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Facile preparation and characterization of the toxin from *Bacillus thuringiensis* var. *kurstaki*

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We report a simple three-step method of generating a homogeneous toxic fragment (toxin) in high yield from *B. thuringiensis* var. *kurstaki*. Purified crystals were digested with trypsin at pH 10.5, followed by $(NH_4)_2SO_4$ precipitation and dialysis. For the HD73 strain the preparation is toxic to eastern-spruce-budworm (*Choristoneura fumiferana*) larvae. It gives a single 66 kDa band on polyacrylamide-gel electrophoresis and a single band with an isoelectric point of 5.5 on an isoelectric-focusing gel. A single isoleucine *N*-terminus was detected, and the first 20 amino acids were found to be identical with those predicted from the gene nucleotide sequence. A single lysine *C*-terminus was detected, and the amino acid composition was in excellent agreement with tryptic cleavages at arginine-28 and lysine-623 of the protoxin. Raman spectroscopic analysis gave values of 20% α-helix, 35% β-sheet and 45% unordered structure. The resistance of the toxin to most proteinases and its susceptibility to proteolysis by papain and Pronases indicates a compact multidomain structure.

INTRODUCTION

The δ-endotoxin that is found as a crystalline inclusion body in *Bacillus thuringiensis* var. *kurstaki* has potent insecticidal activity toward lepidopteran larvae (Dulmage, 1981). The major component of the crystals is a protoxin protein of molecular mass approx. 130–140 kDa. Upon ingestion by insect larvae, the crystals are subjected to the alkaline pH and enzymes of the insect gut and as a result are cleaved with the release of a toxic peptide (Nickerson, 1980; Fast, 1981; Huber & Lüthy, 1981; Aronson *et al.*, 1986). Fragments of 40–70 kDa have been found to be resistant to proteolysis and are major candidates as the ultimate 'toxin' which binds to receptors in the midgut epithelium and disrupts homoeostatic ion regulation by colloid osmotic lysis (Haider & Ellar, 1987; Knowles & Ellar, 1987).

There is currently no established protocol for the preparation of a purified toxin. The few published methods (Chestukhina *et al.*, 1982; Nagamatsu *et al.*, 1984; Andrews *et al.*, 1985; Aronson & Arvidson, 1987) vary considerably and have used. *B. thuringiensis* strains which are known to express, or are suspected of expressing, more than one gene coding for the protoxin (Yamamoto *et al.*, 1988). Toxin is typically generated by solubilizing the crystals at high pH, usually in the presence of reducing agents, and treating with larval gut-juice enzymes or exogenous proteinases, among which trypsin has been widely used. After proteolysis, several chromatographic steps, combined with other standard methods of purification, such as precipitation and dialysis, have been used. For the *B. thuringiensis* strains active against lepidopteran larvae, toxic fragments of different size have been reported, and there are also significant differences in the reports of carbohydrate content, isoelectric point and amino acid composition. From the reported studies, the only clear consensus which has emerged is that toxin is derived from the *N*-terminal portion of the protoxin.

In the present paper we show that stable pure toxin can be produced in high yield by a simple protocol for *kurstaki* strains. The HD73 strain contains a single gene coding for the protoxin (Kronstad & Whitely, 1986). The toxin derived from it has been characterized in detail and has been found to be potent against eastern-spruce-budworm (*Choristoneura fumiferana*) larvae. It provides a suitable starting point for biochemical, biophysical and physiological studies.

EXPERIMENTAL

Fermentation and crystal preparation

B. thuringiensis was cultured in half-strength tryptic soy broth medium at 29 °C for 44 h, and the crystals were purified as described previously (Carey *et al.*, 1986).

Toxin preparation

A crystal suspension containing 100 mg of protein was centrifuged, and the pellet was suspended in 5 ml of 0.1 M-Caps buffer (Sigma), pH 10.5, containing bovine pancreatic tosylphenylalanylchloromethane ('TPCK')-treated trypsin (1 mg/ml; Sigma). After having been stirred overnight at 20 °C, the suspension was centrifuged at 10000 *g* for 30 min. $(NH_4)_2SO_4$ (40%, w/v) was added to the supernatant and stirred at 4 °C for 15 min and then centrifuged. The precipitated protein was suspended in distilled water and thoroughly dialysed against distilled water at 4 °C using 50 kDa-cut-off tubing (Spectrum, Los Angeles, CA, USA). The purified toxin was collected by centrifugation.

Abbreviations used: Caps, 3-(cyclohexyl)propane-1-sulphonic acid; PAGE, polyacrylamide-gel electrophoresis; PFD_{50}, dose resulting in 50% pupal failure; LD_{50}, dose resulting in 50% lethality; μ_{Ser}, mobility relative to serine.

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Protein quantification

The crystal protein or toxin preparation was dissolved in KOH solution, pH 13. Estimates of protein concentration were made from u.v. absorbance at 280 nm, assuming that tryptophan and tyrosine have absorption coefficients of 5700 and 1300 $M^{-1} \cdot cm^{-1}$ respectively (Cantor & Schimmel, 1980). Absorption coefficients of 1.37 ml/mg for the protoxin and 1.45 ml/mg for the purified toxin were calculated. In the case of the purified toxin an absorption coefficient of 1.61 ml/mg at 280 nm was determined by quantitative amino acid analysis.

PAGE

Gels were run on a Pharmacia Phast electrophoresis system with preformed gels and other materials supplied by Pharmacia. Samples were dissolved in sample buffer (8 M-urea/2.5% SDS/5% 2-mercaptoethanol/10 mM-Tris-HCl, pH 8.3), placed in a boiling-water bath for 5 min, and then applied to 10–15% gradient gels. Isoelectrofocusing was carried out by directly applying the native samples to gels with pH ranges from 3 to 9 and from 4 to 6.5. Isoelectrofocusing in urea was carried out by soaking gels in 8 M-urea with appropriate ampholytes for 30 min and then applying the same in 8 M-urea.

Amino acid analysis

Hydrolyses were carried out in 6 M-HCl containing 0.1 M-phenol at 110 °C *in vacuo* for 16, 24 and 48 h. Tryptophan was determined by hydrolysis in 4 M-methanesulphonic acid (Simpson *et al.*, 1976) at 110 °C for 24 h. Serine and threonine were determined by extrapolation to zero time. Leucine, isoleucine, methionine and valine were determined from the 48 h hydrolysate. All other amino acids were determined as the average of the 24 h and 48 h hydrolysates. A Technicon TSM amino acid analyser with a ninhydrin detection system was used for quantification.

N-Terminal identification

(i) Dansylation. A sample of toxin (0.5 mg) was allowed to react with dansyl chloride (Sigma) as described by Gray (1967). The dansyl derivative was identified by high-voltage paper electrophoresis at pH 4.5 and 2.1.

(ii) Isolation of *N*-terminal peptide. The isolation procedure is based on the fact that, after acetylation of the toxin and enzymic digestion, only peptides derived from the *N*-terminus can be neutral at pH 2.1 and therefore readily isolated. Toxin (11 mg) was acetylated with [^{14}C]acetic anhydride (Kaplan *et al.*, 1982) and digested with pepsin. The neutral peptide from pH 2.1 electrophoresis was further purified by paper electrophoresis at pH 3.5 and located by autoradiography.

N-Terminal sequence determination

Toxin from SDS/polyacrylamide gels was electroblotted on to polyvinylidene difluoride membranes (Matsudaira, 1987) and the first 20 *N*-terminal amino acids were sequenced by using a model 470A Applied Biosystems gas-phase sequencer as described by Watson *et al.* (1988).

C-Terminal identification

The procedure described below is based on the rationale that, after [^{14}C]acetylation, the *C*-terminal peptide will be labelled if it contains a lysine residue. The carboxy groups of a sample (9.5 mg) of toxin were coupled with ethanolamine (Means & Feeney, 1971), dialysed and freeze-dried. The coupled protein was allowed to react with [^{14}C]acetic anhydride and digested with pepsin. Radioactive peptides derived from the *C*-terminus were identified using the diagonal electrophoretic procedure described by Duggleby & Kaplan (1975) and autoradiography.

Carbohydrate analysis

Carbohydrate content was determined by the phenol/H_2SO_4 method (Dubois *et al.*, 1956).

Proteolysis of purified toxin

The resistance of toxin to proteolytic cleavage was determined by incubating equimolar amounts of toxin and proteinase in 0.1 M-Caps buffer, pH 10.5, at room temperature for 1 h, followed by gel electrophoresis. Proteinases (Sigma) tested were: α-chymotrypsin type II, elastase type III, thermolysin type X, Pronase type XIV and papain type III.

Molecular-mass determination

The molecular mass of the purified toxin was estimated in two ways: (i) mobility on SDS/PAGE using a standard curve of log(molecular mass) versus relative mobility determined with standard molecular-mass markers; (ii) elution position on gel-permeation chromatography, in 0.1 M-Caps, pH 10.5, employing Sephadex G-200. The elution positions of the standard molecular-mass markers, phosphorylase (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa) and carbonic anhydrase (30 kDa), were determined in 0.1 M-phosphate, pH 7.

Raman spectroscopy

Raman spectral measurements on aqueous and deuterated pellets of purified toxin were performed as previously described (Carey *et al.*, 1986).

Toxicity assays

Toxicity assays were carried out on day-1 sixth-instar larvae of the eastern spruce budworm (*Choristoneura fumiferana*). The larvae were force-fed with toxin sample dissolved in 0.2 M-Caps buffer, pH 10.5. Dose volumes were 4 or 6 μl. After the treatment the larvae were held at 25 °C, 60% relative humidity and 16 h photoperiod. Mortality (LD_{50}) and pupal failure (PFD_{50}) were scored after 8 days.

RESULTS AND DISCUSSION

Fig. 1 demonstrates that an electrophoretically homogeneous protein can be obtained from purified crystals by digestion with trypsin followed by $(NH_4)_2SO_4$ precipitation and dialysis. Under the conditions employed the 130 kDa protoxin is completely digested by trypsin and the resultant toxin produced is solubilized along with some lower-molecular-mass peptides, leaving behind an insoluble core. The solubilized toxin gives a 66 kDa band on SDS/polyacrylamide gels, in both the presence and absence of 2-mercaptoethanol, and 67 kDa on gel filtration. For the soluble material the use of 50 kDa-cut-off dialysis tubing and $(NH_4)_2SO_4$ precipitation removes contaminating peptides and eliminates the need for time-consuming chromatographic procedures. We should obtain close to 50% of the starting protein as toxin, and we

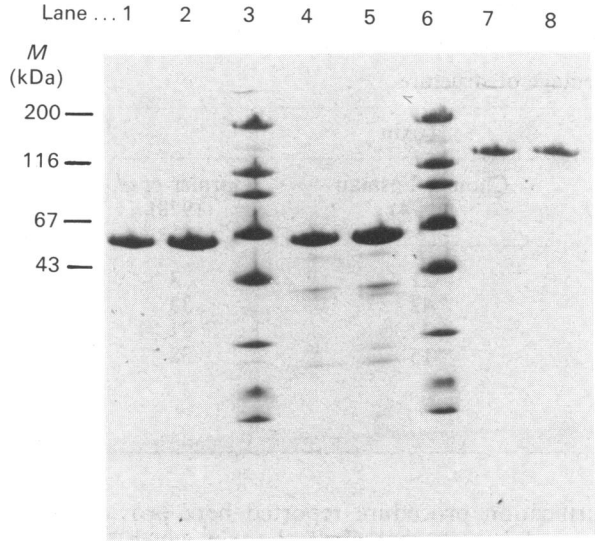

Fig. 1. SDS/PAGE of crystal protein (lanes 7 and 8), supernatant after digestion of crystal protein with trypsin (lanes 4 and 5), purified toxin (lanes 1 and 2) and molecular-mass markers (lanes 3 and 6)

The molecular-mass (*M*) markers were: myosin (200 kDa), β-galactosidase (166 kDa), phosphorylase (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and α-lactalbumin (14 kDa).

obtain 60–70 % of the theoretical maximum. The preparation is toxic to spruce-budworm larvae with an LD_{50} of 1.7 μg/larva and a PFD_{50} of 0.021 μg/larva.

The amino acid composition of the toxin (Table 1) is in excellent agreement with that predicted by the cloned gene nucleotide sequence (Adang *et al.*, 1985) for the 595 residues from positions 29 to 623 of the protoxin, and no carbohydrate was detected. A molecular mass of 66.7 kDa is calculated, which is in excellent agreement with the value obtained experimentally. As predicted by the gene sequence, the notable features of this composition are the low lysine content and lack of cysteine, both of which are relatively abundant in the protoxin. Amino acid analysis of the trypsin-insoluble material showed a high lysine and cysteine content and is therefore composed primarily of the *C*-terminal portion of the protoxin. In the procedure we report here, advantage is taken of the fact that the toxin is soluble at pH 10.5, but the extensive disulphide network of the crystal protein remains intact, rendering the *C*-terminal portion of the protoxin insoluble.

The toxin is soluble at alkaline pH values greater than 9.5 ($\sim$8 mg/ml at pH 10.5) and at acidic values below 3.5 ($\sim$5 mg/ml at pH 3). In the pH range from 5 to 8.5 its solubility is less than 0.5 mg/ml. There is strong evidence that the solubilized protoxin exists as a dimer (Huber & Lüthy, 1981). On gel filtration at pH 10.5 the purified toxin is eluted with an apparent molecular mass of 67 kDa, showing that the solubilized toxin, unlike its precursor protoxin, exists as a monomer.

Further characterization of the toxin and determination of its homogeneity was carried out by *N*-terminal analysis. Only a single fluorescent spot corresponding to isoleucine was detected after the dansylation procedure.

Table 1. Amino acid analysis of toxin

Amino acid	Predicted no. of residues (Ile-29–Lys-623)	Observed no. of residues*
Ala	37	38.6
Arg	43	44.5
Asp + Asn	67	67.2
Cys†	0	0
Glu + Gln	54	57.8
Gly	43	43.2
His	9	9.4
Ile	45	37.5
Leu	48	50.9
Lys	3	2.7
Met	7	5.9
Phe	36	33.4
Pro	30	28.4
Ser	60	61.3
Thr	36	37.3
Trp	10	9.7
Tyr	27	27.9
Val	39	39.3

* Calculated as mol/595 mol; average of two determinations, with the estimated error less than 7 % for all amino acids.

† Determined as cysteic acid after performic acid oxidation.

Also, a single radioactive *N*-terminal peptide from a pepsin digest of the [^{14}C]acetylated toxin was found which had the composition $Thr_1Glu_1Gly_1Ile_1$, corresponding to the sequence IETG following arginine-28. The sequence IETGYTPIDISLSLTQFLLS was obtained with a gas-phase sequencer and corresponds exactly to that predicted by the gene nucleotide sequence. Chestukhina *et al.* (1981) concluded that there was no proteolytic cleavage at the *N*-terminus of the protoxin; our results, however, show that production of toxin involves a proteolytic cleavage at arginine-28. Nagamatsu *et al.* (1984) determined the same cleavage point for a tryptic fragment from the subspecies *dendrolimus*, and Aronson & Arvidson (1987) detected an isoleucine *N*-terminus in the case of the HD263 strain.

The *C*-terminal cleavage site has not been determined for any reported toxic fragment (Aronson *et al.*, 1986). Our analysis (Table 1) indicates three lysine residues per molecule of toxin, which makes the lysine at position 623 a likely cleavage site. A single radiolabelled *C*-terminal peptide is obtained from a peptic digest of [^{14}C]acetylated toxin by the procedure of Duggleby & Kaplan (1975), and therefore lysine is the likely *C*-terminus. The mobility of the peptide (μ_{Ser} = 1.3 at pH 2.1) is that of a dipeptide with a charge of +1. Amino acid analysis gives Glu_1Lys_1ethanolamine$_1$. This result is consistent with the predicted sequence (Adang *et al.*, 1985) Gln-Lys at positions 622 and 623.

Isoelectric focusing gave a single band with an isoelectric point of 5.5 in native gels and 6.0 in urea gels. The presence of only a single band is further evidence of the high purity of our toxin preparation and that the HD73 strain produces a single gene product.

Digestion of the solubilized protoxin with a variety of mammalian, bacterial and larval proteinases has been reported to yield a 60–70 kDa toxic fragment resistant to further proteolysis (Chestukhina *et al.*, 1982; Yamamoto

Table 2. Secondary structure of toxin and protoxin

Secondary structure	Method or reference...	Percentage of structure: Protoxin* Raman spectroscopy	Toxin: Raman spectroscopy	Toxin: Chou & Fasman (1974)	Toxin: Garnier *et al.* (1978)
α-Helix		25	20	21	3
β-Sheet		25	35	42	33
Unordered		50	45	22	21
α or β		—	—	15	38

*Carey *et al.* (1986).

& Iizuki, 1983). We found the purified toxin to be resistant to further proteolysis by chymotrypsin, elastase and thermolysin at pH 10.5. However, the toxin is readily proteolysed to smaller fragments by papain and Pronase. The stability of the toxin to most proteinases shows that it has a tightly folded tertiary structure, but its susceptibility to papain and Pronase indicates that it is composed of several strongly associated domains. Controlled-proteolysis experiments and the isolation and sequencing of fragments need to be carried out, with a view to determining the number and location of the interdomain regions.

The secondary structure of HD73 was estimated by Raman spectroscopy from the polypeptide modes, amide I (1664 cm^{-1}) and amide III (1248 cm^{-1}), whose intensity and position reflect the polypeptide backbone conformation (Carey, 1982). The method of Lippert *et al.* (1976) was used, requiring as input data the spectral intensities at 1240 cm^{-1} for the protein molecule in ^1H_2O and at 1632 cm^{-1} and 1660 cm^{-1} for the protein in ^2H_2O. The results for HD73 toxin as an aqueous pellet gave 20% α-helix, 35% β-sheet and 45% unordered structure. Given the inherent errors of at least ±5% associated with the calculation of the Raman data, these values are not significantly different from that previously reported for the HD73 protoxin (Table 2). This indicates that the overall secondary structure of the toxin is similar to that of the protoxin precursor. The secondary structure was also estimated by the methods of Chou & Fasman (1974) and Garnier *et al.* (1978) for the sequence from position 29 to position 623 of the protoxin. Taken together, the secondary-structure estimations from Raman-spectroscopic and predictive methods concur that the toxin has of the order of 10–20% α-helix, 35–40% β-sheet and large amounts of unordered structure.

It should be emphasized that the use of purified crystals which are free of spores and contaminating endogenous proteinases is critical to the facile production of a homogeneous toxin in high yield. By using the same simplified procedure, a 66 kDa toxic fragment which gives a single band on SDS/polyacrylamide gels, can be produced for the *kurstaki* strains HD1 and NRD12. We have not as yet characterized them to the same extent as the HD73 toxin; however, both these strains have more than one gene coding for the protoxin, and it is possible that each of these bands is a mixture of two or more fragments of similar sequence. For such strains, the purification procedure reported here provides a rapid means for preparing purified toxin which can then be subjected to highly resolving h.p.l.c. methods to separate the similar toxins, if present (Yamamoto *et al.*, 1988). The most straightforward procedure, however, is to use strains which are known to produce a single gene product.

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