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ORIGINAL RESEARCH PAPER

Protease-resistant core form of *Bacillus thuringiensis* Cry1Ie: monomeric and oligomeric forms in solution

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Abstract Cry1Ie is encoded by *cry1Ie*, a gene that is silent in *Bacillus thuringiensis* strains but can be over-expressed in *Escherichia coli*. A protease-resistant core form of Cry1Ie with a molecular weight of approx. 55 kDa was found among the products of trypsin digestion. The oligomer and monomer of the protease-resistant core form were purified separately. The oligomer fraction contained a small quantity of dimer and a large amount of aggregates larger than tetramers. The 50% lethal concentrations (LC_{50}) of the full-length Cry1Ie and both the monomer and oligomer fractions of the protease-resistant core form against *Plutella xylostella* were 14, 21 and 1400 μg/ml, respectively.

Keywords *Bacillus thuringiensis* · Cry1Ie protein · Oligomer · Protease-resistant core form

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Introduction

Crystal (Cry) proteins are produced by *Bacillus thuringiensis* (Bt) during sporulation. Many Cry proteins are synthesized as protoxins with a molecular mass of 130 kDa. Upon ingestion by an insect, the protoxin is activated in the midgut by gut proteases and is converted into a 65 kDa toxin that is derived mainly from the *N*-terminal region of the protoxin (Höfte and Whiteley 1989).

Oligomerization occurs in many bacterial pore-forming toxins. The association of these toxins with their respective receptors is a crucial step in the induction of proteolytic processing by membrane-associated proteases, triggering oligomerization and the subsequent insertion of the toxin into cellular membranes (Rosenberger et al. 2000). Many studies have focused on the oligomerization of Cry proteins (Puntheeranurak et al. 2005; Vie et al. 2001). Moreover, oligomers occur in solutions of Cry proteins (Feng and Becktel 1994; Walters et al. 1994; Guereca and Bravo 1999). Cry1Aa, Cry1Ac, Cry1C, Cry1D and Cry3A toxins exist in solution as a mixture of monomers and high-molecular-mass aggregates with apparent masses greater than 600 kDa, and native Cry toxins do not form oligomers of a defined size (Guereca and Bravo 1999). However, the level of insecticidal activity of oligomers in solution has not been reported.

Many *cry1I*-type genes are silent in *Bacillus thuringiensis* strains because they are often located

downstream of the *cry1* genes and a strong *cry1* transcriptional terminator is present in the intervening sequence between the *cry1* and *cry1I* genes (Gleave et al. 1993). Although the *cry1Ie* genes are silent in Bt strains, they have been over-expressed in *Escherichia coli* BL21(DE3), and the 84 kDa expressed product was toxic to *Plutella xylostella*, *Ostrinia furnacalis* and *Leguminivora glycinivorella* (Song et al. 2003).

In the present study, the protease-resistant core form of Cry1Ie protein was purified, monomers and oligomers were obtained, and the insecticidal activities of both were determined.

Materials and methods

Bacterial strains and plasmids

E. coli BL21(DE3) harboring the recombinant plasmid pET1Ie (Song et al. 2003) was obtained from the Institute of Plant Protection, Chinese Academy of Agricultural Sciences.

Expression and purification of Cry1Ie

E. coli BL21(DE3) harboring the recombinant plasmid pET1Ie was incubated at 37°C in liquid LB medium containing 100 μg ampicillin/ml. The expression of Cry1Ie in the cells was induced with 0.4 mM IPTG at 20°C for approx 15 h. The cells were collected by centrifugation at 5,000×*g* for 10 min and resuspended in lysis buffer (20 mM Tris/HCl, pH 8.0). The cells were lysed by ultrasonication at 200 W with a interval of 15 s on ice, and the inclusion bodies were separated from the soluble supernatant by centrifugation at 15,000×*g* for 10 min at 4°C. The inclusion bodies were washed four times with lysis buffer and three times with distilled water to remove the remaining soluble proteins and then solubilized in 0.5 mM Na_2CO_3 (pH 10.2). The insoluble components were separated by centrifugation at 15,000×*g* for 10 min and discarded. The supernatant was loaded onto a Q-Sepharose Fast Flow column pre-equilibrated with buffer containing 0.5 mM Na_2CO_3 at pH 10.2. The column was then washed with 2–3 column volumes of the Na_2CO_3 buffer and eluted by with a linear gradient of 0–1 M NaCl while the effluent was monitored at 260 and 280 nm. The peak fractions were collected and analyzed by SDS-PAGE.

Trypsin digestion and purification of the protease-resistant core form of Cry1Ie

The Cry1Ie protoxin was digested by treatment with trypsin (TPCK-treated, 1:20 w/w) in 50 mM Na_2CO_3 at 37°C for 1 h. The trypsin-digested products were loaded onto a Superdex-200 column HR (10/30) using a FPLC apparatus. The column was pre-equilibrated with 50 mM Na_2CO_3 at pH 10.2 and eluted at 0.6 ml/min; the eluate was monitored at 260 and 280 nm, and the peak fractions were collected.

N-terminal amino acid residue sequence determination

The purified protease-resistant core form of the protein was identified by SDS-PAGE, and it was subsequently transferred onto polyvinylidene difluoride membrane filters. The membrane-bound proteins, visualized by Coomassie Blue staining, were excised, and the *N*-terminal amino acid residue sequence was determined using the Procise 491 sequencing system from Applied Biosystems.

Glutaraldehyde crosslinking

Glutaraldehyde (25% w/v) was added to the protein solution to give 2% (v/v). After 2 min, the crosslinking reaction was quenched by the addition of solid $NaBH_4$ to give a final $NaBH_4$/glutaraldehyde molar ratio of 10:1. Then, 8 μl 25% deoxycholic acid solution and 25 μl 78% TCA solution were added (Lai et al. 1997). The samples were then centrifuged at 12,000×*g* for 10 min, and the precipitates were washed several times with distilled water and then redissolved in 30 μl of 1.5 M Tris/HCl buffer (pH 8.8) containing 1% (w/v) SDS and 50 mM DTT. Finally, the samples were boiled for 3 min and SDS-PAGE was performed at room temperature using the Tris/glycine buffer system described by Laemmli.

Protein quantification

The protein concentrations were determined by the method of Bradford using BSA as a standard.

Bioassay

Toxicity assays on the second instar larvae of *Plutella xylostella* were conducted on fresh leaf disks using the leaf-dip bioassay (Tabashnik et al. 1993). Disks cut from the leaves of cabbages grown in a greenhouse were used to grow *P. xylostella*. Different concentrations of toxin were brushed on leaves using a clean, autoclaved brush, and then the leaves were dried naturally at room temperature. 50 mM Na_2CO_3 (pH 10.2) was used as negative control. Twenty larvae were placed on a leaf disk and analyzed after 48 h. The 50% lethal concentrations (LC_{50}) were calculated by probit analysis (Finney 1971) after repeating the bioassay at least four times.

Results

Expression and purification of Cry1Ie

Most of the Cry1Ie protein expressed in *E. coli* occurred as an insoluble form in inclusion bodies. The Cry1Ie protein, like many other Cry proteins, can be solubilized in 0.5 mM Na_2CO_3 at pH 10.2. The Cry1Ie protein was further purified by ion-change chromatography and analyzed by SDS-PAGE (Fig. 1, Lane 2).

Trypsin digestion and purification of the protease-resistant core form of Cry1Ie

Cry1Ie was activated by treatment with trypsin from bovine pancreas. During activation, a 66 kDa protein was found which has been previously detected in the activation of many other Cry proteins. Moreover, among the digestion products, a protease-resistant core form with a molecular weight of approximately 55 kDa was observed (Fig. 2, Lane 3). The core form gradually became the main product as the concentration of trypsin was increased. The protease-resistant core form was purified using a Superdex-200 column, and its elution profile was recorded (Fig. 3a). Although peak 1 and peak 2 had different elution volumes, the proteins from both peaks had the same mobility in SDS-PAGE gels, implying that they had the same molecular mass (Fig. 3b).

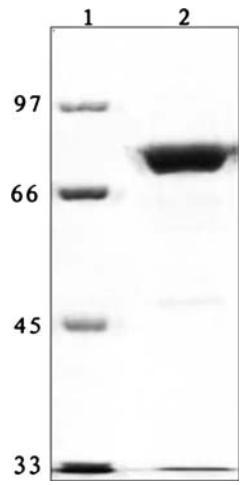

Fig. 1 SDS-PAGE analysis of purified full-length (protoxin) Cry1Ie protein. *Lane 1*, molecular mass marker; *Lane 2*, purified Cry1Ie protein

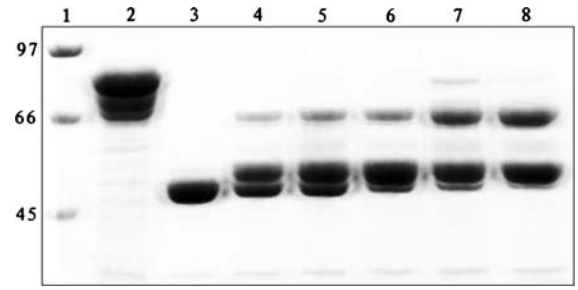

Fig. 2 Digestion of the full-length (protoxin) Cry1Ie with varying concentrations of trypsin at 37°C for 1 h. *Lane 1*, molecular mass marker; *Lane 2*, isolated Cry1Ie; *Lanes 3–8*, products of Cry1Ie digestion with trypsin at protein (weight/weight) ratios of 1:20, 1:30, 1:50, 1:80, 1:100 and 1:150

This result indicated that both a monomer and an oligomer existed in the activated 55-kDa product. Glutaraldehyde was used as a crosslinking reagent to evaluate the oligomeric state of the protease-resistant core form of Cry1Ie (Fig. 4). A product with a molecular mass of 110 kDa was detected on SDS-PAGE after the crosslinking reaction, and a larger amount of protein was observed in a considerably higher oligomeric form, even larger than a tetramer. This result indicated that the oligomer of the core form included a small amount of dimer but was mainly composed of higher order aggregates larger than tetramers. The second peak consisted of the monomer of the core form of the Cry1Ie protein. Comparing the quantities of the collected fractions of the two peaks, we determined that about 40% of the trypsin-digested product existed in the oligomeric form.

In order to determine whether these two forms of the Cry1Ie protease-resistant core are interconvertible, size-exclusion chromatography was used to detect the oligomeric states after incubation at 4°C for different periods of time (Fig. 5). The result shows that the oligomer did not convert into the

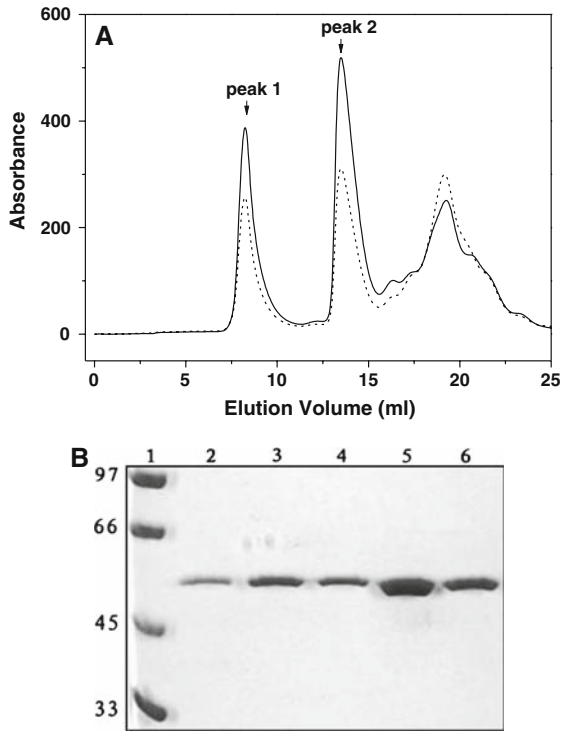

Fig. 3 Purification of the protease-resistant core form of Cry1Ie protein. **a** Elution profile by size-exclusion chromatography of trypsin (1:20, w/w)-digested products. The solid line represents the absorbance at 280 nm, while the dashed line represents the absorbance at 260 nm. The void volume of the column was 8.8 ml as determined using Blue Dextran 2000. The flow rate was 0.6 ml/min. **b** Electrophoretic analysis of the peak fractions. *Lane 1*, molecular mass marker; *Lanes 2–4*, middle fractions of peak 1; *Lanes 5–6*, middle fractions of peak 2

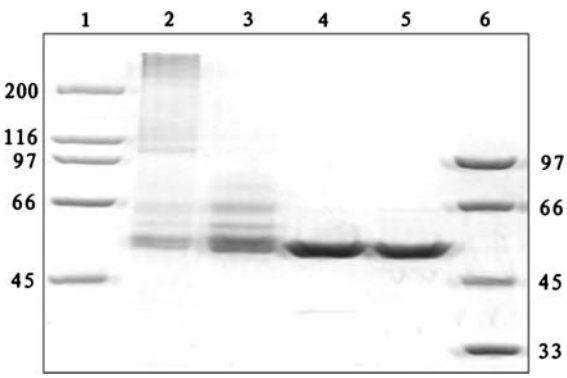

Fig. 4 SDS-PAGE analysis of glutaraldehyde crosslinked proteins of peaks 1 and 2. *Lane 1*, molecular mass marker; *Lane 2*, glutaraldehyde-crosslinked protein of peak 1; *Lane 3*, glutaraldehyde-crosslinked protein of peak 2; *Lane 4*, protein from peak 1; *Lane 5*, protein from peak 2; *Lane 6*, molecular mass marker

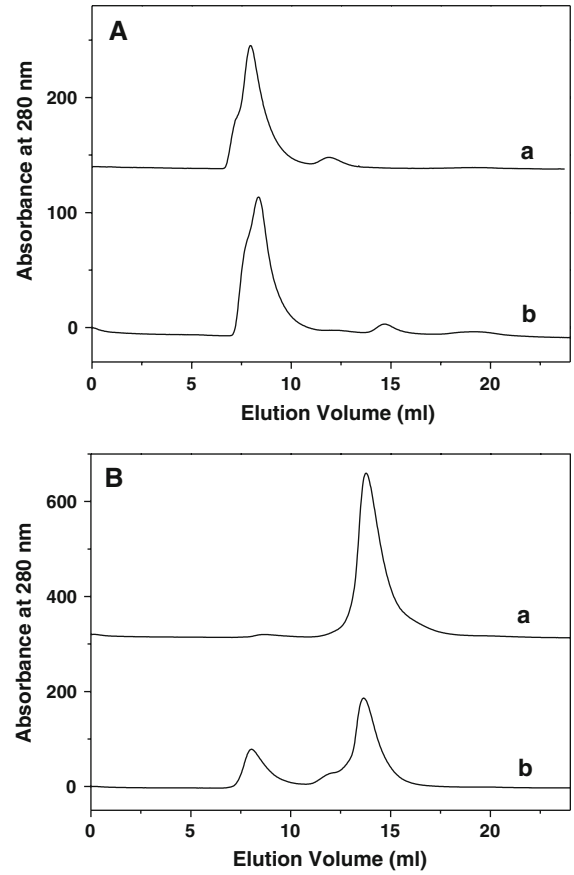

Fig. 5 Elution profiles by size-exclusion chromatography of the oligomer and monomer of the protease-resistant core form of Cry1Ie incubated at 4°C for different periods of time. **A** Elution profiles of the oligomer. *Curve a* represents the newly collected oligomer, while *Curve b* represents the oligomer incubated at 4°C for 3 days. **B** Elution profiles of the monomer. Curve a represents the newly collected monomer, while Curve b represents the monomer incubated at 4°C for 3 days

monomer; however, while the monomer initially remained monomeric, it then partially converted into an oligomer with time. This implies that the oligomer is more stable than the monomer.

The *N*-terminal amino acid residue sequence of the purified protease-resistant core form of the protein was determined to be ATSVV, which begins with amino acid residue 154 of full-length Cry1Ie.

Bioassay of the monomer and oligomer of the protease-resistant core form of Cry1Ie

The LC_{50} of the full-length Cry1Ie protein and both the monomeric and oligomeric forms of the protease-resistant core of Cry1Ie were against *P. xylostella*

Table 1 Bioassay results of the Cry toxin against *Plutella xylostella* larvae

Protein	LC_{50} (μg/ml)	95% fiducial limits
Full-length Cry1Ie	14	10.2–19.2
Monomer of the protease-resistant core form of Cry1Ie	21	14.3–32.3
Oligomer of the protease-resistant core form of Cry1Ie	1,425	840–3,298

determined (see Table 1). The LC_{50} of the monomer was slightly higher than that of full-length Cry1Ie; however, the 95% fiducial limits partially overlap. Since the molecular masses of the full-length Cry1Ie protein and the protease-resistant core form expressed in *E. coli* were 84 kDa (Song et al. 2003) and 55 kDa, respectively, the insecticidal activity of the core form of the Cry1Ie protein was about 35% lower than that of the full-length Cry1Ie protein. The LC_{50} of the oligomer was ~1,400 μg/ml, which is 70 times higher than that of the monomeric form (21 μg/ml), indicating that the insecticidal activity of the oligomer is dramatically lower.

Discussion

Like many other Cry proteins, Cry1Ie yields a 66-kDa protein during activation by trypsin. A protease-resistant core form also exists among the trypsin digestion products, with a molecular weight of approximately 55 kDa. In the core form, the *N*-terminus begins at amino acid 154 of full-length Cry1Ie; this is quite similar to the structure of the core form of Cry1Ia, in which the *N*-terminus starts with amino acid 156 (Sekar et al. 1997). The insecticidal toxicity of the core form of the Cry1Ie protein did not substantially differ from that of full-length Cry1Ie even though it lacks 153 amino acids of the *N*-terminus and some of the C-terminus. This result differed from the analysis of Cry1Ia, in which the core form of Cry1Ia exhibited only ~22% of the toxicity of the full-length protein (Sekar et al. 1997). This difference may in some way be related to the different target larvae used in the bioassay. We used *P. xylostella* larvae while Sekar et al. (1997) used *Ostrinia nubilalis* (European corn borer) larvae.

The crystal structure of the Cry1Aa toxin has been determined (Grochulski et al. 1995). Prediction of the secondary structure of Cry1Ie and its comparison with that of Cry1Aa indicated that the *N*-terminus of the Cry1Ie core is located at the beginning of helix α-4. The α-3 helix in domain I of Cry1Ab was found to contain sequences that could potentially form a coiled-coil structure important for oligomerization (Burkhard et al. 2001; Jimenez-Juarez et al. 2007). Site-directed mutagenesis in helix α-3 resulted in the formation of Cry1Ab mutants that still bound to the Bt-R_1 receptor, similarly to the wild-type toxin; however, oligomerization of the mutants was affected, resulting in the complete loss of pore-forming activity and toxicity against *Manduca sexta* larvae (Jimenez-Juarez et al. 2007). Sequence alignment of Cry1Ab and Cry1Aa indicated that helix α-3 spans amino acids 90–119. Comparison of Cry1Ie with this Cry1Aa region indicated that helix α-3 spans amino acids 118–147 in Cry1Ie, which is outside the sequence of the protease-resistant core form of the Cry1Ie protein. Without helices α1–α3, the protease-resistant core form of Cry1Ie still has a considerable amount of the insecticidal activity like the full-length protein, suggesting that α1–α3 are not absolutely necessary for the process of pore-formation or transmembrane insertion into the midgut of the insect. In this regard, Cry1Ie appears quite different from other Cry proteins.

The insecticidal activity of the oligomer of the core form of Cry1Ie was determined and found to be substantially lower than that of the monomer. We found that the oligomer was formed in solution in absence of receptor. Conversion of oligomer into monomer did not occur to any measurable extent in vitro. The oligomers that form spontaneously in solution most likely differ from the oligomers that form on the membrane of the insect midgut. Aggregation in solution may partially mask the region of the protein that recognizes the membrane receptors. Consequently, the recognition and binding of the protein to its membrane receptors is hindered, and it is difficult for the oligomer that has already formed in solution to bind to the membrane. This may be the reason for the decrease in insecticidal activity of the oligomeric form.

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