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Novel *Bacillus thuringiensis* Insecticidal Crystal Protein with a Silent Activity against Coleopteran Larvae

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A novel *Bacillus thuringiensis* crystal protein with a silent activity against the Colorado potato beetle is described. The crystal proteins are produced as bipyramidal crystals. These crystals contain a protein of 129 kDa with a trypsin-resistant core fragment of 72 kDa. Neither a spore-crystal mixture nor in vitro-solubilized crystals are toxic to any of several Lepidoptera and Coleoptera species tested. In contrast, a trypsin-treated solution containing the 72-kDa tryptic core fragment of the protoxin is highly toxic to Colorado potato beetle larvae. The crystal protein-encoding gene was cloned and sequenced. The inferred amino acid sequence of the putative toxic fragment has 37, 32, and 33% homology to the CryIIIA, CryIIIB, and CryIIID toxins, respectively. Interestingly, the 501 C-terminal amino acids show 41 to 48% amino acid identity with corresponding C-terminal amino acid sequences of other crystal proteins. Because of the toxicity of the fragment to the Colorado potato beetle and because of the distinct similarities of the toxic fragment with the other CryIII proteins, this gene was given a new subclass name (*cryIIIC*) within the CryIII class of coleopteran-active crystal proteins. CryIIIC represents the first example of a crystal protein with a silent activity towards coleopteran insect larvae. Natural CryIIIC crystals are not toxic. Toxicity is revealed only after an in vitro solubilization and activation step.

Bacillus thuringiensis is a gram-positive bacterium which may be toxic to certain insects. The insecticidal activity and spectrum are determined by proteins, produced in large amounts during sporulation, which aggregate to form parasporal crystals. Most *B. thuringiensis* isolates are toxic to several lepidopteran larvae (7, 21). A few strains have also been reported to be toxic to dipteran larvae, such as mosquito and black fly larvae (9, 24). More recently, *B. thuringiensis* strains which are highly active against coleopteran larvae such as Colorado potato beetle larvae have been isolated (6, 11, 18, 19, 29).

The crystal protein genes of several *B. thuringiensis* strains have been characterized and cloned (for a review, see reference 16). To date, between 25 and 30 distinct crystal protein gene sequences have been published either in the general scientific literature or in patent applications. The *cryIIIA* gene and its corresponding crystal protein have been identified in three *B. thuringiensis* strains. The coding sequences of the *cryIIIA* gene are exactly the same in the three independent strains in which the gene was found (6, 10, 28). The *cryIIIB* gene, which was recently identified in a *B. thuringiensis* subsp. *tolworthi* strain (29), was also found in a *B. thuringiensis* subsp. *pakistani* strain in our laboratory. The *cryIIID* gene was found in a *B. thuringiensis* subsp. *kurstaki* strain (19). Here, we report the full characterization of CryIIIC and its bioactivity.

The *cryIIIC* gene was described previously in a patent application (26), and our findings were partly communicated at the First International Conference on the Molecular Biology of *Bacillus thuringiensis*, San Francisco, Calif., 1991.)

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MATERIALS AND METHODS

Isolation of *B. thuringiensis* BTS137J. *B. thuringiensis* BTS137J was isolated as described previously by Travers et al. (31) by selective sample enrichment in a buffer containing sodium acetate, heat treatment, and then plating out on agar plates. The BTS137J strain reported here was isolated as a single colony from a cow dung sample collected in Zephyrhills, Fla. The strain was deposited at the Deutsche Sammlung von Mikroorganismen (DSM 5132).

Serotyping. BTS137J belongs to the serovar H5 (galleriae) as determined by J. F. Charles at the Institut Pasteur (Paris, France).

Preparation and purification of the crystal protein. The BTS137J strain was grown until sporulation in liquid T3 medium (31). A mixture of spores and crystals was harvested by centrifugation, and purified crystals were prepared as described by Mahillon and Delcour (22). Crystal proteins were dissolved for 3 h at 37°C in alkaline buffer (50 mM $NaHCO_3$–5 mM dithiothreitol, pH 10), after which the pH of the solution was adjusted to 8 with a 1/10 volume of 0.1 N HCl, and the crystal proteins were trypsinized overnight by adding 1 μg of trypsin per 25 μg of protein. Trypsinized crystal proteins were purified by chromatography as described by Höfte et al. (12). The N-terminal amino acid sequences of the full-length protein and the trypsin-resistant, insecticidal fragment were determined with a pulsed-liquid Sequenator (model 470A; Applied Biosystems Inc., Foster City, Calif.).

Immunological analysis. Enzyme-linked immunosorbent assay (ELISA) plates were coated overnight at 4°C with 100 μl of solubilized crystal proteins (0.5 μg/100 μl). The ELISA was performed as described by Höfte et al. (15). In addition to the previously described antibodies (15), a polyclonal antiserum and monoclonal antibodies specific for the CryIIIA protein were used.

For Western blotting (immunoblotting) analysis, protein extracts or trypsinized protein extracts were subjected to

sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and gels were blotted onto nitrocellulose filters (25). Nitrocellulose filters were blocked by being rinsed for 5 min in 2% Tween 20 (Sigma, St. Louis, Mo.) in TBS (50 mM Tris–150 mM NaCl, pH 7.5). All further washing steps were done with TBS containing 0.05% Tween 20 (TBST). Primary and secondary antibodies were diluted in TBST. The filters were incubated for 1 h with the CryIIIA-specific polyclonal antibody (1/5,000, vol/vol), rinsed with tap water, incubated for 1 h with an alkaline phosphatase-conjugated secondary antibody (1/1,000, vol/vol), and again rinsed with tap water. The protein-antibody complexes were visualized by incubating the filters in a color solution (50 μl of 4-nitroblue tetrazolium chloride and 35 μl of 5-bromo-4-chloro-3-indolyl phosphate per 10 ml of 0.1 mM Tris-HCl–0.05 mM $MgCl_2$ [pH 9.5] buffer). Nonimmunoreactive proteins were visualized by India ink staining: the membranes were washed once in 1% potassium hydroxide for 5 min and three times in phosphate-buffered saline (PBS) (Oxoid, Columbia, Md.)-methanol (90%/10%, vol/vol) for 5 min each and finally stained with a solution of PBS-Tween 20-India ink solution (100%/0.3%/0.05%, vol/vol/vol) until proteins were clearly visible.

Proteolytic processing by gut proteases. Pure gut secretions of Colorado potato beetles were prepared by forcing larvae to vomit (by gently pressing their heads). For the preparation of gut secretions of *Manduca sexta*, two midguts of L_5 larvae were dissected and suspended in 1 ml of sterile water. The suspensions were gently shaken and centrifuged. The supernatant, which contained the diluted gut juices, was used for further experiments. Proteolytic activities of both solutions were tested by spotting 2 μl of each solution onto Ilford Technical Film (white contrast latitude orthochromatic film) (Ilford Ltd., Mobberley, Cheshire, England) and incubating the film overnight in a moist atmosphere. Proteolytic activity is revealed by halo formation.

A mixture of 10^7 spores and crystals was mixed either with 3 μl of gut secretions collected from Colorado potato beetle larvae–100 μl of PBS or with 20 μl of gut secretions of tobacco hornworm larvae and incubated overnight at 28°C. Soluble and insoluble fractions were prepared by centrifugation. Solubilized CryIIIC crystal proteins (10 μg in 74 μl of $NaHCO_3$ buffer) were treated with 1 μg of trypsin, 3 μl of gut secretions of Colorado potato beetles–100 μl of PBS, or 10 μg (20 μl) of gut secretions of tobacco hornworms. Nonincubated and incubated (but untreated) samples of solubilized CryIIIC and gut secretions of Colorado potato beetles or tobacco hornworms were included as controls. Solubilization and processing of the crystals or crystal proteins were analyzed by SDS-PAGE and immunoblotting. All samples used in lanes 1 to 3, 6 to 10, 12, and 15 of Fig. 4 were prepared by trichloroacetic acid precipitation and subsequent solubilization of protein pellets in SDS buffer solution (2.5% SDS–0.125 M mercaptoethanol–150 mM Tris-HCl [pH 8.8]–4 mM EDTA–0.75 M sucrose–0.075% bromophenol blue). Samples used in lanes 4, 5, 13, and 14 of Fig. 4 were prepared by direct solubilization in SDS buffer solution.

DNA library construction, screening, and sequencing. Total DNA of BTS137J was partially digested with *Sau*3A and size fractionated on a sucrose gradient. Fractions of DNA of between 5 and 10 kb were ligated to *Bam*HI-digested and dephosphorylated pUC18 (36). The recombinant plasmids were transformed into *Escherichia coli* MC1061 cells. Transformed *E. coli* MC1061 cells (10,000) were plated on agar plates (1,000 colonies per plate) and grown overnight. Nitrocellulose filters were moistened in alkaline buffer and placed on top of the agar plates for 2 h, after which they were then lifted and processed as for Western blots as described above. Cell colonies which gave positive results were further analyzed. One of these, which carried a subclone with the full-length crystal protein, was sequenced by the chemical modification method of Maxam and Gilbert (23).

Alignment of crystal proteins. The amino acid sequence of CryIIIC was deduced from the nucleotide sequence and aligned with the sequences of the following crystal proteins: CryIA(a) (27), CryIA(b) (12), CryIA(c) (1), CryIB (2), CryIC (17), CryID (14), CryIE (32), CryIIA (5), CryIIB (35), CryIIIA (13), CryIIIB (29), CryIIID (19), CryIVA (34), CryIVB (3), CryIVC (30), CryIVD (4), and CytA (33). This was done for the putative toxic fragments (from residue 1 to the C-terminal residue of the fifth conserved sequence block [16] and for the C-terminal fragments [from the end of the fifth conserved block to the last amino acid]). For the calculation of the percentages, pairs of amino acids were aligned by using the Genalign program (IntelliGenetics, Mountain View, Calif.), with no gap penalty. The number of matched amino acids was divided by the mean number of residues of the two sequences.

Insect bioassays. A spore-crystal mixture, solubilized crystals, and trypsinized crystal proteins were tested against larvae of the following lepidopteran and coleopteran insects: *Anthonomus grandis* Boheman (cotton boll weevil), *Diabrotica undecimpunctata howardi* Barber (corn rootworm), *Leptinotarsa decemlineata* (Say) (Colorado potato beetle), *Heliothis virescens* (Fabricius) (tobacco budworm), *M. sexta* (Johannson) (tobacco hornworm), and *Spodoptera littoralis* (Boisduval) (cotton leafworm). Bioassays were done at 25°C in 60 to 70% humidity with a 16-h-light, 8-h-dark cycle. For all assays except the Colorado potato beetle bioassay, artificial diets were coated with the various preparations of the crystal protein and the tests were scored after 5 days (6 days for the cotton leafworm). In the case of the Colorado potato beetle assay, fresh potato leaves were dipped in a suspension of a spore-crystal mixture or in a solution containing the crystal protein. One-day-old larvae (three groups of 24) were put on the coated leaf for 24 h, after which the coated leaf was replaced with fresh, untreated leaves. Mortality was recorded 3 days later. Toxicity data were analyzed by probit analysis (8).

Nucleotide sequence accession number. The *cryIIIC* nucleotide sequence accession number at GenBank is M64478.

RESULTS

Insecticidal activity. Initially, a sporulated culture (containing spores and crystals) of the strain was tested against several lepidopteran and coleopteran larvae, but no toxic activity was detected. There seemed to be only a weak and rather variable toxicity against the Colorado potato beetle larvae. Bioassays with the solubilized crystals gave virtually the same results. When trypsinized crystal proteins were tested, a high level of toxicity to Colorado potato beetle larvae was observed. However, no activity against any of the lepidopteran insects was detected. The 50% lethal concentration for the Colorado potato beetle was approximately four times higher than the 50% lethal concentration of trypsinized crystal protein solutions of CryIIIA (Table 1).

Identification of the crystal protein. The BTS137J strain produced bipyramidal crystals (Fig. 1) very similar to those formed by CryI crystal proteins. SDS-PAGE analysis of extracts of a sporulated culture showed a prominent protein band of approximately 129 kDa, corresponding to the protein

TABLE 1. Toxicity of purified CryIIIA, and purified and trypsinized CryIIIC proteins against Colorado potato beetle larvae

Protein	LC_{50}[a] (μg of protein/ml)	FL_{95}[b]		Slope
		Minimum	Maximum	
CryIIIA	3.5	2.2	5.5	2.1
CryIIIC	13.1	5.5	30.9	2.1

[a] 50% lethal concentration.
[b] 95% fiducial limit.

present in purified crystals (Fig. 2). The crystals were dissolved in alkaline buffer, and upon trypsin digestion, a protein of about 72 kDa which was highly toxic to Colorado potato beetle larvae was identified (Fig. 2).

Polyclonal antisera and monoclonal antibodies generated against CryIA, CryIB, CryIC, and CryIVB proteins (15) were used in an ELISA with the solubilized crystal protein, but no immunoreaction could be detected. The crystal protein was not reactive in an ELISA with a set of 24 monoclonal antibodies directed against different regions of the CryIIIA protein. However, the polyclonal antiserum generated against CryIIIA was strongly reactive with the solubilized crystal protein. Immunoblotting of SDS extracts from the sporulated strain and of purified crystals with the anti-CryIIIA serum (Fig. 3) showed a single reactive band of approximately 129 kDa. The 72-kDa protein band, generated by trypsin digestion of solubilized crystal proteins, was also recognized by the anti-CryIIIA serum. The fact that the toxic fragment of the crystal protein was immunologically related to CryIIIA indicated a structural relationship with the crystal proteins of the CryIII family. Because at the time of its identification this was the third crystal protein found to be toxic to Colorado potato beetle larvae and because of the low level of homology of the toxic fragment, this protein was given a new subclass name within the CryIII class (which includes Coleoptera toxins), CryIIIC, by the recently founded *B. thuringiensis* Nomenclature Committee. (This committee was founded at the First International Conference on the Molecular Biology of *Bacillus thuringiensis*, 26 to 28 July 1991. The committee is presided over by D. Dean from Ohio State University, Columbus.)

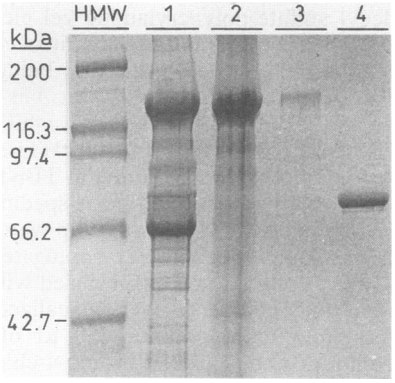

FIG. 2. Results of SDS-PAGE of sporulated cultures (lane 2), solubilized crystals (lane 3), and trypsin-digested crystal proteins (lane 4) and a protein extract of a sporulated culture of strain HD-1 (from a collector housed at the National Center for Agricultural Utilization Research, Peoria, Ill.) (lane 1). HMW, high-molecular-weight markers.

Proteolytic processing of CryIIIC by gut proteases. Since the native crystal protein was not toxic to Colorado potato beetle larvae and became toxic only after trypsin treatment, it appeared that the crystals did not solubilize in the larval guts. Therefore, spore-crystal mixtures or solubilized crystals were mixed with gut secretions collected from Colorado potato beetle larvae and, for comparison, from tobacco hornworm larvae, and the solubilization and processing of

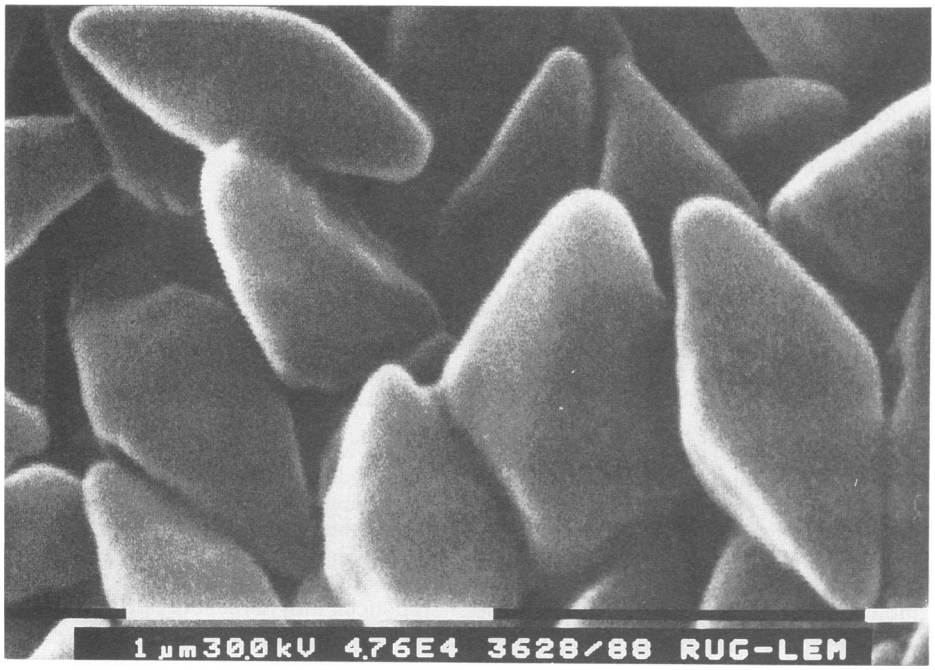

FIG. 1. Scanning electron micrograph of purified crystals of BTS137J. One bar unit represents 1 μm.

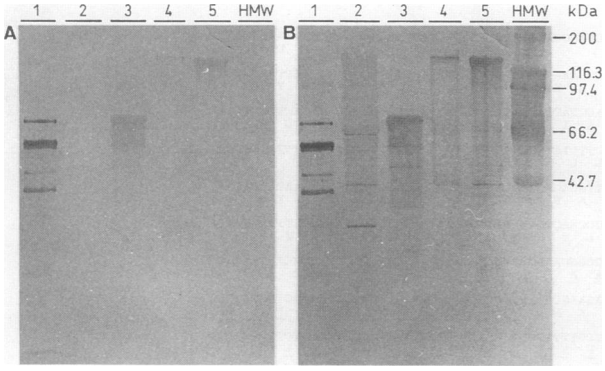

FIG. 3. (A) Immunoblot of BTS137J spore-crystal protein extracts (lane 5), solubilized BTS137J crystals (lane 4), trypsinized BTS137J crystal proteins (lane 3), spore-crystal extracts of HD-1 (lane 2), and trypsinized *B. thuringiensis* subsp. *tenebrionis* crystal proteins (lane 1) with a CryIIIA-specific antiserum. (B) Visualization of total proteins by India ink staining of the same blot. HMW, high-molecular-weight markers.

the crystal proteins were analyzed by SDS-PAGE and immunoblotting (Fig. 4). Processed crystal proteins were tested against the Colorado potato beetle and the tobacco hornworm. Initially, a mixture of spores and crystals was incubated overnight in pure gut secretions of Colorado potato beetles. The soluble material was separated from insoluble material by centrifugation and analyzed. As demonstrated in Fig. 4A and B (lanes 14 and 15), all immunoreactive material representing the CryIIIC crystal protein remained in the pellet, indicating that it was not solubilized in the gut secretions of Colorado potato beetles. The same procedure was repeated with tobacco hornworm gut secretions. In contrast to the findings for the Colorado potato beetle, for the tobacco hornworm, all of the crystal protein was found in the soluble fraction (Fig. 4, lane 12) as a fragment with a molecular weight identical to that of the trypsin-resistant fragment (lane 7). This protease-resistant fragment was toxic to the Colorado potato beetle. When CryIIIC was solubilized in alkaline buffer, only partial processing was observed after overnight incubation (Fig. 4, lane 8). Full processing was observed only after treatment with gut secretions of Colorado potato beetles, which resulted in the appearance of a protease-resistant fragment that is toxic to the Colorado potato beetle (Fig. 4, lane 6). When solubilized CryIIIC is treated with gut secretions of tobacco hornworms, it seems to be totally digested, as shown by the absence of immunoreactive protein (Fig. 4, lane 3).

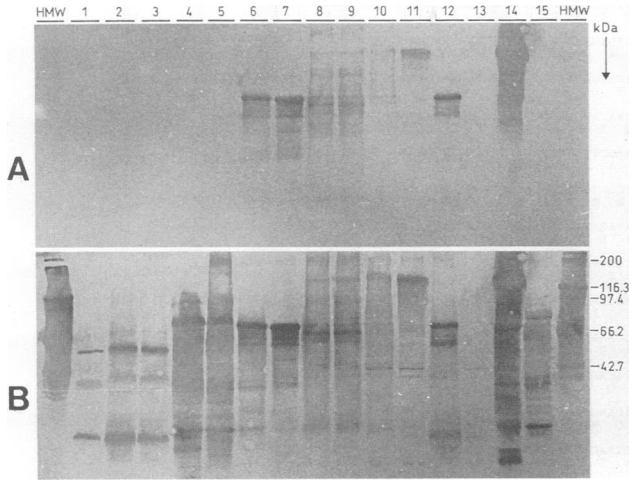

FIG. 4. Immunoblot of various preparations and treatments of BTS137J crystals with a CryIIIA-specific polyclonal antiserum (A) and visualization of total proteins by Indian ink staining (B) (see Materials and Methods for details of the preparation of the samples). Lanes: 1 and 2, gut secretions of tobacco hornworms, incubated overnight or not incubated, respectively; 3, solubilized CryIIIC crystals treated overnight with gut secretions of tobacco hornworms; 4 and 5, gut secretions of Colorado potato beetles, incubated overnight or not incubated, respectively; 6 and 7, solubilized CryIIIC crystals treated overnight with gut secretions of Colorado potato beetles (neutral pH) or with trypsin, respectively; 8 and 9, solubilized CryIIIC crystals, incubated overnight or not incubated, respectively; 10, CryIIIC crystals solubilized in $NaHCO_3$ buffer containing EDTA; 11, spore-crystal extract; 12 and 13, soluble and insoluble fractions, respectively, of an overnight-incubated mixture of spores and crystals suspended in gut juices of tobacco hornworms; 14 and 15, insoluble and soluble fractions, respectively, of an overnight-incubated mixture of spores and crystals suspended in gut juices of Colorado potato beetles and PBS. HMW, high-molecular-weight markers.

Cloning and sequencing of the *cryIIIC* gene. Colony hybridization and Southern blot analysis of the BTS137J strain showed no hybridization (even under low-stringency conditions) with a 2.9-kb *Hin*dIII DNA fragment from *B. thuringiensis* subsp. *tenebrionis* containing the *cryIIIA* gene. Other probes derived from different DNA fragments from different *cry* genes were used, but again, no hybridization could be detected. Since a DNA probe with sufficient homology to the *cryIIIC* gene could not be found, it was decided to screen the genomic library (see Materials and Methods) with antibodies recognizing the IPTG (isopropyl-β-D-thiogalactopyranoside)-induced crystal protein fragments. Of a total of 79 positive colonies, 48 were purified, replated, and reprobed with the anti-CryIIIA serum. Total protein preparations from highly positive colonies were analyzed by immunoblotting with the same antiserum. Twenty-four clones expressing an immunoreactive protein corresponding to the crystal protein of the strain were selected for restriction enzyme analysis of the plasmid DNA. One clone (pOH221) expressing an immunoreactive protein of 129 kDa was selected for further analysis. It contained a DNA insert of 5,050 bp, of which 4,004 nucleotides were sequenced.

Nucleotide and deduced amino acid sequences of the *cryIIIC* gene. The nucleotide and deduced amino acid sequences of the *cryIIIC* gene are shown in Fig. 5. The DNA sequence contains an open reading frame of 1,138 amino acids with a predicted molecular weight of 129,399. The translation initiation site was confirmed by NH_2-terminal amino acid sequencing of the purified crystal protein.

Sequence similarities with other Cry proteins. The N-terminal amino acid sequence up to residue 637, corresponding to the putative toxic fragment, showed only limited homology to other Cry proteins (Table 2). The highest degree of homology (37%) was found with CryIIIA. The five conserved amino acid blocks described by Höfte and Whiteley (16) were also highly conserved in CryIIIC (Fig. 5). A comparison of the toxic fragments of the three CryIII proteins did not reveal any additional highly conserved amino acid blocks. The C-terminal fragment, from amino acid 638 to the end, was clearly homologous to corresponding fragments from other Cry proteins, with the level of homology ranging between 41 and 45% (Table 2).

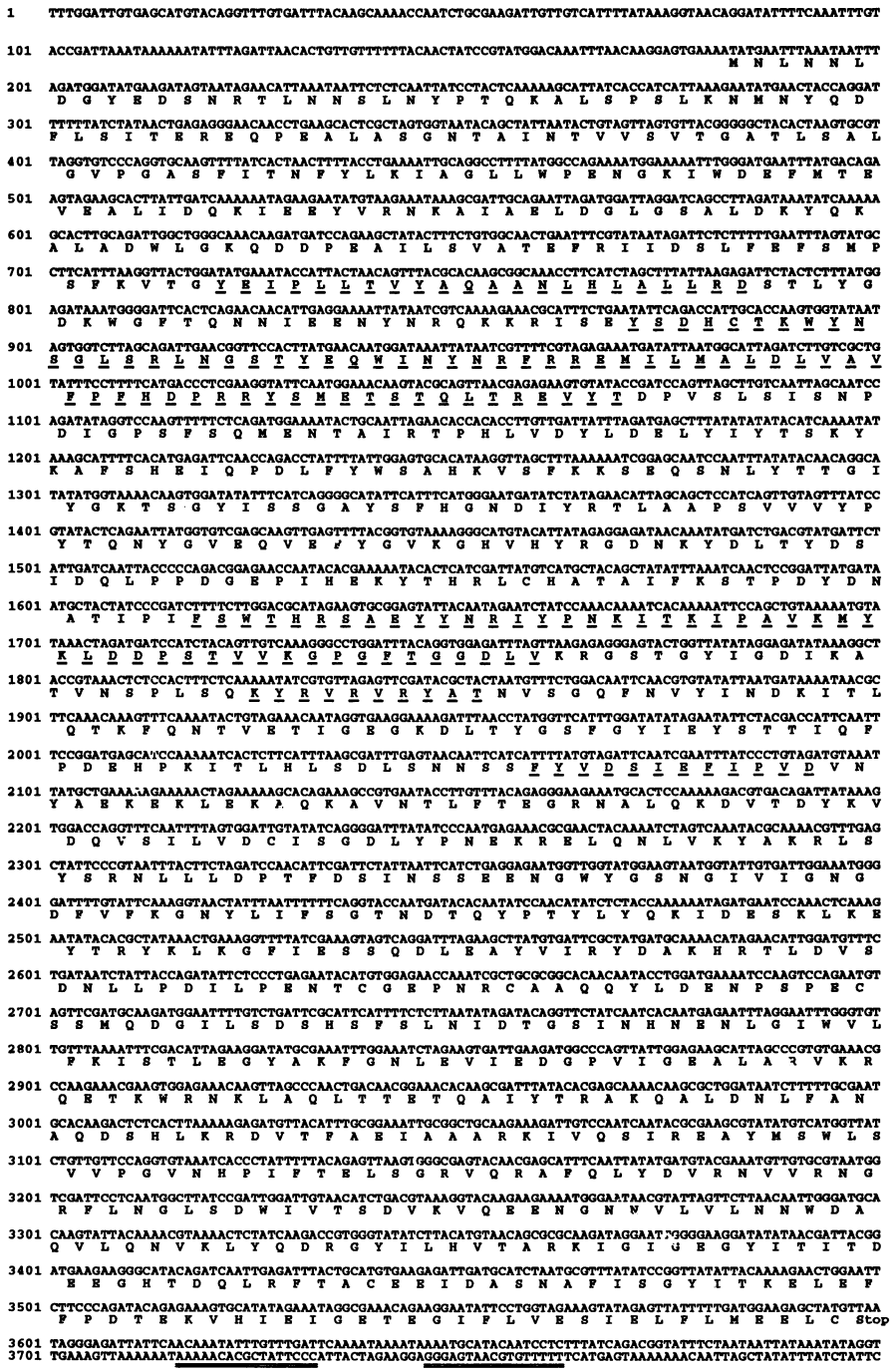

FIG. 5. Nucleotide sequence and deduced amino acid sequence of the *cryIIIC* gene. The derived amino acid sequence is shown in the one-letter code. Regions in the toxic fragment homologous to the conserved regions defined by Höfte and Whiteley (15) are underlined. The inverted sequence at the 3′ end is double underlined.

DISCUSSION

All insecticidal crystal proteins which have been isolated which are toxic to coleopteran insects (CryIIIA, CryIIIB, and CryIIID) share similar characteristics. They are produced as rhomboid crystals (6, 18, 19, 29) which contain crystal proteins of ca. 73 kDa. These proteins appear to be naturally truncated since, in comparison with crystal proteins toxic to lepidopteran insects, they lack ca. 547 to 582 amino acids at their C terminus (16). The BTS137J strain is the first *B. thuringiensis* isolate found to produce a coleopteran toxin packaged in a bipyramidal crystal. The bipyramidal crystals contain a single protein of ca. 129 kDa with a trypsin-resistant toxic core fragment of ca. 72 kDa. The amino acid sequence of the toxic fragment shows some homology with the other three coleopteran toxins. However, the homology is surprisingly limited and is at best 37%.

TABLE 2. Percent amino acid identity between putative N-terminal toxic fragments of CryIIIC and other Cry proteins and the remaining C-terminal fragments

Protein	% Amino acid identity[a]	
	N	C
CryIA(a)	29	45
CryIA(b)	28	44
CryIA(c)	29	45
CryIB	31	41
CryIC	32	44
CryID	30	45
CryIE	29	48
CryIIA	15	NA[b]
CryIIB	16	NA
CryIIIA	37	NA
CryIIIB	32	NA
CryIIID	33	NA
CryIVA	18	43
CryIVB	25	41
CryIVC	20	NA
CryIVD	19	NA

[a] N, putative toxic N-terminal fragments of CryIIIC and Cry proteins; C, C-terminal fragments. For details, see Materials and Methods.
[b] NA, not applicable.

Despite the limited homology and the significant structural differences between CryIIIC and other CryIII proteins, the CryIIIC trypsin core fragment is also active against the Colorado potato beetle.

The most novel feature of the CryIIIC crystal protein is that the insecticidal activity for Colorado potato beetle larvae is generated only after in vitro solubilization and proteolytic activation with Colorado potato beetle gut secretions or trypsin, while in its natural, crystallized form it is not toxic. The proteolytic processing experiments show that the CryIIIC crystals are not solubilized in purified gut secretions of Colorado potato beetles but can be processed to yield a toxic fragment only after solubilization. Although there is no evidence that this can be extended to the in vivo situation, it is likely that the nontoxicity is due to the insolubility of the crystals in the guts of the Colorado potato beetle. The insolubility is probably related to the acidic pH of the gut environment. Nearly all crystal proteins that contain a typical C-terminal fragment are solubilized only at a high pH. This is confirmed by the observation that CryIIIC crystals are solubilized in gut secretions of the tobacco hornworm, which has an alkaline gut environment. The total digestion of in vitro-solubilized CryIIIC crystal proteins after treatment with tobacco hornworm gut secretions (Fig. 4) is probably due to extended unfolding of the crystal protein in the alkaline buffer, which renders the protein accessible to the proteases present in the gut secretions. These data suggest that the Colorado potato beetle may not be the natural target for this crystal protein. The natural target insect for this insecticidal crystal protein remains unknown. This is the first example of a coleopteran-active crystal protein with a silent toxic activity. A similar phenomenon was previously observed with lepidopteran insect larvae. Lecadet and Martouret (20) have described the unmasking of a highly specific activity against lepidopteran insects through enzymatic treatment of a *B. thuringiensis* subsp. *dendrolimus* crystal.

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