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Proteolytic processing of a coleopteran-specific δ-endotoxin produced by *Bacillus thuringiensis* var. *tenebrionis*

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Insecticidal protein δ-endotoxin crystals harvested from sporulated cultures of *Bacillus thuringiensis* var. *tenebrionis* contain a major polypeptide of 67 kDa and minor polypeptides of 73, 72, 55 and 46 kDa. During sporulation, only the 73 kDa polypeptide could be detected at stage I. The 67 kDa polypeptide was first detected at stage II and increased in concentration throughout the later stages of sporulation and after crystal release, with a concomitant decrease in the 73 kDa polypeptide. This change could be blocked by the addition of proteinase inhibitors. Trypsin or insect-gut-extract treatment of the δ-endotoxin crystals after solubilization resulted in a cleavage product of 55 kDa with asparagine-159 of the deduced amino acid sequence of the toxin [Höfte, Seurinck, van Houtven & Vaeck (1987) Nucleic Acids Res. **15**, 71–83; Sekar, Thompson, Maroney, Bookland & Adang (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 7036–7040; McPherson, Perlak, Fuchs, Marrone, Lavrik & Fischhoff (1988) Biotechnology **6**, 61–66] at the *N*-terminus. This polypeptide was found to be as toxic *in vivo* as native δ-endotoxin.

INTRODUCTION

Bacillus thuringiensis is an aerobic spore-forming Gram-positive bacterium that synthesizes a crystalline protein δ-endotoxin during sporulation (Bechtel & Bulla, 1976). The crystal δ-endotoxin is specifically pathogenic to insect larvae. A total of five pathotypes have been suggested to classify the different subspecies of *B. thuringiensis*: pathotype A, specific for lepidoptera, (butterflies and moths), pathotype B, specific for diptera (flies), pathotype C, active only against coleoptera (beetles) (Krieg *et al.*, 1983), pathotype D, toxic to both lepidoptera and diptera and pathotype E, examples of which produce the crystal δ-endotoxin but have not been found to be toxic to any insect species tested so far (Ellar *et al.*, 1986).

After the isolation of a coleopteran-specific δ-endotoxin (Krieg *et al.*, 1983; Herrnstadt *et al.*, 1986), the initial analysis of the crystalline toxin inclusions by SDS/polyacrylamide-gel electrophoresis (Bernhard, 1986; Herrnstadt *et al.*, 1986) revealed a major polypeptide of 68 or 64 kDa. No toxic polypeptides smaller than 64 kDa were obtained when this protein was treated with trypsin or gut extracts from a susceptible larva (Herrnstadt *et al.*, 1986). This finding would appear to distinguish the coleopteran toxins from all other δ-endotoxins so far studied, which have been shown to be synthesized initially as protoxins that are activated by proteolytic cleavage in the larval gut.

We have therefore re-examined this finding and report here the identification and partial sequencing of a 55 kDa fragment of the *B. thuringiensis* var. *tenebrionis* coleopteran-specific δ-endotoxin generated by treatment with trypsin or insect gut enzymes, which shows identical toxicity *in vivo* with the native toxin.

In addition, in the light of recent suggestions that var. *tenebrionis* proteinases may be acting on the δ-endotoxin either during synthesis or after crystal release (Sekar *et al.*, 1987) we have examined the effect of the addition of proteinase inhibitors during both these events.

EXPERIMENTAL

Bacterial subspecies and growth conditions

The bacterial isolate used during the present study was selected as an atypical colony variant after heat treatment of purified spores of *Bacillus thuringiensis* var. *tenebrionis* (Krieg *et al.*, 1983) and designated as '*B. thuringiensis* var. *tenebrionis* 1911' (Northrop & Slepecky, 1967). Growth and sporulation conditions were as described for *Bacillus megaterium* KM (Stewart *et al.*, 1981).

Purification of the crystal δ-endotoxin

The crystal δ-endotoxin was purified as described by Thomas & Ellar (1983) by using gradients of 67, 72 and 82% (w/v) sucrose in distilled water. In some instances crystals were isolated from cultures to which EDTA (3.72 mg/ml; Sigma), PMSF (1.74 mg/ml; Sigma) and NEM (1.25 mg/ml; Sigma) had been added at early stage VI. Sucrose solutions used for gradient centrifugation of these crystals were made up in 50 mM-Tris/HCl, pH 7.5, containing 10 mM-KCl and 4 mM-EDTA.

Analysis of crystal production during growth and sporulation

B. thuringiensis var. *tenebrionis* 1911 was grown in PWYE medium overnight, diluted into fresh PWYE

Abbreviations used: CCY, casein/casein/yeast-extract sporulation medium; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; P.g.e., *Pieris brassicae* (cabbage white butterfly) gut extract; PMSF, phenylmethanesulphonyl fluoride; PWYE, peptone/water/yeast extract; T.g.e., *Tenebrio molitor* (mealworm) gut extract; LC_{50} is defined in detail in the text.

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medium and grown to mid-exponential phase before diluting into CCY medium (Stewart *et al.*, 1981). All cultures were incubated at 30 °C and shaken at 200 rev./min. This procedure gives a high degree of sporulation synchrony (Ellar & Posgate, 1974). Growth and sporulation in CCY medium were monitored by A_{600} measurements and phase-contrast microscopy. Cells were harvested during exponential growth, early stationary phase and throughout sporulation. Sporulation stages referred to in the text designate the recognizable morphological events in spore formation (Ellar, 1978). Axial filament formation (stage I) is followed by an asymmetric division to form the forespore compartment (stage II), membrane proliferation to give an immature forespore free within the mother cell (stage III), spore cortex (stage IV) and spore coat formation (stages V and early VI) and finally release of mature spores after cell lysis (stage VI). Lysates were obtained by essentially the same protocol as that described by Ward & Ellar (1986), except that PMSF (10 mM) and NEM (10 mM) were added in addition to EDTA. The step involving DNAase and $MgSO_4$ to remove DNA was omitted.

Protein determination

Protein concentrations were determined by the method of Lowry *et al.* (1951) with bovine serum albumin (Sigma) as a standard.

Electrophoresis

SDS/polyacrylamide-gel electrophoresis was carried out by a method modified from that of Laemmli & Favre (1973) as described by Thomas & Ellar (1983). Protein samples were solubilized before loading by incubation at 100 °C for 5 min in gel sample buffer [50 mM-Tris/HCl (pH 7.5)/1% (w/v) SDS/25 mM-DTT/2 mM-PMSF/1 mM-EDTA/10% (w/v) glycerol/Bromophenol Blue (0.0025%, w/v)].

Samples were run on a 10% gel with a 6% stacking gel at 20 mA constant current until the dye marker reached the main separating gel, then at a constant current of 25 mA. Gels were stained overnight at room temperature with 0.1% (w/v) Coomassie Brilliant Blue R (Sigma) in 50% (v/v) methanol/10% (v/v) acetic acid and destained with several changes of 10% (v/v) methanol/10% (v/v) acetic acid.

Enzymic digestion of solubilized δ-endotoxin

Crystals were solubilized by resuspending them in either 3.3 M-NaBr or 50 mM-Na_2CO_3/HCl, pH 10.5, at 37 °C for 60 min. The insoluble material was removed by centrifugation at 10000 *g* for 15 min at room temperature. NaBr-solubilized δ-endotoxin (2 mg/ml) was made 10% (v/v) in 50 mM-NaH_2PO_4, pH 7.5 (pH adjusted with NaOH), and the effect of trypsin (2:1, w/w, enzyme/toxin) and *Tenebrio molitor* (meal-worm; coleoptera) gut extract (T.g.e.) (10% v/v) was determined after incubation at 37 °C, by SDS/polyacrylamide-gel electrophoresis. The effect of *Pieris brassicae* (cabbage white butterfly) gut extract (P.g.e.; 10%, v/v) was observed after solubilization of δ-endotoxin (1 mg/ml) in 50 mM-Na_2CO_3/HCl, pH 10.5, and incubation at 37 °C.

P.g.e. was prepared by the method of Knowles *et al.* (1984). Essentially the same method was used for T.g.e., except that 50 mM-NaH_2PO_4 (pH 7.5, adjusted with NaOH) minus DTT replaced 50 mM-Na_2CO_3/HCl, pH 9.5, plus DTT.

Dialysis of enzyme-treated δ-endotoxin was carried out against deionized water at 4 °C and resulted in the recrystallization of the δ-endotoxin protein. The crystals formed were washed with deionized water in order to remove the proteinases.

Peptide mapping

Peptide mapping by limited proteolysis was performed by a method modified from that of Cleveland *et al.* (1977) as described by Stewart & Ellar (1983).

Antiserum preparation and immunoblotting

Antibodies were raised to a gel-purified (Walker *et al.*, 1980) 67 kDa polypeptide of *B. thuringiensis* var. *tenebrionis* 1911 δ-endotoxin in New Zealand White rabbits as described previously (Ward *et al.*, 1984).

Proteins separated by SDS/polyacrylamide-gel electrophoresis were electrophoretically transferred to nitrocellulose paper (Schleicher and Schüll) in a Bio-Rad Transblot apparatus by the method of Towbin *et al.* (1979). Non-specific sites were blocked with 3% (w/v) bovine haemoglobin (Sigma) in Tris-buffered saline (10 mM-Tris/HCl, pH 7.4, containing 0.9% NaCl) and the nitrocellulose paper was then incubated with var. *tenebrionis* 1911 antiserum. Bound antibodies were detected after incubation with second antibody (goat anti-rabbit immunoglobins, peroxidase-conjugated) and developed with 4-chloro-1-naphthol and H_2O_2 (Hawkes *et al.*, 1982).

Protein sequencing

Proteins were blotted on to Immobilon membranes (Millipore) as described by Matsudaira (1987). The pH of the blotting buffer {10 mM-Caps [3-(cyclohexylamino) propane-1-sulphonic acid]} was, however, 6.5. Excised Coomassie Blue-stained bands were applied to the sample cartridge of an Applied Biosystem 470A gas-phase sequencer. Sequencer analysis was performed by using standard RUN 470–1 software under the control of a 900A data/controller module. Analysis of the amino acid phenylthiohydantoin derivatives was performed on-line using a 120A PTA amino acid analyser. The anilinothiazolinone derivatives of histidine and arginine failed to be eluted from the membrane in detectable yield; these residues are designated X and appear in parentheses in the sequence.

Bioassay

Bioassays *in vivo* using *Phaedon cochleariae* (mustard beetle; coleoptera) larvae were carried out by painting turnip (*Brassica*) leaves with a suspension of crystal δ-endotoxin. Three replicates of eight or ten larvae were carried out and larval mortality was recorded after 120 h. LC_{50} measurements refer to the concentration of δ-endotoxin protein which, when applied as a uniform covering on a leaf disc, produces 50% mortality. Larvae were kindly provided by Mr. B. Betts, May and Baker, Ongar, Essex, U.K.

Bioassays using *Tenebrio molitor* (coleoptera) larvae were carried out by involuntary feeding of 5 μl of Na_2CO_3-solubilized δ-endotoxin (1 mg/ml) and P.g.e.-treated solubilized δ-endotoxin (1 mg/ml) every 24 h. Weight gain by batches of ten larvae was recorded after 120 h. Two replicates were performed. *T. molitor* larvae

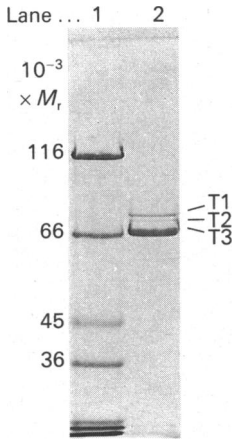

Fig. 1. Polypeptide profile of *B. thuringiensis* var. *tenebrionis* δ-endotoxin

An SDS/10%-polyacrylamide gel (Coomassie Blue-stained) is shown. Lane 1, M_r standards; lane 2, *B. thuringiensis* var. *tenebrionis* δ-endotoxin (10 μg).

were obtained from Xenopus Ltd., Holmesdale Nursery, South Nutfield, Redhill, Surrey. Appropriate controls were carried out in each case.

RESULTS

SDS/polyacrylamide-gel electrophoresis of the purified crystalline δ-endotoxin isolated after release from *B. thuringiensis* var. *tenebrionis* 1911, in the absence of proteinase inhibitors, revealed polypeptides of 73, 72 and 67 kDa, designated 'T1', 'T2' and 'T3' respectively (Fig. 1). Two minor polypeptides with apparent molecular masses of 58 and 46 kDa ('T4' and 'T5' respectively) were occasionally visible in gels of purified crystals at higher loadings (results not shown). A crystal preparation showing this polypeptide pattern is subsequently refered to as 'native toxin' throughout the present paper.

Peptide mapping by limited proteolysis (results not shown) suggested that the major polypeptides (T1/2 and T3) exhibit a significant degree of sequence similarity (T1 and T2 could not be separated in this analysis). Since the polypeptides T4 and T5 are only present in very small amounts in the native crystal, their peptides were not visible after peptide mapping. *N*-Terminal

Table 1. System for classifying the polypeptides reported in native *B. thuringiensis* var. *tenebrionis* δ-endotoxin

References: a, McPherson *et al.* (1988); b, Höfte *et al.* (1987); *c*, the present paper.

Polypeptide	Molecular mass (kDa)	*N*-Terminus	Reference(s)
T1	73	Methionine 1	
T2	72	Glutamic acid 16	a
T3a	67	Aspartic acid 58	a, b, c
T3b	67	Methionine 48	a
T3c	67	Alanine 50	a
T4	55	Asparagine 159	c
T5	46	?	

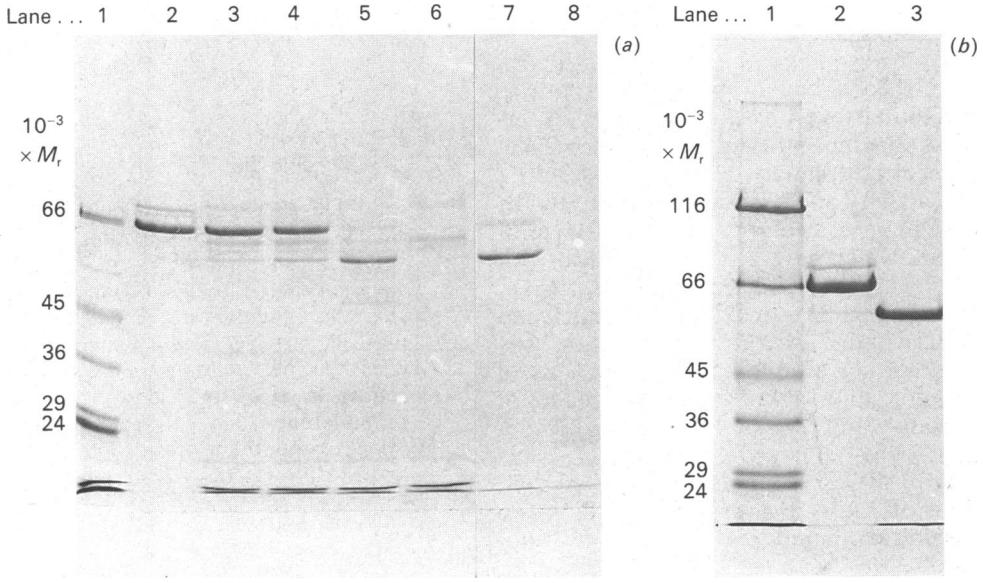

Fig. 2. Enzymic digestion of *B. thuringiensis* var. *tenebrionis* δ-endotoxin

SDS/10%-polyacrylamide gels (Coomassie Blue-stained) are shown. (*a*) Lane 1, M_r standards; lane 2, 10 μg of NaBr-soluble var. *tenebrionis* δ-endotoxin; lane 3, 10 μg of NaBr-soluble var. *tenebrionis* δ-endotoxin made 10% (v/v) in 50 mM-NaH_2PO_4 (pH 7.5, adjusted with NaOH) and treated with T.g.e. (10%, v/v) for 1 h at 37 °C; lane 4, as lane 3, but treated for 3 h; lane 5, as lane 3, but treated for 24 h; lane 6, T.g.e. control; lane 7, 10 μg of NaBr-soluble var. *tenebrionis* δ-endotoxin made 10% (v/v) in 50 mM-NaH_2PO_4 (pH 7.5, adjusted with NaOH) and treated with trypsin (1:2, toxin/enzyme) at 37 °C for 24 h; lane 8, trypsin (5 μg). (*b*) Lane 1, M_r standards; lane 2, 10 μg of Na_2CO_3/HCl, pH 10.5, soluble var. *tenebrionis* δ-endotoxin; lane 3, 10 μg of Na_2CO_3/HCl (pH 10.5)-soluble δ-endotoxin treated with P.g.e. (10%, v/v) for 1 h at 37 °C.

Table 2. Toxicity of native *B. thuringiensis* var. *tenebrionis* δ-endotoxin and a 55 kDa product derived by enzymic cleavage, against coleopteran larvae

Second-instar *P. cochleariae* larvae were assayed using a 200 μg/ml suspension of native crystal δ-endotoxin or trypsin-treated and recrystallized δ-endotoxin in 0.02% Triton X-100 (1) or 200 μg/ml of solubilized δ-endotoxin and solubilized toxin treated with P.g.e. (2), painted on turnip leaves. *T. molitor* larvae were involuntarily fed 5 μl of a 1 mg/ml solution of δ-endotoxin or solubilized toxin treated with P.g.e. For details of solubilization and enzyme treatment of *B. thuringiensis* var. *tenebrionis* δ-endotoxin see the Experimental section. Toxicity towards *P. cochleariae* larvae and *T. molitor* larvae was assessed after 120 h and classified from weak (+) to strong (++++).

Species	Treatment of toxin	Toxin...	Toxicity: Native (67 kDa)	Toxicity: 55 kDa	Toxicity: Control
Phaedon cochleariae	(1)		++++	++++	–
Tenebrio molitor	(2)		++++	++++	–
			+	+	–

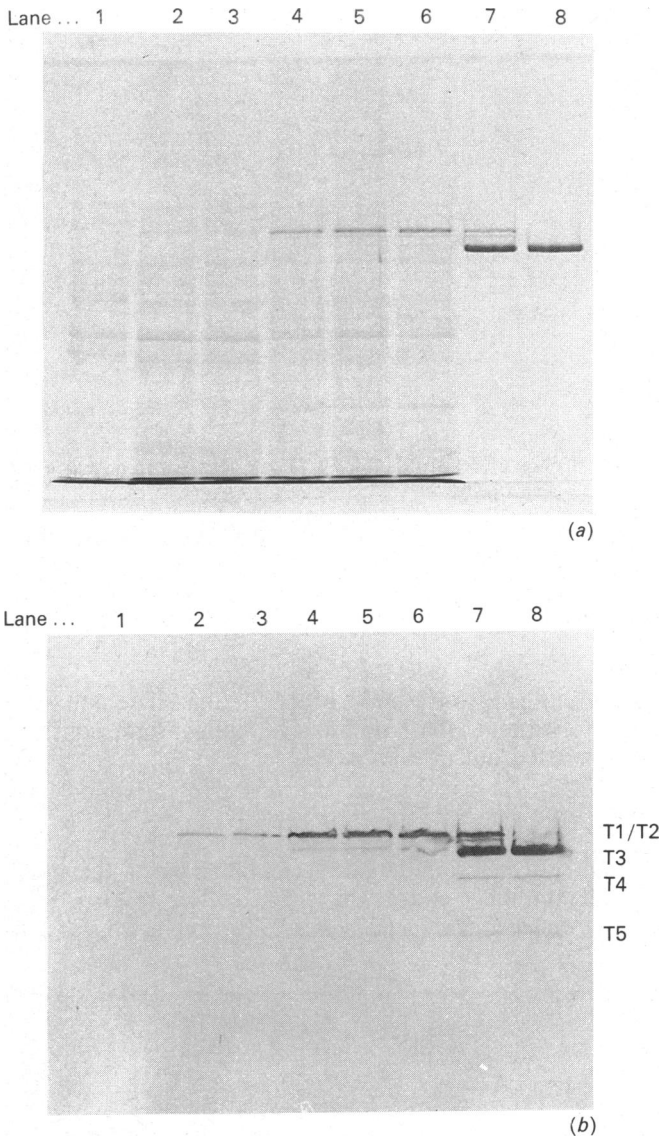

Fig. 3. Expression of *B. thuringiensis* var. *tenebrionis* δ-endotoxin

(*a*) SDS/10%-polyacrylamide gel (Coomassie Blue-stained) of lysates made from var. *tenebrionis* cells harvested at the following growth stages: lane 1, early stationary; lane 2, stage II; lane 3, stage III; lane 4, stage IV; lane 5, stage V; lane 6, stage VI; lane 7, 10 μg of purified var. *tenebrionis* δ-endotoxin harvested at 90% release; lane 8, 10 μg of purified var. *tenebrionis* δ-endotoxin harvested 36 h after complete release. (*b*) Immunoblot using antibodies raised against var. *tenebrionis* δ-endotoxin of an SDS/10%-polyacrylamide gel loaded as in (*a*) and transferred to nitrocellulose paper.

analysis of T3 and T4 (obtained by overloading of the gel) in native δ-endotoxin yielded the sequences DSSTTKDVIQKGI and NP(X)SQG repectively. Both can be located in the deduced amino acid sequence of the var. *tenebrionis* δ-endotoxin (Höfte *et al.*, 1987; Sekar *et al.*, 1987; McPherson *et al.*, 1988) corresponding to positions Asp-58 and Asn-159, and have deduced molecular masses of 66512 and 55441 Da. Table 1 shows a proposed system of reference to the polypeptides of the var. *tenebrionis* δ-endotoxin.

Treatment of solubilized native δ-endotoxin with trypsin, T.g.e. or P.g.e. yielded a major polypeptide that was observed to correspond to T4 as determined by *N*-terminal analysis and apparent molecular mass after SDS/polyacrylamide-gel electrophoresis (Figs. 2*a* and 2*b*).

The insecticidal activity of T4 was assayed by using the recrystallized protein generated after dialysis of trypsin-treated δ-endotoxin, which gave a profile identical with track 7, Fig. 2(*a*), or using solubilized and P.g.e.-treated δ-endotoxin (track 3, Fig. 2*b*). In both cases toxicity to *Phaedon cochleariae* and *Tenebrio molitor* larvae was identical with that of corresponding concentrations of native crystal δ-endotoxin (Table 2). The LC_{50} value for Na_2CO_3-soluble native and trypsin-digested (55 kDa) δ-endotoxin against newly emerged first-instar *P. cochleariae* larvae was 10–50 μg/ml. It should be noted that, when preparations of T4 were run at high loadings on 10–20% gradient polyacrylamide/SDS gels, a faint doublet of approx. 6 kDa was observed (results not shown). Since the ratio of these low-molecular-mass components to the T4 band was invariably 1:15–20, we think it unlikely that they play any part in the toxicity mechanism.

A high degree of sporulation synchrony was achieved during growth of var. *tenebrionis*, with more than 90% of cells reaching any given stage within 2 h. Sporulation stages are as defined by Ellar (1978) and described in the Experimental section. After the culture reached stage VI, a period of 25–30 h elapsed before 90–100% crystal release was observed. The appearance of the δ-endotoxin during growth and sporulation was monitored by using SDS/polyacrylamide-gel electrophoresis and immunoblotting of the proteins in lysates of var. *tenebrionis* 1911 cells prepared by sonication at different stages of sporulation (Figs. 3*a* and 3*b*).

When δ-endotoxin synthesis was monitored by immunoblotting (Fig. 3*b*), T1 was the first polypeptide detected, followed later by T3 and a polypeptide of 46 kDa (T5). At high sample loadings (results not

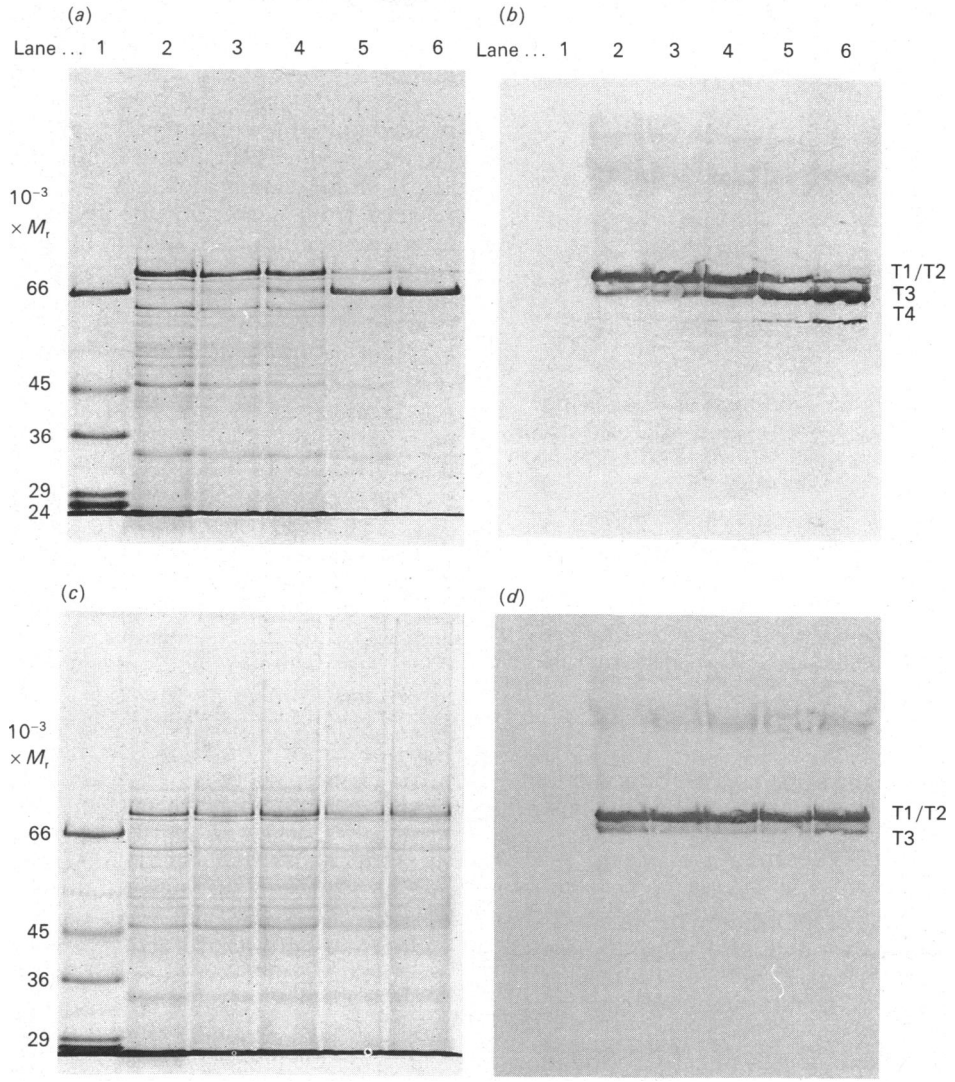

Fig. 4. Expression of *B. thuringiensis* var. *tenebrionis* δ-endotoxin during stage VI of sporulation, with or without the addition of proteinase inhibitors (10 mM-EDTA, 10 mM-PMSF and 10 mM-NEM) at the onset of stage VI

(*a*) SDS/10%-polyacrylamide gel (Coomassie Blue stained) of lysates made from var. *tenebrionis* cells harvested at the following growth stages: lane 1, M_r standards; lane 2, stage VI (0% crystal release); lane 3, stage VI (5% crystal release); lane 4, stage VI (10% crystal release); lane 5, stage VI (90% crystal release); lane 6, 24 h after 100% crystal release. (*b*) Immunoblot using antibodies raised against var. *tenebrionis* δ-endotoxin of an SDS/10%-polyacrylamide gel loaded as in (*a*) and transferred to nitrocellulose paper. (*c*) SDS/10%-polyacrylamide gel (Coomassie Blue-stained) of lysates made from var. *tenebrionis* cells (plus proteinase inhibitors) harvested at the same stages as in (*a*). Lanes are as for (*a*). (*d*) Immunoblot using antibodies raised against var. *tenebrionis* δ-endotoxin of an SDS/10%-polyacrylamide gel loaded as in (*c*) and transferred to nitrocellulose paper.

shown), T1 was first detected during stage I (early stationary phase) with T3 appearing later at stage II. A significant increase in the proportion of T3 and the appearance of T2 and T4 can be observed in purified crystals obtained after spontaneous release (Fig. 3*b*, tracks 7 and 8), compared with crystals isolated after disruption of stage VI sporulating cells (Fig. 3*b*, track 6).

A more detailed analysis of the δ-endotoxin in the cell during stage VI and during the release phase (Figs. 4*a* and 4*b*) again showed the presence of T3 together with T1 before crystal release. The proportions of these polypeptides altered during the period of crystal release, the amount of T1 declining with a concomitant increase in T3. T4 was again apparent only after crystals began to be released. Both the large increase in T3 and the post-release appearance of T4 could be blocked by the addition of proteinase inhibitors at stage VI (Figs. 4*c* and 4*d*). Inclusions were isolated by sucrose-density gradients from cultures treated with proteinase inhibitors. When analysed by SDS/polyacrylamide-gel electrophoresis, these inclusions gave a polypeptide profile with a major T1/2 band and minor T3 band (Fig. 5, track 3). These inclusions were soluble in 3.3 M-NaBr and, after recrystallization by dialysis against deionized water, gave the same polypeptide profile (Fig. 5, track 4).

X-ray powder patterns from spontaneously released crystals harvested in the absence of proteinase inhibitors have been obtained (Li *et al.*, 1988). When this X-ray analysis was repeated using crystals harvested in the presence of proteinase inhibitors, the powder patterns

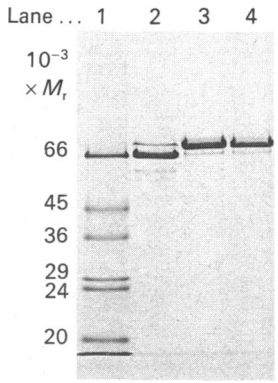

Fig. 5. Comparison of the crystal δ-endotoxin of *B. thuringiensis* var. *tenebrionis* isolated from cultures with or without added proteinase inhibitors (10 mM-EDTA, 10 mM-PMSF, and 10 mM-NEM) at stage VI

An SDS/13%-polyacrylamide gel (Coomassie Blue-stained) is shown. Lane 1, M_r standards; lane 2, crystal δ-endotoxin from cultures without proteinase inhibitors; lane 3, crystal δ-endotoxin from cultures with added proteinase inhibitors; lane 4, crystal δ-endotoxin formed after dialysis of NaBr-soluble crystals isolated from inhibited cultures.

indicated that they have the same crystal form and unit-cell dimensions as native inclusions (results not shown).

DISCUSSION

Throughout stages I–VI of sporulation the δ-endotoxin of *B. thuringiensis* var. *tenebrionis* 1911 is produced as a 73 kDa polypeptide (T1) (Figs. 3*a* and 3*b*). From stage II onwards in sporulation a second immunoreactive polypeptide of 67 kDa (T3) appears. This is comparable with an immunoblot of sporulating cells in early sporulation stages shown by Sekar (1988).

N-Terminal analysis suggests that the increase in amount of a band in the approximate position of T3 observed when crystals begin to be released from the cell and during subsequent incubation in the culture media is the result of proteolysis of T1 to T3a (Table 1) with Asp-58 as the *N*-terminal residue. This is in agreement with the findings of Höfte *et al.* (1987). The observation that no polypeptides corresponding to T5 or of lower molecular mass than T5 were produced when proteinase inhibitors were added at stage VI of sporulation strongly suggests that these are all the result of proteolysis of T1 and T3.

Var. *tenebrionis* δ-endotoxin crystals have been previously reported to contain polypeptides of 74 and 68 kDa (Krieg *et al.*, 1987) or 68 and 55 kDa (Bernhard, 1986). Our data indicate that this discrepancy could have resulted from harvesting the crystals at different times after sporulation. As shown in Figs. 3(*a*) and 3(*b*), crystals harvested early will contain the 73 and 72 kDa polypeptides (T1 and T2), whereas if harvesting is delayed, T1 and T2 will be gradually proteolysed and may not be apparent in some δ-endotoxin preparations (Fig. 3*a*, track 8). Sekar *et al.* (1987) reported that minor polypeptides of 73 and 56 kDa could be observed in addition to the major polypeptide of 67 kDa when high concentrations of δ-endotoxin were analysed by SDS/polyacrylamide-gel electrophoresis and immunoblotting.

The crystalline nature of the δ-endotoxin inclusion does not preclude limited proteolysis in the cell or in the culture medium after release, since the thin plate-like form of the native crystal would provide a large surface area accessible to endogenous proteinases. Sekar *et al.* (1987) also showed that a greater amount of the var. *tenebrionis* 73 kDa polypeptide was present at stage III/IV compared with released inclusions and suggested that the major 67 kDa polypeptide in native inclusions arose from proteolysis of the 73 kDa polypeptide either before or after crystal formation. The observation that, at stage VI, when crystal formation is apparently complete, the major polypeptide present is 73 kDa (Figs. 4*a* and 4*b*) leads to the suggestion that proteolysis to the 67 kDa (T3a) polypeptide is the result of associated endogenous proteinase activity (Bernhard, 1986) after crystal release. This increase in T3a during crystal release could be effectively inhibited by using proteinase inhibitors (Figs. 4*c* and 4*d*), and inclusions containing a major 73 kDa polypeptide isolated (Fig. 6). Endogenous proteinase activity could also account for the appearance of T4 after crystal release.

Our finding that limited proteolysis of the native δ-endotoxin by trypsin or insect gut extract yields a stable product (T4) with full insecticidal activity, differs from that of Herrnstadt *et al.* (1986), who observed only very small non-toxic peptides when solubilized crystals were exposed to these enzymes. We have considered the possibility that peptides cleaved from the native δ-endotoxin could remain associated with T4, and a minor

Source of gene	Residue no.	Amino acid sequence	
kurstaki HD1	576	FSNGSSVFTL SAHVFNSGNE VYIDRIEFVP AEVTFEA--- EYDLERAQKA	622
kurstaki HD73	578	AFTSSLGNIV GVRNFSGTAG VIIDRFEFIP VTATLEA--- EYNLERAQKA	622
berliner	577	FSNGSSVFTL SAHVFNSGNE VYIDRIEFVP AEVTFEA--- EYDLERAQKA	623
tenebrionis	613	FELSGNNLQI GVTGLSAGDK VYIDKIEFIP VN end	644
israelensis 1	647	APNQNISLVF NRSDVYTNTT VLIDKIEFLP ITRSIREDRE KQKLETVQQI	696
israelensis 2	603	SSNQLITIAI QPLNMTSNNQ VIIDRIEIIP ITQSVLDETE NQNLESEREV	652

Fig. 6. Comparison of deduced amino acid sequences of *Bacillus thuringiensis* δ-endotoxins

--- Represent introduced gaps in the amino acid sequence. References: *kurstaki* HD 1 (Schnepf *et al.*, 1985); *kurstaki* HD 73 (Adang *et al.*, 1985); *berliner* (Höfte *et al.*, 1986); *tenebrionis* (Höfte *et al.*, 1987); *israelensis* 1 (Ward & Ellar, 1987); *israelensis* 2 (Tungpradubkul *et al.*, 1988).

doublet of 6 kDa can be observed by SDS/polyacrylamide-gel electrophoresis. However, the lack of 1:1 stoichiometry between T4 and the 6 kDa doublet that we detected in the proteinase digests suggests the latter peptides have no role in the toxic mechanism, although at this stage we cannot rule out the possibility that these peptides function in some other way to influence toxicity.

In the case of the 130 kDa lepidopteran toxins, the major proteolytic event in the insect gut is the removal of almost 600 amino acids from the *C*-terminus, which is a prerequisite for conversion of the inactive protoxin into the toxic form (Aronson *et al.*, 1986). Other experiments (Nagamatsu *et al.*, 1984; Höfte *et al.*, 1986) showed that only 28 amino acids were removed from the *N*-terminus during the activation of the lepidopteran toxins. Studies on deletion mutants suggested that processing beyond this point would result in loss of activity *in vivo* (Schnepf & Whiteley, 1985; Höfte *et al.*, 1986). In contrast, we have observed that the toxicity of the coleopteran-specific var. *tenebrionis* protein is unchanged after extensive *N*-terminal processing. To determine if this processing is essential for toxicity, assay systems need to be developed by which the activity of the 73 kDa polypeptide can be measured in the absence of proteinase activity.

Although the *B. thuringiensis* δ-endotoxins differ widely in insecticidal specificity, sequence comparisons reveal several regions of similarity which could therefore be important in the toxic mechanism. Interestingly, despite the extensive *N*-terminal processing of the var. *tenebrionis* δ-endotoxin, all these regions are retained in the smallest toxic fragment that we have obtained to date. On the basis of sequence similarities, Höfte *et al.* (1987) have suggested that the 73 kDa var. *tenebrionis* toxin could be considered as a naturally truncated version of the lepidopteran toxins. Recently Chungjatupornchia *et al.* (1988) observed a conserved region at the extreme *C*-terminus of a dipteran- and lepidopteran-specific δ-endotoxin active fragment and noted that this region also occurred in var. *tenebrionis* δ-endotoxin. As shown in more detail in Fig. 6, a block of ten amino acids at the extreme *C*-terminus of the var. *tenebrionis* toxin is found to be almost identical in five other 130 kDa toxins, including three specific for lepidoptera and two active against diptera. Deletion mutagenesis with several of these 130 kDa toxins has shown that this region is essential for toxicity (Schnepf & Whiteley, 1985; Höfte *et al.*, 1986) and, if this is true for the var. *tenebrionis* toxin, it would preclude any significant *C*-terminal processing in the insect gut.

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