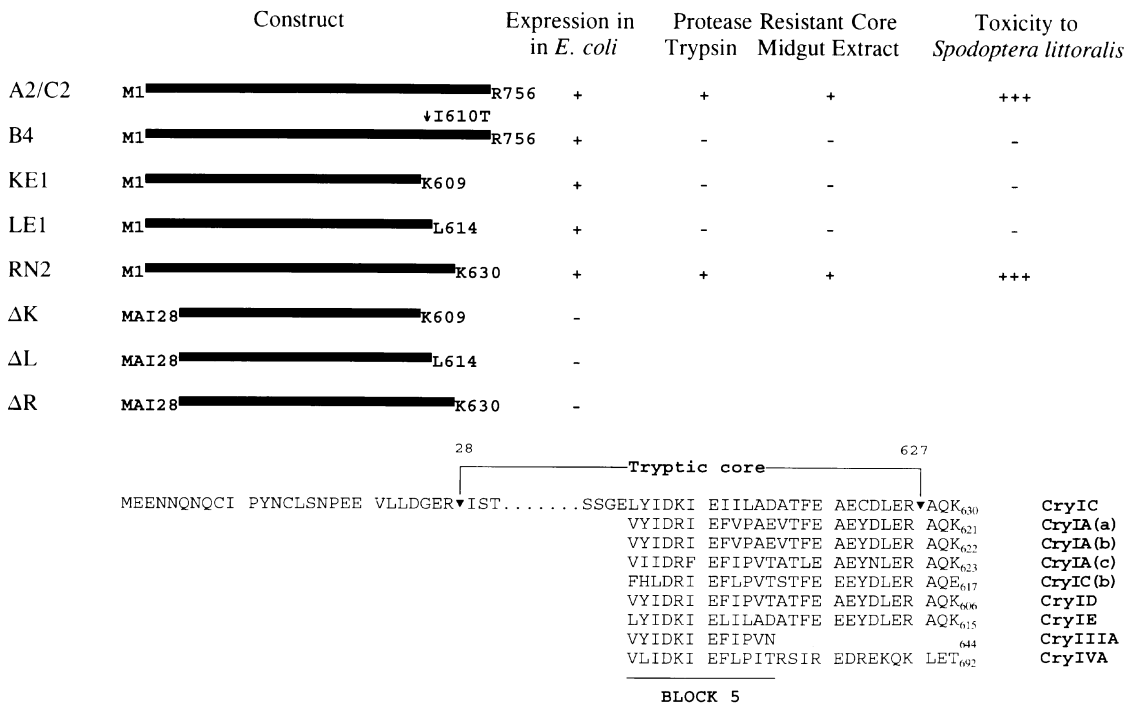


Fig. 1 Mapping the boundaries of active CryIC toxin. Expression plasmids coding for CryIC proteins with intact N-termini, MA residues added to the proteolytic cleavage site at position I28, and C-termini extending to residues 609, 614, 630, and 756 (depicted schematically at the left) were constructed. In addition, mutations causing amino acid replacements D735G and I610T were introduced into *cryIC* expression plasmids A2 and B4, respectively. After assaying their production in *Escherichia coli*, truncated CryIC proteins accumulating in inclusion bodies were purified and used as substrates in proteolysis assays with trypsin and midgut extracts from *Spodoptera littoralis*. Insecticidal activity of truncated CryIC proteins was tested in bioassays with *S. littoralis* neonate larvae. The proteolysis data indicate that CryIC is cleaved by trypsin at residues R27 and R627. In block 5, representing conserved C-terminal sequences of Cry toxins (compiled at the bottm), residue A615 marks the possible C-terminal boundary of active CryIC toxin, since truncated toxins carrying I610T replacement or L614 at the C-terminus are non-toxic and sensitive to trypsin as well as to *S. littoralis* midgut proteases

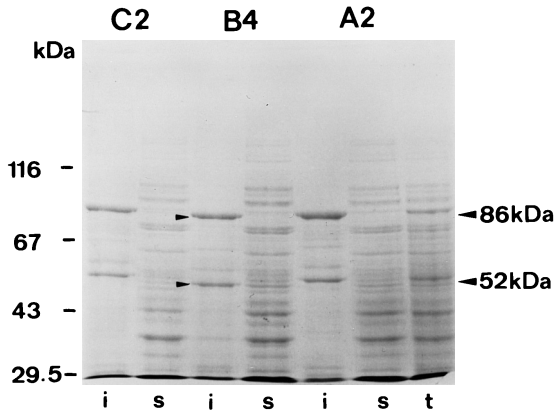
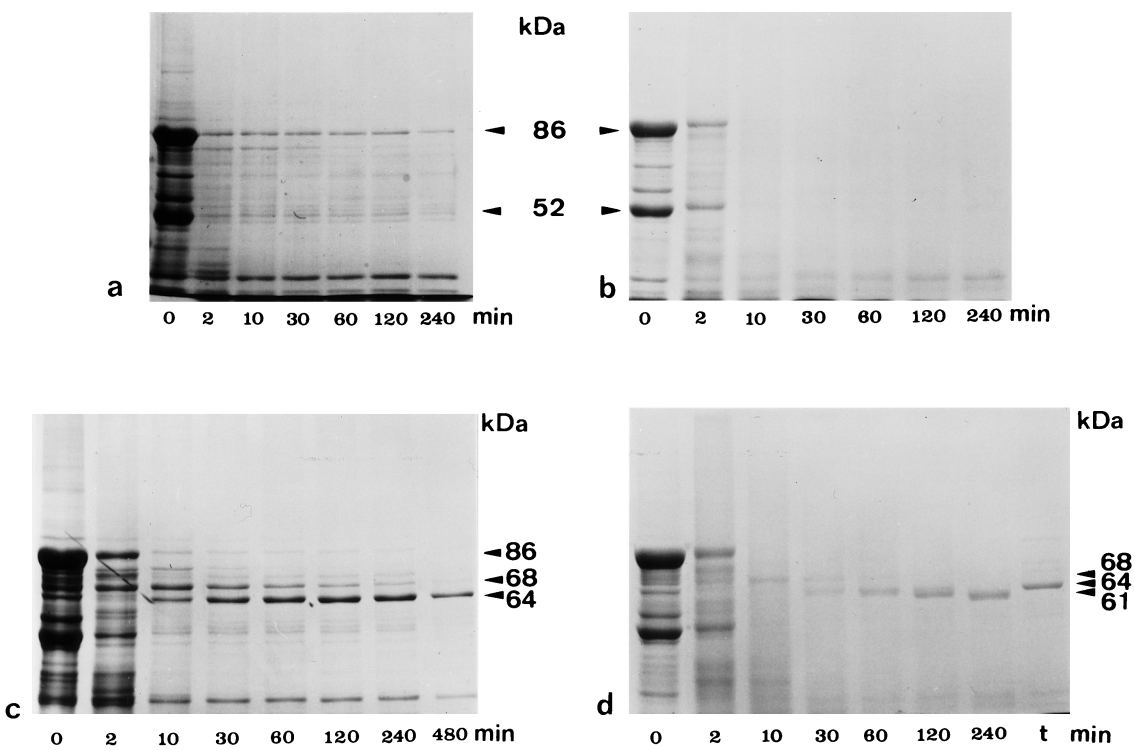


Fig. 2 Truncated CryIC toxins A2, B4, and C2 expressed in *E. coli* accumulate in inclusion bodies. Protein samples (3 µg) from soluble (*s*) and purified inclusion body (*i*) fractions of total *E. coli* cell extracts (shown for A2 in *lane t*) were resolved in 7.5% SDS-polyacrylamide gels. Toxins translated from methionine located at positions 1 and 290 of CryIC and resulting in bands with apparent M_r of 86 and 52 kDa are marked by *arrows* at the right, whereas corresponding B4 proteins, showing slightly increased mobility, are labeled by *small* arrows within the lane

sequence of the 68-kDa intermediate was found to be identical with the N-terminus of the 86-kDa substrate, demonstrating that the first, rapid phase of proteolytic hydrolysis involved the removal of C-terminal peptide sequences. The core toxin of 64 kDa carried an N-terminal ISTGNSSIDIS sequence, indicating that slow cleavage of the N-terminal peptide of 27 amino acids occurred precisely at the amino acid residue I28, as determined previously for other CryI toxins (Höfte et al. 1986; Bietlot et al. 1989). In contrast to the 86-kDa toxin fragment, the N-terminally truncated CryIC protein of 52 kDa was completely degraded by trypsin.

Processing of the CryIC proteins by midgut extracts showed a similar pattern, including fast (30 min at an approximate enzyme : substrate ratio of 1:15) accumulation of an intermediate of 68 kDa, which was slowly converted to a core toxin fragment of 61 kDa. A fuzzy appearance of the 61 kDa product in SDS-PAGE and a failure to obtain a unique terminal amino acid sequence of this polypeptide suggested that exopeptidases, or other proteases with yet undefined specificity, may contribute in midgut extracts to the formation of the 61-kDa end-product during the final phase of proteolytic processing.

Fig. 3a–d Proteolysis of B4 and A2 mutant CryIC proteins. **a** Proteins from purified inclusion bodies (500 µg, containing about 350 µg of B4 mutant toxin of 756 amino acids) were digested by trypsin (2.5 µg) in a total volume of 0.5 ml at 37° C. At time intervals indicated, 50-µl aliquots were precipitated by TCA, dissolved in sample buffer, and separated by 7.5% SDS-PAGE. **b** Inclusion body proteins were incubated with *S. littoralis* midgut extract (33 µg) and samples were processed as described in **a**. **c** Inclusion body fraction (1 mg, containing about 700 µg of A2 mutant CryIC toxin of 86 kDa) was treated with trypsin (5 µg) as described in **a**. The positions of the 86-kDa substrate, 68-kDa intermediate, and 64-kDa end-product of tryptic hydrolysis resolved by SDS-PAGE (7.5%) are labeled by *arrows*. **d** Hydrolysis of A2 proteins was performed with *S. littoralis* midgut extract (66 µg) and the products were separated as in **c**. *Lane t* contains trypsin-digested A2 toxin, to compare its mobility to those of intermediate (68 kDa) and end-products (61 kDa) of proteolysis with midgut extract



Minimal size of CyrIC toxin required for production of a protease-resistant core and stable expression in *E. coli*

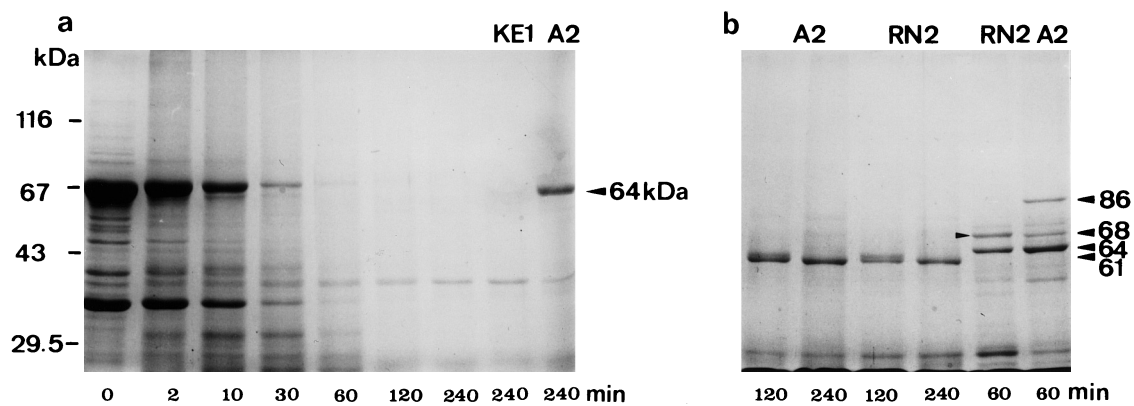
To examine the stability of truncated CryIC toxins in *E. coli*, methionine and alanine residues were added to the N-terminal proteolytic cleavage site at I28 by cloning of PCR-amplified *cryIC* fragments in pET-11d (see Materials and methods). Because the C-terminal I610T replacement appeared to affect the proteolytic stability of B4 mutant CryIC protein, the C-terminal boundary of active core toxin was mapped by introducing stop codons after amino acid positions 609, 614, and 630 bordering two potential tryptic cleavage sites. These C-terminal deletions were combined with an intact N-terminus in expression plasmids pKE1 (M1-K609), pLE1 (M1-L614), and pRN2 (M1-K630) or with a modified N-terminus carrying a deletion of the first 28 amino acids in vectors pΔK (MAI28-K609), pΔL (MAI28-L614), and pΔR (MAI28-K630) (Fig. 1). The latter three constructs failed to result in IPTG-inducible production of any protein (data not shown), suggesting that the N-terminally truncated CryIC toxins were either not translated efficiently or are unstable in *E. coli* . By contrast, CryIC derivatives carrying an intact N-terminus were stably expressed and accumulated to similar quantities in inclusion bodies. In addition to truncated toxins of the expected size, the inclusion bodies contained smaller CryIC products (e.g., 36 kDa for pRN2), the translation of which was initiated from an internal methionine at CryIC position 290.

CryIC proteins with C-terminal K609 (KE1, data not shown) and L614 (LE1) residues were completely hydrolyzed both by trypsin and *S. littoralis* midgut proteases (Fig. 4a), as was the B4 mutant protein (Fig. 3b), indicating that the C-terminal boundary of the proteolytically stable CryIC toxin lays downstream of amino acid position 614. This excluded the assumption that L609 could be a C-terminal tryptic cleavage site (Visser et al. 1990). Tryptic digestion of the RN2 protein, extending to the C-terminal K630 residue, resulted in a trypsin-resistant fragment of about 64 kDa. The size of the intact RN2 protein was slightly larger than the proteolytic intermediate of 68 kDa obtained by C-terminal tryptic digestion of the A2 protein (Fig. 4b). Since the RN2 and A2 proteins yielded trypsin-resistant cores of identical size (64 kDa), this size difference indicated that the C-terminal tryptic cleavage occurred at residue R627, preceding the C-terminus of intact RN2 protein by three amino acid residues. When digested by midgut proteases, both RN2 and A2 proteins resulted in a protease-resistant core of 61 kDa (Fig. 4b).

Insecticidal activity of truncated CryIC toxins

To confirm the data of in vitro protease assays, truncated CryIC toxins stably produced in *E. coli*, were added at different concentrations to an artificial diet of *S. littoralis* neonate larvae. The concentration of CryIC toxin fragments carrying methionine at position 1 was determined by laser scanning of purified inclusion body proteins resolved in SDS-PAGE. EC_{50} values (reflecting a 50% effective concentration of truncated CryIC proteins) showed that those CryIC toxin fragments, which resulted in the formation of protease-resistant core by digestion with trypsin and *S. littoralis* midgut proteases in vitro, were equally efficient in insectidal assays with *S. littoralis* neonate larvae. Thus, the A2, C2, and RN2 CryIC proteins yielded similar EC_{50} values of 0.5 $\mu g\,ml^{-1}$. As expected, truncated CryIC derivatives B4, KE1, and LE1, which showed protease

Fig. 4a, b Proteolysis of C-terminally truncated CryIC toxins. **a** Inclusion body fraction (1 mg containing about 80% LE1 mutant CryIC toxin of apparent M_r 67 kDa) was digested with *S. littoralis* midgut extract and samples were treated as described in Fig. 3. Aliquots of KE1 and A2 toxins hydrolyzed by trypsin for 240 min were resolved in the last two lanes by SDS-PAGE (10%). **b** A2 and RN2 proteins (each 1 mg) were digested with *S. littoralis* midgut extract and the hydrolysis products obtained after 2 and 4 h of incubation were resolved by SDS-PAGE (7.5%) as described in Fig. 2. As controls, the last two lanes show RN2 and A2 proteins hydrolyzed by trypsin for 1 h. An *arrow* in the fifth lane marks the band corresponding to the intact RN2 protein, in comparison to *arrows* labeling the intact A2 protein (86 kDa), tryptic intermediate of A2 (68 kDa), tryptic end-products of RN2 and A2 (64 kDa), as well as the end-product of A2 and RN2 hydrolysis (61 kDa) with midgut extract



sensitivity in vitro, proved to be non-toxic also in the insect bioassays, yielding EC_{50} values higher than 30 $\mu g\,ml^{-1}$.

Discussion

N-terminal processing of all CryIA proteins is believed to occur at the conserved R28 residue (Höfte et al. 1986; Bietlot et al. 1989), which corresponds to R27 in CryIC, as demonstrated by our data. The C-terminus of core CryIA(c) toxin was identified as K623 (Bietlot et al. 1989), whereas R601 was initially thought to be the C-terminal tryptic cleavage site in CryIA(b) (Höfte et al. 1986). However, protease sensitivity of truncated toxins terminating at F604 suggested that A607 may represent the C-terminus of CryIA(b) (Martens et al. 1995). By analogy, K609 was proposed to be a potential C-terminal protease cleavage site in CryIC (Visser et al. 1990). Our data clearly exclude this possibility, since the C-terminally truncated CryIC proteins KE1 and LE1, which terminated, respectively, at residues K609 and L614, are non-toxic and are degraded by both trypsin and *S. littoralis* midgut proteases. Residues K609 and L614 are part of the conserved sequence block 5 of CryI proteins (Höfte and Whiteley 1989). In block 5, the position of the P606 residue of CryIA(b) corresponds to the L614 residue of CryIC (Fig. 1). Whereas our data demonstrate that a C-terminal truncation of CryIC to L614 results in protease sensitivity, recent mapping data show that a CryIA(b) toxin carrying a C-terminal P606 residue is active (Wabiko and Yasuda 1995). This remarkable difference is probably due to a slight diversity of amino acid sequences in the block 5 region of these proteins. X-ray crystallography data show that the corresponding A606 residue in CryIA(a) forms a hydrogen bond with R523 of block 4, whereas the preceding amino acids D599, R600, and E602 of block 5 (represented by D608, K609, and E611 in CryIC) interact with the neighboring β-strands of conserved blocks 3 and 4. Block 5 thus provides a network of amino acid interactions stabilizing the conformation of processed Cry toxins (Li et al. 1991; Grochulski et al. 1995). Therefore, it is possible that A615 of block 5 represents the functional boundary of active CryIC toxin, which is also suggested by instability of the LE1 mutant toxin that carries a C-terminal L614 residue. Conformational alteration induced by the I610T replacement, causing protease sensitivity of the B4 mutant protein, also shows that mutations of block 5 result in structural destabilization of the toxin. Mutations in the "arginine face" of block 4 (R523 and R525 residues of strand β17) are known to alter interactions with block 5 and yield CryIA(a) proteins poorly expressed in *E. coli* (Chen et al., 1993). Similarly, replacements of charged D670 and E673 residues of block 5 (corresponding to D608 and E611 in CryIC) result in CryIVA toxins with reduced conformational stability (Nishimoto et al. 1994). In common with the I610T exchange in the B4 mutant, a R610Q replacement in CryIA(b) (corresponding to K609 of CryIC; Nakamura et al. 1992) affects the common DR/KIE sequence of block 5 (Fig. 1) and abolishes protease resistance.

As indicated by these data, the C-terminus of CryI toxins processed by trypsin-like insect proteases may not actually correspond to the functional boundary of active toxin mapped by mutagenesis studies. Choma et al. (1990) noticed that processing of the CryIA(c) protoxin is a biphasic reaction. By characterization of a CryIC proteolytic intermediate of 68 kDa we observed that the last, slow phase of tryptic processing involves the removal of an N-terminal peptide of 27 amino acids. The observation that the RN2 protein (terminating at K630) and the A2 protein (extending to position 756) result in tryptic products of identical size (64 kDa) indicates that the tryptic cleavage site is located at residue R627 of CryIC. Since an intermediate of 68 kDa is detectable also during proteolysis with *S. littoralis* midgut extracts (Fig. 3d), it is possible that trypsin-like insect proteases use the same C-terminal tryptic cleavage site. However, proteolysis with midgut extracts yields a final product of 61 kDa, which approaches the M_r value calculated for a CryIC protein with functional boundaries at residues 28 and 615. It is very unlikely that aspecific proteolysis by non-serine or exoproteases would shorten the processed N-terminus, because the removal of the first α-helix (residues 35–47) would affect the ion-channel formation required for toxicity (Li et al. 1991; Grochulski et al. 1995). In fact, a deletion of eight amino acids downstream of the N-terminal R27/28 cleavage site was shown to yield an inactive CryIA(b) toxin (Höfte et al. 1986), whereas the N-terminus of CryIA(b) toxin processed by *S. litura* extracts was found to carry the expected I29 residue (Nakamura et al. 1990). Therefore, a difference in the size of CryIC digestion products obtained by trypsin and *S. littoralis* midgut proteases probably reflects an extended proteolysis at the C-terminus between residues 615 and 627.

Unexpectedly, our data also revealed that the N-terminal peptide of CryIC located between positions 1 and 28 is required for the production of toxin in *E. coli*. Whether this N-terminal peptide is essential for proper folding of the toxin during translation in bacteria or protects the cells from toxicity resulting from ion channel formation by the activated N-terminus (Martens et al. 1995) remains to be determined.

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