Microbiology (1994), 140, 1869–1880 Printed in Great Britain

Biochemical characterization of *Bacillus thuringiensis* cytolytic δ-endotoxins

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The entomocidal δ-endotoxins CytA and CytB produced by *Bacillus thuringiensis* (*Bt*) subspecies *israelensis* and *kyushuensis* respectively showed a similar level of toxicity to mosquito larvae but were not toxic to the larvae of the lepidopteran *Manduca sexta*. CytA and CytB are also similar in sequence, predicted secondary structure and α-helical content, the only obvious difference being a C-terminal fifteen residue 'tail' on CytB. Investigations of the function, if any, of the CytB C-terminal 'tail' showed that this δ-endotoxin is highly expressed and forms inclusions in an acrystalliferous *Bt* mutant without the aid of the 20 kDa 'helper' protein from *Bt* subspecies *israelensis* which is essential for CytA inclusion formation. After proteinase K treatment, CytA and CytB were processed to virtually the same points in a sequence alignment and were equally haemolytic *in vitro*. However, the results suggested that unprocessed CytB differs from unprocessed CytA in that the former is not haemolytic.

Keywords: *Bacillus thuringiensis*, δ-endotoxins, CytA, CytB

INTRODUCTION

Bacillus thuringiensis (*Bt*) is a Gram-positive bacterium which produces an insecticidal cytoplasmic protein inclusion during sporulation (Lüthy *et al.*, 1982). Depending upon the subspecies, inclusions may be composed of one or more δ-endotoxins which are variously toxic to the larvae of Lepidoptera, Diptera, Coleoptera or some combination thereof (Höfte & Whiteley, 1989). The inclusions are ingested by susceptible larvae, solubilized by the high pH of the larval midgut (in the case of Lepidoptera and Diptera), and the protoxin is activated by proteases (Lüthy *et al.*, 1982). The activated δ-endotoxin then interacts with midgut epithelial cells via insect-specific receptors, creating membrane pores that cause cell swelling and lysis (Knowles & Ellar, 1987).

Whilst most δ-endotoxins are members of a family of sequence-related Cry δ-endotoxins (Höfte & Whiteley, 1989), at least three are not. These are two CytA δ-endotoxins, from *Bt* subsp. *israelensis* (Ward & Ellar, 1984; Waalwijk *et al.*, 1985) and subsp. *morrisoni* PG14 (Earp & Ellar, 1987), and CytB from subsp. *kyushuensis* (Koni & Ellar, 1993). Unlike Cry δ-endotoxins, Cyt δ-endotoxins display broad cytolytic activity *in vitro* (Thomas & Ellar, 1983a; Chilcott & Ellar, 1988; Knowles *et al.*, 1992) as well as being specific to dipteran larvae *in vivo* (Armstrong *et al.*, 1985; Knowles *et al.*, 1990b). There is limited evidence that, besides being toxic to dipteran larvae, CytB causes feeding inhibition of the larvae of two lepidopteran species, *Manduca sexta* (Held *et al.*, 1990) and *Pieris brassicae* (Knowles *et al.*, 1992). This work was not conclusive however as these workers used either whole subsp. *kyushuensis* inclusions (Held *et al.*, 1990), of which CytB comprises only about 30%, or CytB partially purified from subsp. *kyushuensis* inclusions (Knowles *et al.*, 1992) of which CytB comprises only approximately 60%.

The broad cytolytic activity of CytA *in vitro* has been attributed to its high hydrophobicity and its ability to bind to certain ubiquitous membrane lipids (Thomas & Ellar, 1983b). Site-directed mutagenesis (Ward *et al.*, 1988) suggested that although such receptor-independent membrane insertion can occur under certain conditions (Knowles *et al.*, 1990a), an insect-specific receptor may be essential for toxicity *in vivo*. Support for the existence of a receptor comes from the demonstration of specific binding to mosquito midgut epithelial cell apical brush border membranes (Ravoahangimalala *et al.*, 1993).

The X-ray crystallographic structure of the CryIIIA δ-endotoxin revealed that the sequence blocks conserved

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The EMBL accession number for the amino acid sequence reported in this paper is Z14147.

among Cry δ-endotoxins are distributed between three distinct structural domains and suggested a functional role for each domain (Li *et al.*, 1991). Although the X-ray crystallographic structure of a Cyt δ-endotoxin has not yet been determined, the recent cloning and sequencing of a second Cyt δ-endotoxin gene (Koni & Ellar, 1993) enabled a predicted secondary structure model for Cyt δ-endotoxins (Ward *et al.*, 1988) to be further refined (Koni & Ellar, 1993).

Further information on the structure of Cyt δ-endotoxins has been obtained in this work by a combination of circular dichroism studies and comparison of proteolytic cleavage sites in CytA and CytB. Expression of *cytB* in *Bt* has enabled a comprehensive comparison of the properties and toxicity of the products of both cloned *cytB* and cloned *cytA* to be carried out. *In vivo* assays of Cyt δ-endotoxins against dipteran and lepidopteran larvae were used to investigate the possibility that the broad *in vitro* cytolytic activity of these δ-endotoxins was paralleled *in vivo*.

METHODS

Bacterial strains, plasmids, media and culture conditions. *Bt* strain IPS78/11 (Crickmore & Ellar, 1992) was grown to late exponential growth phase by overnight incubation in Luria–Bertani (LB) broth or agar plates (Sambrook *et al.*, 1989) at 30 °C. To obtain synchronous sporulation, IPS78/11 was grown in casein/casein/yeast extract (CCY) broth (Stewart *et al.*, 1981) for 36–48 h using a 0·1% inoculum of cell culture grown to exponential growth phase in LB broth using a heat-shocked spore suspension (Ellar & Posgate, 1974). *Escherichia coli* TG2 (Sambrook *et al.*, 1989) was grown on LB broth or agar plates (Sambrook *et al.*, 1989) at 37 °C overnight. For *lacZα* promoter induction, 1 mM IPTG was incorporated.

The *Bt*/*E. coli* shuttle vector used for subcloning was pSV2 (Crickmore & Ellar, 1992). Other plasmids used were pCYTBXba1 and pCYTB (both containing the CytB protein gene; Koni & Ellar, 1993) and cam2027 (possessing both *cytA* and the 20 kDa 'helper' protein gene; Crickmore *et al.*, 1990). Plasmids were selected for in cultures with either ampicillin (100 μg ml^{-1}) or chloramphenicol (5 μg ml^{-1}) as appropriate.

DNA manipulations. Plasmid DNA was obtained from cells by standard alkali lysis techniques (Sambrook *et al.*, 1989), except that *Bt* cells were treated with lysozyme (10 mg ml^{-1}) at 37 °C for 30 min prior to alkali lysis. DNA modification and restriction enzymes were obtained from New England Biolabs and Gibco BRL. Restriction digests and DNA ligations were performed using Gibco BRL buffers. DNA was routinely electrophoresed on horizontal 0·5 × TBE [45 mM Tris, 45 mM boric acid, 1 mM EDTA (pH 8·0)] agarose gels and visualized with ethidium bromide (Sambrook *et al.*, 1989). If necessary, DNA fragments were purified from 1 × TAE [40 mM Tris, 1·14% (v/v) glacial acetic acid, 1 mM EDTA (pH 8·0)] agarose gels by the Geneclean II technique (BIO101) or by electroelution (Sambrook *et al.*, 1989). *E. coli* TG2 was transformed by electroporation (Dower *et al.*, 1988) as described previously (Koni & Ellar, 1993). IPS78/11 was also transformed by electroporation (Bone & Ellar, 1989) using settings of 1·8 kV, 600 Ω, 25 μF and a gap length of 0·4 cm.

Expression plasmids for *cytB* in IPS78/11. pBTCYTB1 was made by ligating *cytB* on a *SacI*/*SalI* fragment from pCYTB (Koni & Ellar, 1993) into the *SstI* and *SalI* sites of pSV2. pBTCYTB2 was produced by blunt-end ligation of *cytB* into the *SmaI* site of pSV2 on a Klenow end-filled *DdeI* fragment from pCYTBXba1 (Koni & Ellar, 1993). Both constructs were made by using *E. coli* TG2 as host and were then electroporated into IPS78/11. Prior to this, *E. coli* colonies transformed with pBTCYTB2 were screened by restriction mapping to select clones which, like pBTCYTB1, had *cytB* in the same orientation as the vector chloramphenicol resistance gene.

Electron microscopy. Cell culture samples were fixed in 2% (v/v) glutaraldehyde/growth media mix (Agar Scientific) for 2 h before being washed three times for 5 min each in 50 mM sodium cacodylate, pH 7·3 (Hopwood & Milne, 1991). The sample was then fixed with 2% (w/v) osmium tetroxide (Agar Scientific) for 1 h and washed as above. Fixed samples were then dehydrated, first with 50% (v/v) ethanol and finally 100% ethanol, and embedded in Spurr's epoxy resin (Fisons Polaron). Thin sections were cut on a Sorvall Porter-Blum ultramicrotome MT-2 and dried down onto copper 200 square mesh support grids (Gilder Grids). Sections were stained for 30 min with 2% (w/v) uranyl acetate, for 2 min with Reynolds' lead citrate (Smith & Croft, 1991) and then dried before viewing on a Philips EM300 transmission electron microscope at an accelerating voltage of 80 kV.

Isolation and manipulation of protein inclusions. CytB inclusions were released from *E. coli* cells by sonication. Inclusions were purified from lysed *Bt* and *E. coli* cell cultures by discontinuous sucrose density gradient centrifugation. To prevent aggregation of inclusions with spores prior to and during sucrose gradient centrifugation, inclusion/spore suspensions were not concentrated more than 100-fold relative to the culture volume. Also, inclusion/spore suspensions and sucrose solutions were made in 0·1 M Tris (pH 7·5)/0·1 M KCl. Inclusions were stored in water at −20 °C at concentrations of 1–10 mg ml^{-1} until required. Inclusions were solubilized at 37 °C by incubation in either 50 mM Na_2CO_3, pH 10·0 or 10·5, or in 50 mM NH_4HCO_3, pH 10·5, with or without 10 mM DTT. Unsolubilized material was removed by centrifugation at 12000 ***g*** in a microfuge for 15 min. For proteolytic processing, solubilized material was treated with 0·1–50% (w/w) proteinase K at 37 °C for various times. For SDS-PAGE, soluble material was precipitated and the protease inactivated by adding 0·5 vols of 60% (v/v) TCA and holding at −20 °C for 2 h. Precipitated material was prepared for subsequent gel electrophoresis by centrifugation at 12000 ***g*** in a microfuge for 30 min and washing once with acetone before vacuum desiccation. Citrate-precipitation of proteins prior to mosquito larvae (filter feeding) bioassays was carried out by a modification of the method of Chilcott & Ellar (1988). Citric acid was added to lower the pH to approximately 4·5 and the sample then stored at −20 °C for at least 2 h until required. Typically, 0·1 vols of 11% (w/v) citric acid were added to δ-endotoxin solubilized in 50 mM Na_2CO_3, pH 10·5, with or without 10 mM DTT. The material was then either used directly in mosquito larvae bioassays without further manipulation or repurified by centrifugation at 12000 ***g*** in a microfuge for 15 min and washed once with distilled water. The precipitate was then resuspended in distilled water and protein mass determined prior to use.

Protein mass determination. Inclusion/soluble protein mass refers to protein as estimated by the Lowry method using BSA fraction V (Sigma) as a standard. Where stated, inclusion protein mass was estimated by UV light absorbance at 280 nm by consideration of the number of moles of tyrosine and tryptophan per mole of protein and their extinction coefficients (Cantor & Schimmel, 1980). To this end, protein inclusions were denatured using 8 M urea/6 M guanidine.HCl/50 mM

Tris (pH 8)/1 mM EDTA and the absorbance at 280 nm measured either immediately after the instantaneous solubilization of the inclusions, or after incubation at 37 °C for 1 h. An absorbance of 1·0 is equivalent to 48·8 μM (1·3342 mg ml^{-1}) CytA and 34·7 μM (1·015 mg ml^{-1}) CytB.

Protein mass and composition were also determined by amino acid analysis of approximately 2 nmol (about 50 μg according to Lowry protein determination) of inclusions solubilized in NH_4HCO_3 (pH 10·5)/10 mM DTT. Protein samples were lyophilized, hydrolysed in an argon atmosphere by the vapour from 6 M HCl containing 1% (v/v) 2-mercapoacetic acid at 110 °C for 24 h, relyophilized and then analysed using a Pharmacia LKB Alpha Plus II sodium ion exchange system.

SDS-PAGE & immunoblot analysis. SDS-PAGE was carried out using the discontinuous buffer system (Laemmli & Favre, 1973) as modified by Thomas & Ellar (1983a). Before loading, an equal volume of loading buffer [50 mM Tris (pH 7·5)/0·5 mM EDTA/0·5% SDS/5% (v/v) glycerol/0·05% bromophenol blue/25 mM DTT] was added to each sample and then boiled for 5 min. Protein size markers used were SDS-7 with added β-galactosidase (both from Sigma). Proteins were visualized by staining in 0·1% Coomassie blue/10% (v/v) methanol/10% glacial acetic acid and then destaining in 10% methanol/10% acetic acid. Alternatively, proteins were transferred to nitrocellulose (Schleicher and Schuell) using an LKB Transblot semi-dry blotter, as described by the manufacturer. The success of the blotting was judged by staining the protein bands on the filter with 1% (w/v) Ponceau S/5% glacial acetic acid and then destaining with several washes of water. The membrane was then treated with 10 mM Tris (pH 7·4)/15 mM NaCl/3% (w/v) BSA at room temperature with gentle shaking for 1 h before anti-CytB antiserum (Koni & Ellar, 1993) was added at a 1:2000 dilution and left for a further 2 h. The membrane was then washed five times with 10 mM Tris (pH 7·4)/15 mM NaCl before being incubated in 10 mM Tris (pH 7·4)/15 mM NaCl/3% BSA and secondary antiserum (horseradish-peroxidase-conjugated anti-rabbit IgG; Sigma) with gentle shaking for 1 h. Secondary antiserum specifically bound to the filter was visualized by oxidation of 4-chloro-1-naphthol with hydrogen peroxide after washing as above.

N-terminal protein sequencing. Proteins were subjected to SDS-PAGE in the presence of 2 mM mercaptoacetic acid and then transferred onto Problot membrane (Applied Biosystems) using an LKB Transblot semi-dry blotter as described by the manufacturer. Proteins were visualized by Coomassie blue staining as described earlier and destaining with 50% methanol. After washing the membrane with water and then drying at room temperature, protein bands were excised and subjected to N-terminal sequencing using an ABI 477A Applied Biosystems automated pulse-liquid sequencer.

Mass spectrometry. Electrospray mass spectrometry was performed using a BIO-Q quadrupole analyser (Loo *et al.*, 1989) by VG Instruments connected to an Intel computer running on Lab-Base software. The mobile phase was pumped to the mass spectrometer using an Applied Biosystems 130A syringe pump, with a flow rate of 4 μl min^{-1} maintained while samples were analysed. Gramicidin-S was used as an internal standard at 2 mg l^{-1}. The mass range scanned was calibrated using 20 μM myoglobin (horse heart). After loading of the injection loop, samples were sprayed into the mass spectrometer source using a 34 gauge stainless steel capillary needle with a source temperature of 50 °C. For positive ion analysis, the mobile phase and the myoglobin calibration solvent was 50% methanol/1% acetic acid, the needle voltage was 4 kV and samples were made to 50% methanol before analysis. For negative ion analysis, the mobile phase and the myoglobin calibration solvent was 50% acetonitrile, the needle voltage was 3 kV and samples were made to 50% (v/v) acetonitrile.

Circular dichroism spectroscopy. Circular dichroism was performed on a Jobin-Yvon CD 6 machine connected to an IBM PC Personal System/2, running CD6 and PROTEIN CONTIN-fit (Provencher & Glockner, 1981; Provencher, 1982). Samples assayed were 1–2 mg δ-endotoxin ml^{-1} solubilized in 50 mM NH_4CO_3 (pH 10·5) with or without 10 mM DTT, from which an aliquot was set aside to accurately determine protein content by amino acid analysis. For processing of δ-endotoxins, incubation was at 37 °C for 16 h with 0·1% proteinase K. About 35 μl of sample was loaded into a 0·1 mm gap cuvette and scanned seven times between 195 nm and 250 nm with an interval of 0·5 nm and 3 s for each reading. The average spectrum was then smoothed by averaging using a window of 5 points (2·5 nm). After subtraction of buffer/protease ellipticity, the δ-endotoxin ellipticity was converted to molecular ellipticity for the purpose of CONTIN-fit α-helical content determination by consideration of the molar concentration of the sample in terms of residues.

-SH group determination. -SH group content was determined by the method of Ellman (1959) after solubilizing 0·5–1·0 mg of inclusions in 1 ml of denaturant and determining the protein concentration by UV light absorbance.

Bioassays. Mosquito larvae bioassays were performed using 1–5-d-old *Aedes aegypti* larvae (reared from eggs supplied by Shell Research), *Anopheles gambiae* and *Culex pipiens* larvae (both obtained from the London School of Hygiene and Tropical Medicine as eggs). Both rearing and bioassays were performed at 30 °C in a humidified incubator. Batches of 1000–4000 larvae, depending upon the success rate of egg hatching, were reared in plastic containers 25 × 25 × 15 cm deep with about 5 l of distilled water and Farley's Farex baby food as diet. Bioassays were performed in triplicate using 20 larvae in 1 cm diameter microtitre plate wells containing 0·9 ml of the water in which the larvae were reared and 0·1 ml of δ-endotoxin. Negative controls contained only water. If, however, the δ-endotoxin was citrate-precipitated and not repurified, negative controls contained a 90 μl aliquot of 50 mM Na_2CO_3 (pH 10·5) combined with a 10 μl aliquot of 11% citric acid. CytA was used in parallel with CytB both as a positive control and also for comparison. Mortality was recorded after 24 h.

M. sexta larvae bioassays were performed using 3-week-old larvae (first instar, 0·15–0·20 g in weight) reared on an artificial solid diet (J. Cayley, personal communication) from eggs supplied by Dr Stuart Reynolds (University of Bath). Both rearing and bioassays were performed at 25 °C in a humidified incubator. Larvae were fed involuntarily with 2 μl of δ-endotoxin and then allowed to feed on unlimited solid diet for several days, during which body weight was monitored daily. Alternatively, larvae were allowed to feed voluntarily on a 3 mm high, 1 cm diameter disc of solid diet onto which 20 μl of δ-endotoxin had been adsorbed. This disc was consumed within 1–2 d. The remains of this food were replaced with new, δ-endotoxin-treated food each day and body weight monitored for several days. In all *M. sexta* bioassays, CryIA(c) and water were used as positive and negative controls, respectively.

Haemolysis assays were in microtitre plate wells at 25 °C as described by Thomas & Ellar (1983a) except that a 5% (v/v) erythrocyte suspension was used. The erythrocytes used were prepared from New Zealand White rabbit blood, which was stored at 4 °C in Alsevers' solution (0·1 M trisodium citrate/40 mM glucose) until use. δ-Endotoxin was solubilized

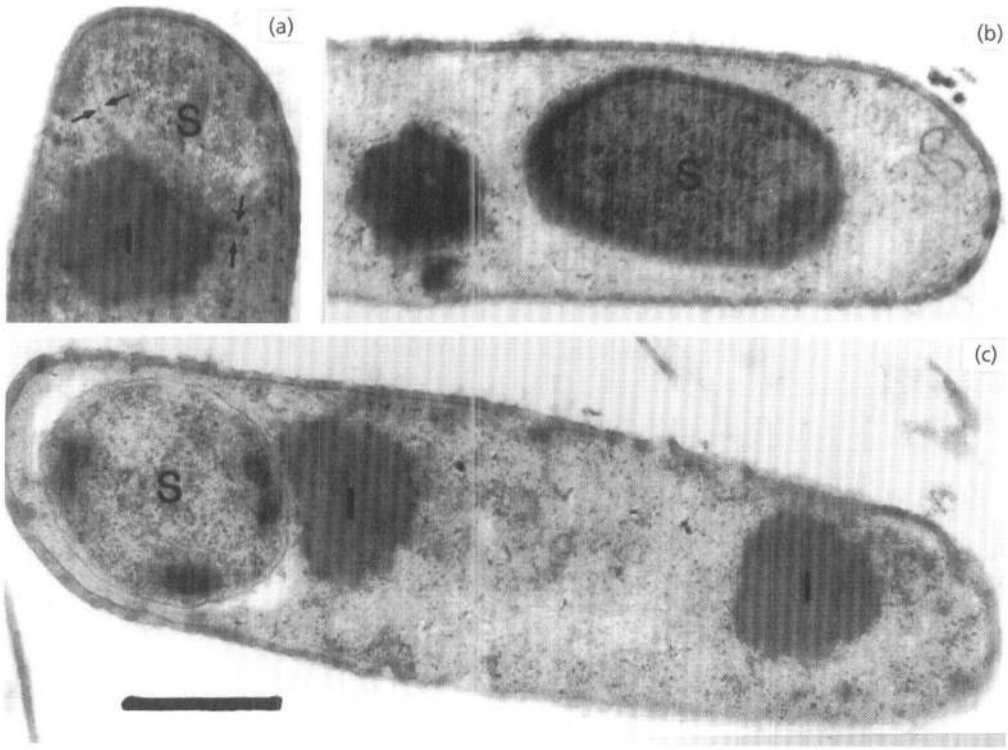

Fig. 1. Electron micrographs of CytB inclusions formed within IPS78/11 containing pBTCYTB1 during stages II (a), III (c) and IV (b) of sporulation (Bechtel & Bulla, 1976). Spores and inclusions are marked by 'S' and 'I', respectively. The arrows shown on (a) delineate the forespore membrane. The size bar on (c) applies to all three micrographs and represents 0·5 μm.

and proteolytically processed with 10% proteinase K in 50 mM Na_2CO_3, pH 10·5, with or without 10 mM DTT as described earlier, and was then diluted 10-fold in haemolysis buffer [50% (v/v) phosphate-buffered saline/2·25% (w/v) glucose/0·05% gelatin] prior to use. The end-point of haemolysis was judged after 3 h as the last dilution at which colouration of the liquid was still visible to the naked eye.

RESULTS

Expression in *Bt*

Cytoplasmic inclusions of CytA are formed in the acrystalliferous *Bt* subsp. *israelensis* (*Bti*) strain IPS78/11 (Crickmore & Ellar, 1992) when *cytA* is expressed together with the *Bti* 20 kDa 'helper' protein gene using the construct cam2027 (Crickmore *et al.*, 1990). The latter has the 'helper' protein gene with its own promoter, mapped by Adams *et al.* (1989).

To express *cytB* in IPS78/11, two plasmids were constructed (see Methods) containing one or both of the putative promoter sequences identified previously (Koni & Ellar, 1993) which are similar to sporulation-specific promoter sequences (Brown & Whiteley, 1988; 1990). The first (pBTCYTB1) possessed only the middle–late sporulation-specific putative promoter upstream of the *cytB* coding sequence whereas the second (pBTCYTB2) contained both the early–middle and middle–late sporulation-specific putative promoters. Both constructs resulted in high *cytB* expression and inclusion formation during sporulation of IPS78/11. Fig. 1 shows that inclusions were seen as early as stage II of sporulation (Bechtel & Bulla, 1976) even with pBTCYTB1. Immunoblot analysis of both vegetative cell cultures and the sedimentable fraction of lysed, sporulated cell cultures of IPS78/11 containing pBTCYTB1 and IPS78/11 containing pSV2 revealed that only sporulated cell cultures of IPS78/11 containing pBTCYTB1 produced sedimentable material of the size expected of CytB that cross-reacted with anti-CytB antiserum (Fig. 2, track 5). Both the purified *E. coli* and IPS78/11 inclusions (see next section) also showed a band approximately twice the size of CytB that cross-reacted with anti-CytB antiserum (Fig. 2, tracks 1 and 6), which is thought to be CytB dimer.

Inclusion purification

Both CytA and CytB inclusions from IPS78/11 and CytB inclusions from *E. coli* (Koni & Ellar, 1993) were recovered at the interface between sucrose concentrations of 1·9 M and 2·0 M. Small quantities of CytA and CytB inclusions from IPS78/11 were also present at the interface between sucrose concentrations of 2·0 M and 2·2 M but were not harvested because of extensive contamination with spores. Recovery of both CytA and CytB inclusions from IPS78/11 was 25–35 mg protein l^{-1} whilst recovery of CytB inclusions from *E. coli* was

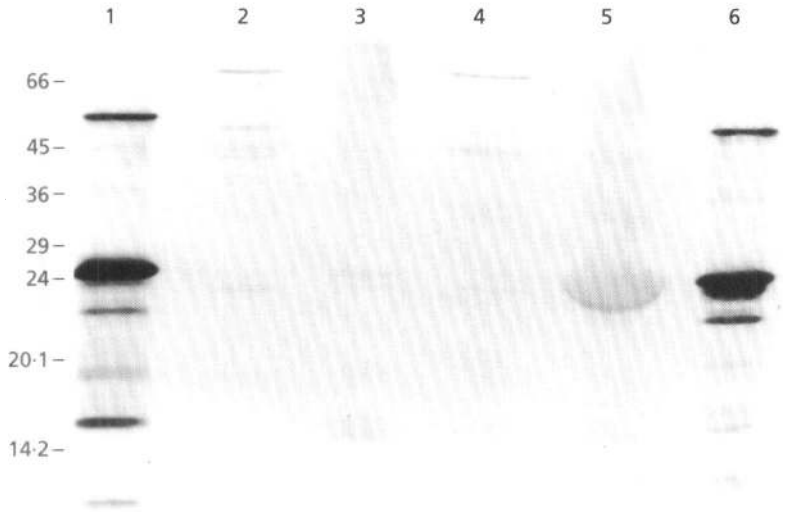

Fig. 2. Immunoblot analysis of vegetative cell cultures (tracks 2 and 4) and the sedimentable fraction of lysed, sporulated cell cultures (3 and 5) of IPS78/11 containing pSV2 (2 and 3) and IPS78/11 containing pBTCYTB1 (4 and 5). Cell cultures (0·5 ml) were sedimented by centrifugation at 12000 ***g*** in a microfuge for 15 min. Tracks 1 and 6 contain approximately 4 μg of *E. coli* and IPS78/11 CytB inclusions, respectively. The positions of protein size markers are shown alongside with their size given in kDa.

150 mg protein l^{-1}, of which CytB accounted for about 40% (i.e. 60 mg protein l^{-1}).

Inclusion solubilization

CytB inclusions from *E. coli* were found to contain a significant amount of *E. coli* protein besides CytB (Fig. 3, track 2). However, CytB could be solubilized at concentrations as high as 5 mg inclusions ml^{-1} with very little solubilization of the *E. coli* protein by incubation at 37 °C for 1 h in either 50 mM Na_2CO_3, pH 10·0 or 10·5 (Fig. 3, track 4), or in 50 mM NH_4HCO_3, pH 10·5. Proteolysis was not seen unless 10 mM DTT was present (Fig. 3, track 6).

CytA and CytB inclusions from IPS78/11 were solubilized at concentrations as high as 2 mg ml^{-1} by incubation at 37 °C for 1 h in 50 mM Na_2CO_3, pH 10·0 or 10·5, with 10 mM DTT (Fig. 4) or in 50 mM NH_4HCO_3 (pH 10·5)/10 mM DTT. Some proteolytic degradation of both CytA and CytB was observed due to the presence of contaminating *Bt* proteases associated with the inclusions. However, this endogenous proteolysis was largely overcome by solubilizing the inclusions for 30 min on ice instead of at 37 °C (Fig. 5).

N-terminal sequencing established the N-terminus of endogenously processed CytB to be residue 5 (see later, Fig. 7). There is a lysine residue at position 4, suggesting the presence of a trypsin-like protease. Although the C-terminus was not established, it would appear from the size of endogenously processed CytB (Fig. 5, track 11) that some C-terminal processing may also have occurred. Loss of the first four N-terminal residues from unprocessed CytB should result in a reduction in size of only 542 Da but endogenously processed CytB appears to be about 2 kDa smaller than unprocessed CytB (Fig. 5).

Unlike the CytB inclusions from both *E. coli* and IPS78/11, CytA inclusions were almost insoluble at pH 10·0 in the absence of DTT and only about 20% soluble at pH 10·5 without DTT after 1 h incubation at 37 °C (Fig. 4). *In vivo*, inclusions may be solubilized by a combined effect of the alkaline gut pH and gut proteases. Therefore, CytA inclusions were incubated at 1 mg ml^{-1} in 50 mM Na_2CO_3, pH 10·5, with 10% proteinase K. No significant solubilization was seen after 5 h at 37 °C.

Determination of molecular size from IPS78/11 inclusions

Like native CytB from *Bt* subsp. *kyushuensis* inclusions (Earp *et al.*, 1987), CytB from *E. coli* (Fig. 3) and IPS78/11 (Fig. 4b) appeared to be about 3 kDa smaller than CytA by SDS-PAGE (Fig. 4a). This was despite the fact that the deduced coding sequence of CytB (Koni & Ellar, 1993) revealed that CytB is actually 2 kDa larger than CytA (Ward & Ellar, 1984; Waalwijk *et al.*, 1985). In order to determine whether or not the size of CytA and CytB in inclusions corresponded to a full length sequence, electrospray mass spectrometry was employed.

IPS78/11 CytB inclusions were solubilized at 1 mg ml^{-1} in 50% methanol/10% acetic acid. Positive ion analysis revealed one major peak series whose molecular size was determined to be 58484 ± 12 Da (average from 16 peaks). This is precisely double the expected size of CytB, suggesting that CytB was present in the inclusions as a dimer. In fact, a small amount of CytB dimer could be seen even by SDS-PAGE (Fig. 4b) and immunoblot analysis (Fig. 2, tracks 1 and 6). The mass spectrometry was repeated with inclusions solubilized on ice for 30 min with 50 mM NH_4HCO_3 (pH 10·5)/10 mM DTT instead of acetic acid. Again, CytB was found to be a dimer. However, negative ion mass spectrometry of CytB inclusions solubilized in 30% (v/v) NH_4OH revealed CytB to be in a monomeric form at 29293 ± 16 Da (average from 14 peaks). When further analysed, a minute quantity of material slightly smaller than full length CytB was revealed.

Positive ion mass spectrometry of CytA did not prove possible. Negative ion analysis was possible after solubilizing inclusions in 30% NH_4OH/10 mM DTT for 30 min, revealing CytA to be mostly in a full length

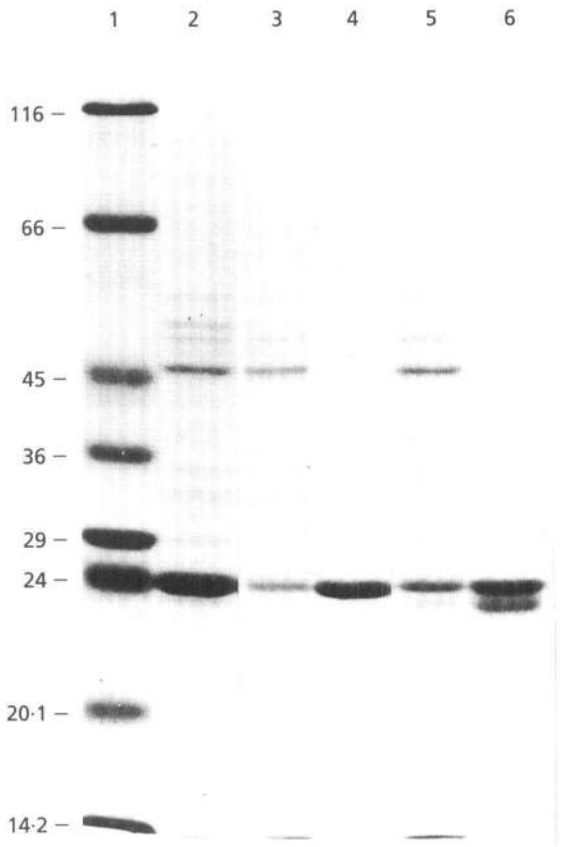

Fig. 3. Solubilization of *E. coli* CytB inclusions. Aliquots of inclusions (50 μg) were solubilized at 37 °C for 1 h at 5 mg ml^{-1} in 50 mM Na_2CO_3 (pH 10·5) with (tracks 5 and 6) or without (3 and 4) 10 mM DTT. Sedimentable (3 and 5) and solubilized (4 and 6) fractions were then separated as described in the text and subjected to SDS-PAGE. Protein size markers and 50 μg untreated inclusions are shown in tracks 1 and 2, respectively. The size of each marker is indicated alongside in kDa.

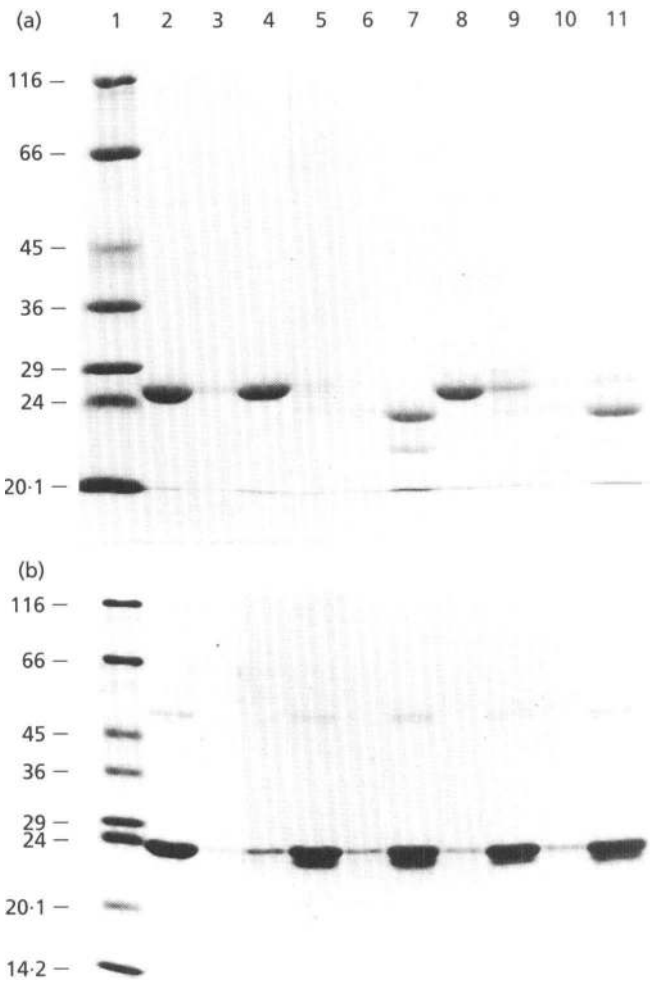

Fig. 4. Solubilization of CytA (a) and CytB (b) inclusions from IPS78/11. Aliquots of inclusions (20 μg) were solubilized at 37 °C for one h at 2 mg ml^{-1} in 50 mM Na_2CO_3, pH 10·0 (tracks 4–7) or pH 10·5 (8–11), with (6–7 and 10–11) or without (4–5 and 8–9) 10 mM DTT. Alternatively, inclusions were suspended in deionised water alone (2 and 3). Sedimentable fractions (even-numbered tracks) and solubilized fractions (odd-numbered tracks) were then separated as described in the text and subjected to SDS-PAGE. Track 1 contains protein size markers, the sizes shown alongside in kDa.

monomeric form of 27231 ± 19 Da (average from 12 peaks). As with CytB, a small quantity of material that was not full length CytA was also present.

-SH group content of IPS78/11 inclusions

-SH group determination was carried out for both CytA and CytB inclusions from IPS78/11 on three separate occasions. Using protein mass as determined by UV absorbance for each individual assay, the -SH group content of CytA and CytB was found to be 36·7 ± 2·3 % and 21·7 ± 4·3 % of the theoretical maximum, respectively. Even if the determination of protein content was made by amino acid analysis as opposed to UV absorbance, the average -SH group content of CytA was still less than 47 % whilst that of CytB was little changed. Control assays done with CryIIIA inclusions, which lack disulphide bonds (Carroll, 1990; MacIntosh *et al.*, 1990), revealed at least 92 % of -SH groups. These results suggested that both CytA and CytB exist in a largely disulphide-bonded state.

Proteolytic processing

Both CytA (Armstrong *et al.*, 1985) and CytB (Knowles *et al.*, 1992) are known to be protoxins. When solubilized at 1 mg ml^{-1} in 50 mM Na_2CO_3 (pH 10·5)/10 mM DTT on ice and then incubated at 37 °C in the presence of 0·1 % proteinase K, both CytA and CytB were completely processed to a single product in 1–3 h. This processing was also achieved by incubation with 10 % proteinase K for 5–10 min. However, upon prolonged (3 h) incubation in 10 % proteinase K the CytB product became unstable and was further processed to a second, smaller, stable product and eventually a third, even smaller product. Complete conversion to the smallest species was seen after

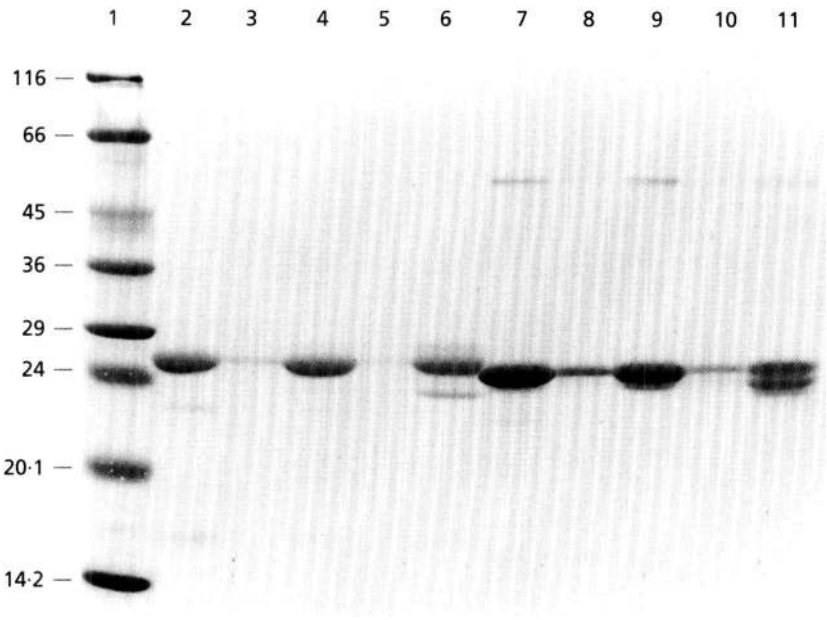

Fig. 5. Solubilization of CytA (tracks 2–6) and CytB (7–11) inclusions from IPS78/11 at 4 °C. Aliquots (20 μg) were solubilized at 4 °C (tracks 3–4 and 8–9) or 37 °C (5–6 and 10–11) for 30 min at 2 mg ml^{-1} in 50 mM Na_2CO_3 (pH 10·5)/10 mM DTT. Sedimentable (tracks 3, 5, 8 and 10) and solubilized (4, 6, 9 and 11) fractions were then separated as described in the text but at 4 °C, and subjected to SDS-PAGE. Protein size markers, untreated CytA inclusions and untreated CytB inclusions are shown in tracks 1, 2 and 7, respectively. The size of each marker is indicated alongside in kDa.

Table 1. Determination of Cyt δ-endotoxin proteolytic processing sites

δ-Endotoxin was used at a concentration of 1 mg ml^{-1}. The three δ-endotoxin sizes shown are the size estimated to the nearest 100 Da by SDS-PAGE; the size determined by mass spectrometry to the nearest Da; and the hypothetical size calculated using the Staden PIP program (Staden, 1987) to the nearest Da based on the known N-terminus and the hypothetical C-terminus. The SD of the size as determined by mass spectrometry is of the order of that quoted in the text.

Toxin	Protease amount (%, w/w)	Proteolytic processing time (h)	Known N-terminal residue	Hypothetical C-terminal residue	Size (Da)		
					Protein gel	Mass spec.	Hypothetical C-terminal
CytA	–	–	1	249	27400	27321	27341
	10	0·2–0·5	31	233	22500	22355	22365
	10	0·2–0·5	31	233	22500	22369	22365
	50	16	–	–	22500	–	–
CytB	–	–	1	259	24000	29242*	29236
	10	0·2–0·5	34	238	21300	22865	22863
	10	> 16	–	–	20400	–	–
	50	16	38	230	20400	21520	21522

* Size of unprocessed CytB when the size as determined by positive ion mass spectrometry is divided by two.

incubation in 50 % proteinase K at 37 °C for 16 h (Fig. 6, track 6). Based on SDS-PAGE, the CytA product appeared unchanged even after treatment with 50 % proteinase K for 16 h.

A combination of N-terminal sequencing and positive ion electrospray mass spectrometry was used to determine the N- and C-termini of the proteolytically-processed δ-endotoxins. For mass spectrometry, IPS78/11 inclusions were solubilized at 1 mg ml^{-1} in 50 mM NH_4HCO_3 (pH 10·5)/10 mM DTT on ice for 1 h. Mass spectrometry of processed CytA proved to be very difficult but a single species was detected on two separate occasions, albeit at a relatively low signal intensity. The average molecular size observed by mass spectrometry was compared to the expected molecular size based on the determined N-terminus and the hypothetical C-terminus (Table 1). Clearly, the expected and observed molecular sizes are very similar, and deviate by much less than the size of a single residue.

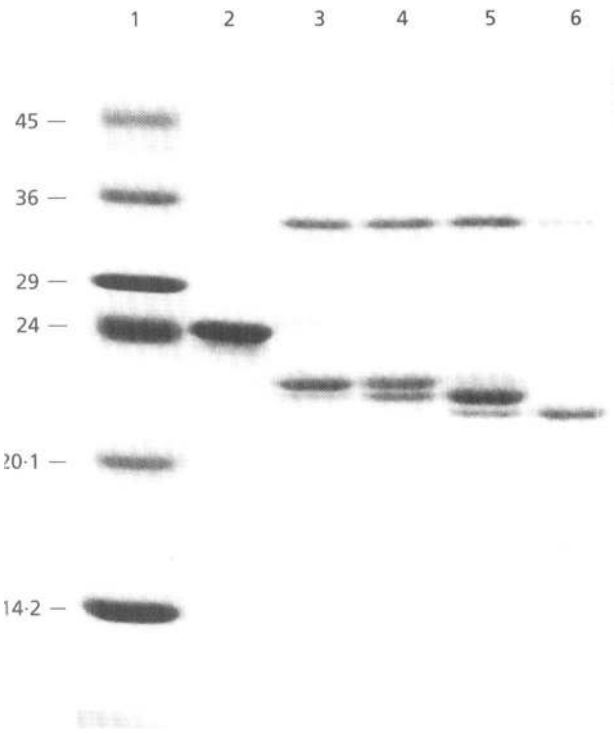

Fig. 6. Prolonged proteolytic processing of CytB inclusions from IPS78/11. Aliquots (10 μg) were solubilized at 4 °C for 30 min at 1 mg ml^{-1} in 50 mM Na_2CO_3, pH 10·5, with 10 mM DTT. Aliquots were then treated by addition of proteinase K to 50% (i.e. 0·5 mg ml^{-1}). Incubation was at 37 °C for 30 min (track 3), 60 min (4), 3 h (5) and 16 h (6) before the reaction was stopped by TCA-precipitation. Samples were repurified as described in Methods and then subjected to SDS-PAGE. Tracks 1 and 2 contain protein size markers and untreated CytB, respectively. The size of each marker is shown alongside in kDa. The intense band in tracks 3–6 of about 35 kDa in size is proteinase K.

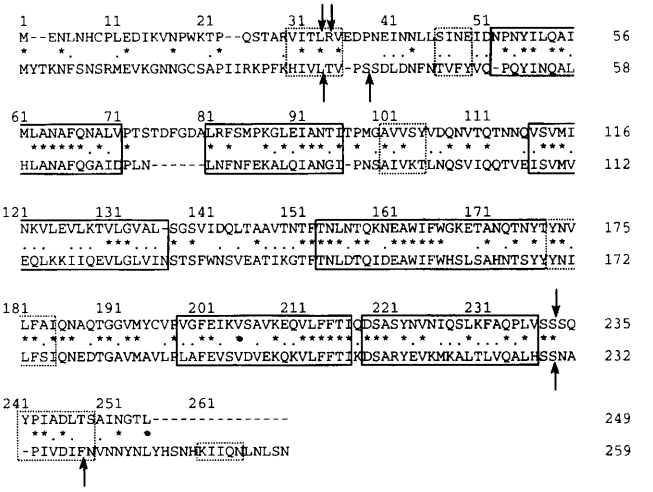

Fig. 7. Sequence alignment between CytA (upper sequence) and CytB, with identical residue positions shown by asterisks and residues which are members of the same physicochemical group or which substitute well for each other (Bordo & Argos, 1991) indicated by dots (Koni & Ellar, 1993). Numbers above the alignment give an indication of the alignment position including the gaps, whereas those at the side are of the last residue on each line for the individual proteins. The regions in solid and dashed boxes are predicted to be conserved α-helices and β-strands, respectively. The predicted secondary structure in the CytB additional C-terminal sequence is also shown. N- and C-terminal proteolytic processing sites are indicated by arrows. The outer N-terminal arrow above the CytA sequence is that for a minor species (about 5%) after processing for 30 min with 10% (w/w) proteinase K, whilst the inner arrow is that for the species referred to in Table 1. The outer N- and C-terminal arrows below the CytB sequence are the cleavage sites after 30 min of 10% (w/w) proteinase K treatment whereas the inner arrows mark the final product termini.

In Fig. 7, the sites of proteolytic cleavage for CytA and CytB are superimposed on the CytA/CytB sequence alignment and consensus predicted secondary structure (Koni & Ellar, 1993). This clearly reveals that CytA and CytB were processed to approximately the same points in the sequence alignment. Also, processing at the N-terminal end occurred just as readily in CytB as in CytA (Table 1). The prolonged period of time required for full processing of CytB appeared to be due to resistance to proteolysis at the C-terminal end of the first stable processed product, at alignment position 247 (Fig. 7). Processing of the additional 15 residue C-terminal sequence itself appears to have occurred readily.

Circular dichroism spectroscopy

Amino acid analysis was performed in triplicate in order to establish an accurate protein concentration for samples which were to be used for circular dichroism, but at the same time revealed that the observed composition of CytA and CytB was in close agreement to that expected (Waalwijk *et al.*, 1985; Koni & Ellar, 1993).

Circular dichroism in triplicate revealed processed CytA and processed CytB from IPS78/11 inclusions to be 57 ± 1·8% (115·7 ± 3·7 residues) and 49 ± 6·1% (100 ± 12·4 residues) α-helical. This compares to 45 ± 2·1% (91·8 ± 4·3 residues) for processed *E. coli* CytB and 43 ± 1·3% (111·4 ± 3·4 residues) for unprocessed CytB. These approximate to the expected value of 116 residues based on the consensus-predicted secondary structure model (Koni & Ellar, 1993; Fig. 7).

Toxicity to mosquito larvae

Toxicity was tested against 2–5-d-old *A. aegypti* and *C. pipiens* larvae and 4–5-d-old *A. gambiae* larvae. The data show that there is very little difference between CytA and CytB inclusions from IPS78/11 (Table 2), especially in view of the fact that 1 μg CytB is equivalent to only about 0·93 μg CytA in terms of moles of δ-endotoxin.

The efficiency of recovery of solubilized, unprocessed (i.e. solubilized at 4 °C and not exposed to proteinase K) CytA and CytB by citrate-precipitation was established to be virtually 100% and 75% respectively by protein mass determination after repurification. The lower recovery of CytB may not be too surprising in view of the fact that unprocessed CytB has a relatively high theoretical isoelectric point of about 7 (Koni & Ellar, 1993). Com-

Table 2. Mosquito larvae bioassays with IPS78/11 δ-endotoxin inclusions

Figures represent the range of δ-endotoxin concentrations for which approximately 50% mortality was seen, using a twofold serial dilution from 8 μg ml^{-1}. Probit analysis was not applied since the change in toxicity from one δ-endotoxin dose to the next was often quite dramatic. At least five experiments were conducted for each δ-endotoxin concentration, each experiment comprising assays in triplicate.

Species	Age (d)	Approx. LC_{50} (μg ml^{-1})	
		CytA	CytB
A. aegypti	2	0·5–1·0	1·0
	3 and 4	0·5–1·0	1·0–2·0
	5	1·0	2·0–4·0
A. gambiae	4	1·0	1·0
	5	2·0	2·0
C. pipiens	2	0·5–1·0	0·5–1·0
	3	1·0	1·0–2·0
	4 and 5	2·0	2·0–4·0

parison of the toxicity of citrate-precipitated unprocessed δ-endotoxin compared to intact inclusions revealed the former to be as toxic as whole inclusions but no more so.

After precipitation with citric acid (Chilcott & Ellar, 1988), processed CytA and all of the CytB proteolytic products seen were all equally as toxic to 2-d-old *A. aegypti* larvae as unprocessed CytA and unprocessed CytB.

Toxicity to *M. sexta* larvae

Involuntary feeding and inclusion-supplemented food assays were performed with CytA and CytB inclusions from IPS78/11. Inclusion-supplemented food assays were also performed with unprocessed *E. coli* CytB solubilized in 50 mM Na_2CO_3 (pH 10·5) and with CytA and CytB solubilized in 50 mM Na_2CO_3 (pH 10·5)/10 mM DTT and processed with 10% proteinase K for 30 min. Ten larvae were assayed at each δ-endotoxin dose. As little as 5 ng (0·038 pmoles) of CryIA(c) caused complete feeding inhibition whereas none of the CytA nor CytB preparations caused any obvious feeding inhibition when 10 μg (about 350 pmoles) was used. When inclusion-supplemented food assays were repeated using 100 μg of δ-endotoxin inclusions, again there was no obvious feeding inhibition.

Haemolysis *in vitro*

In order to study the *in vitro* cytolytic activity of Cyt δ-endotoxins, microtitre plate haemolysis assays were employed. In experiments using essentially solubilized, unprocessed (i.e. solubilized at 4 °C and not exposed to proteinase K) δ-endotoxin from IPS78/11 inclusions, both CytA and CytB were found to be haemolytic (Table 3). 'Unprocessed' CytB from *E. coli* inclusions was not haemolytic until processed with proteinase K, even when used at a concentration more than 40-fold higher than that used for 'unprocessed' CytB from IPS78/11 inclusions. This suggested that the problem of endogenous proteolysis of CytB from IPS78/11 inclusions seen after solubilization at 37 °C had not been completely overcome by solubilization at 4 °C. Thus, although others have demonstrated unprocessed CytA to be haemolytic (Chilcott & Ellar, 1988), this cannot be confirmed with any certainty by the work here as 'unprocessed' CytA may also have been processed slightly by endogenous proteases.

Table 3. Haemolytic activity of unprocessed ('unknown') and processed ('endogenous' and 'full') Cyt δ-endotoxins

Results were identical in three separate experiments. Alkali/protease negative controls were not haemolytic.

Toxin	Source	Processing	Haemolysis end-point (μg ml^{-1})*
CytA	IPS78/11	Unknown	0·8
		Endogenous	0·4
		Full	0·2
CytB	IPS78/11	Unknown	3–6
		Endogenous	0·4
		Full	0·2
CytB	*E. coli*	Unknown	> 250
		Full	0·2

* Amount of unprocessed toxin used to carry out the assay (rather than the mass of processed δ-endotoxin after protease treatment).

Proteolytically processed CytA was only 4-fold more haemolytic than unprocessed CytA (Table 3). Processed CytB from both IPS78/11 and *E. coli* inclusions was at least 1200 times more haemolytic than unprocessed CytB from *E. coli* inclusions (Table 3). All of the proteinase K-generated products were equally haemolytic.

DISCUSSION

Both in the *Bt* strain IPS78/11 (this study) and *E. coli* (Koni & Ellar, 1993), *cytB* was highly expressed and formed inclusions without the aid of the *Bti* 20 kDa 'helper' protein (Adams *et al.*, 1989) or any other *Bt* subspecies *kyushuensis* gene. In contrast, *cytA* showed very low expression in both *E. coli* and IPS78/11 (a plasmid-cured derivative of its native host) in the absence of the *Bti* 20 kDa 'helper' protein (Adams *et al.*, 1989; Crickmore *et al.*, 1990). Even with the 20 kDa 'helper' protein gene, *cytA* expression in *E. coli* was low (Adams *et al.*, 1989; Visick & Whiteley, 1991), whilst in *Bt* high expression and inclusion formation occurred (Crickmore *et al.*, 1990; Chang *et al.*, 1993; Wu & Federici, 1993).

CytA is a mosquito-larvicidal δ-endotoxin with a reported 50% lethal concentration of 0·1 μg ml^{-1} (Ward *et al.*,

1988) to greater than 1·0 μg ml^{-1} (Visser *et al.*, 1986). This variation may have been partly due to the fact that toxicity varied with the age of the larvae (this work). In the present comparison between CytA and CytB, there was very little difference between the two when in either their inclusion or citrate-precipitated forms. Since the data suggested that young larvae were more susceptible than older larvae, it may be that *A. gambiae* larvae less than 4 d old are also more sensitive. If this were true, *A. gambiae* would therefore be more susceptible than either *A. aegypti* or *C. pipiens*, the latter being least sensitive. *A. gambiae* larvae less than 4 d old were not employed because of the high mortality seen in negative controls with such young larvae.

Assays here with *M. sexta* larvae showed that as little as 5 ng of CryIA(c) led to complete feeding inhibition whereas 100000 times more (100 μg) of CytA or CytB than CryIA(c) (in terms of moles) had no effect. Thus, the broad cytolytic activity of Cyt δ-endotoxins *in vitro* is not paralleled *in vivo*. As mentioned in the Introduction, the growth inhibition of lepidopteran larvae observed by Held *et al.* (1990) and Knowles *et al.* (1992) may have been a result of the fact that these workers were using whole *Bt* subsp. *kyushuensis* inclusions in the former case and CytB partially purified from such inclusions in the latter case. Conceivably, the other proteins present in *Bt* subsp. *kyushuensis* inclusions (Earp *et al.*, 1987) may prove to have lepidopteran toxicity.

The fact that CytA and CytB were proteolytically processed to the same site in the sequence alignment suggested that the two share the same general structure. However, it was noteworthy that the time required for processing to the same C-terminal point was much longer for CytB than for CytA. If the additional C-terminal sequence in CytB (Koni & Ellar, 1993) served to protect a CytB region against proteolysis, its absence in CytA could account for the different rates of proteolysis. This itself may partly explain why *cytA* was not highly expressed without the aid of the *Bti* 20 kDa 'helper' protein. Rapid proteolysis of CytA at the C-terminal end may have prevented inclusion formation in the absence of the *Bti* 20 kDa 'helper' protein and therefore prolonged exposure to proteases, leading to complete degradation. However, it has been shown that the additional 15 residue C-terminal sequence of CytB was not itself the most protease resistant element but that the high protease resistance lay in a region of the sequence alignment that CytA and CytB apparently shared. Also, it has recently been shown that the additional 15 residue C-terminal sequence can be removed from CytB at the genetic level and that this truncated CytB is still highly expressed in *E. coli* and is not haemolytic until proteolytically processed (P. A. Koni, M. Adams & D. J. Ellar, unpublished). Nevertheless, it remains possible that CytB possesses a secondary structure in its C-terminal, proteolytically-removed region which is not in common with CytA and which is responsible for the observed proteolytic resistance.

Since CytB possesses only one cysteine residue (in the N-terminal region that is removed by proteolytic processing), disulphide bonding would only result in the formation of dimers. CytA, on the other hand, possesses two cysteine residues (cysteine-7 in the N-terminal region that is removed by proteolytic processing, and cysteine-190) and conceivably could form a large, insoluble network of molecules by intermolecular disulphide bonding. Thus it is interesting that CytA inclusions were not solubilized at pH 10·5 or in 30% NH_4OH unless DTT was included, in 10% acetic acid or in HCl, pH 1 whereas CytB inclusions from IPS78/11 were solubilized (data not shown). This behaviour reflects that of CytA from native *Bti* inclusions in that only about 40% of the CytA content of *Bti* inclusions solubilized in 50 mM Na_2CO_3, pH 10·5 (Thomas & Ellar, 1983a). Furthermore, *Bti* inclusions contain disulphide bonds (Couche *et al.*, 1987). Thus, the -SH content of both CytA and CytB inclusions from IPS78/11 were determined and found to be 36·7 ± 2·3% and 21·7 ± 4·3%, respectively.

If the insolubility of CytA inclusions were due to disulphide bonding, then both cysteine-7 and cysteine-190 would have to be involved. Certainly, surface exposure of cysteine-190 would be in agreement with work by Chow *et al.* (1989) who were able to modify proteolytically processed CytA with the cysteine modifier mercuryl chloride. Also, this region contains the epitope for a monoclonal antibody that inhibits toxin binding (Chow *et al.*, 1989).

The significance, if any, of disulphide bonding in CytA toxicity is not known. When solubilized and then citrate-precipitated, CytA was as toxic as whole inclusions but no more so. Thus, it would appear that disulphide bonding is not an impediment to toxicity *in vivo*. It may be that disulphide bonds play a role in maintaining the inclusion state until ingestion by preventing solubilization under mild conditions, although there is no evidence to support this.

ACKNOWLEDGEMENTS

We would like to thank the Agricultural and Food Research Council for financial assistance; the Cambridge Centre for Molecular Recognition for help with protein sequencing, amino acid analysis, circular dichroism spectroscopy and electrospray mass spectrometry; Dr Neil Crickmore for providing pSV2 and cam2027; Mr Said Al-Yahyaee for determining the N-terminus of processed CytA; Mr Trevor Sawyer for hatching *M. sexta* eggs and providing CryIA(c); and Mr Dan Hill for assistance with electron microscopy.

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Received 8 September 1993; revised 26 January 1994; accepted 7 February 1994.