Maximal toxicity of cloned CytA δ -endotoxin from *Bacillus thuringiensis* subsp. *israelensis* requires proteolytic processing from both the N- and C-termini

Said A. S. Al-yahyaee† and David J. Ellar

Author for correspondence: David J. Ellar. Tel: +44 1223 333651. Fax: +44 1223 333345.
e-mail: DJE1@MOLE.BIO.CAM.AC.UK

Department of
Biochemistry, University of
Cambridge, Tennis Court
Road, Cambridge CB2
1QW, UK

Unlike the CytA toxin from native *Bacillus thuringiensis* subsp. *israelensis* (Bti) crystals, the inclusions of cloned CytA produced by Bti IPS 78/11 in the presence of the 20 kDa 'helper' protein require a reducing agent in addition to a highly alkaline pH for complete solubilization. Activation of the solubilized CytA with a range of proteases produced 25–22 kDa products. SDS-PAGE analysis and N-terminal amino acid sequencing revealed that CytA was processed very similarly at both termini by proteinase K or by *Anopheles* or *Culex* gut extracts. Trypsin, by contrast, processed CytA predominantly at the N-terminus. *In vitro* cytolytic assays against *Aedes aegypti* cells, and haemolytic assays against rat erythrocytes, showed that CytA processed at both termini by proteinase K was the most active form. Thus CytA, like other Bt δ -endotoxins, is processed to a well-defined protease-resistant product and this processing enhances the toxicity *in vitro* and possibly *in vivo*.

Keywords: *Bacillus thuringiensis*, δ -endotoxins, CytA, haemolytic assay

INTRODUCTION

The crystalline inclusions produced by *Bacillus thuringiensis* subsp. *israelensis* (Bti) (Goldberg & Margalit, 1977) have been shown by SDS-PAGE to contain four different proteins (Tyrell *et al.*, 1981; Thomas & Ellar, 1983). These four proteins were designated CytA, CryIVD, CryIVB and CryIVA, with molecular masses of 27, 65, 135 and 128 kDa, respectively (Hofte & Whiteley, 1989). The most abundant of the four is CytA, which constitutes 40% of total crystal protein. Thomas & Ellar (1983) partially solubilized CytA by incubating the crystals with 50 mM Na₂CO₃/HCl (pH 10.5), for 30 min at 37 °C. When the alkali-solubilized protein was assayed against various cell lines and erythrocytes, it showed a broad *in vitro* cytolytic and haemolytic activity.

Armstrong *et al.* (1985) solubilized CytA from native Bti crystals by incubating them in deionized water brought to pH 12 with NaOH for 3 h at 37 °C. The solubilized product was then treated with trypsin for 2 h followed by proteinase K for another 2 h at 37 °C. This activation

regime produced a 25 kDa protease-resistant protein. After purification, this 25 kDa protein was shown to be active against mosquito cell lines and erythrocytes as well as being lethal to 6-week-old BALB/c mice when 100 µg was injected intraperitoneally. A similar study was reported by Gill *et al.* (1987) who, in addition to the 25 kDa protein, purified a 24 kDa protein which was antigenically similar and had an identical N-terminus. Both proteins lysed human erythrocytes and cultured mosquito cells, the 24 kDa protein being the more toxic.

Chilcott & Ellar (1988) employed partial solubilization as described by Thomas & Ellar (1983) and Mono Q ion-exchange chromatography to purify the 27 kDa CytA from Bti crystals; the preparation was then treated with trypsin and reappplied to the Mono Q column. The trypsin-treated product was eluted with 10–600 mM NH₄HCO₃ (pH 8.5) and shown to have a molecular mass of 25 kDa. This purified product was similar in size and properties to that reported by Armstrong *et al.* (1985) and Gill *et al.* (1987).

The cloning of the *cytA* gene (Ward *et al.*, 1984) and subsequent expression in *B. subtilis* (Ward *et al.*, 1986) and acrySTALLIFEROUS Bti IPS 78/11 (Crickmore *et al.*, 1990) made it possible to obtain pure CytA crystals, which were shown by Ward *et al.* (1986) to be alkali soluble and active

† **Present address:** Department of Biochemistry, College of Medicine, Sultan Qaboos University, PO Box 35, Al-Khod 123, Sultanate of Oman.

Abbreviation: Bti, *Bacillus thuringiensis* subsp. *israelensis*.

against mosquito cell lines and erythrocytes with the same LC_{50} value as CytA derived from native Bti δ -endotoxin crystals. When the solubilized product was activated with *Aedes aegypti* gut extract it retained toxicity against mosquito cell lines and erythrocytes (Ward *et al.*, 1988).

Soluble CytA, unlike CytB (Knowles *et al.*, 1992; Koni & Ellar, 1994) or Cry toxins (Hofte & Whiteley, 1989) is probably active without prior processing. However, the high susceptibility of the CytA N-terminus to endogenous proteases hindered previous attempts to assay the unprocessed CytA. In this paper we show that the N-terminal processing of CytA by endogenous proteases can be minimized by solubilizing CytA at pH 12. We also show the relative contribution of the N-terminal processing and the N-terminal and C-terminal processing to CytA cytolytic activity.

METHODS

Bacterial strain, media and growth conditions. The Bt strain used was subspecies *israelensis* IPS 78/11 harbouring the *cytA* gene and the 20 kDa 'helper' protein gene (Crickmore *et al.*, 1990). The strain was grown to late exponential phase in Luria-Bertani (LB) broth or on LB agar plates (Sambrook *et al.*, 1989) at 30 °C, overnight. For synchronous sporulation, IPS 78/11 was grown in casein/casein/yeast extract (CCY) broth (Stewart *et al.*, 1981) for 24–36 h using a 0.1 % inoculum of cell culture grown to exponential phase in 5 % peptone, 0.1 % yeast extract, 0.5 % NaCl (PWYE) from a heat-shocked spore suspension (Ellar & Posgate, 1974). For the selection of plasmids encoding chloramphenicol acetyltransferase, 5 µg chloramphenicol ml^{-1} was included.

Purification and solubilization of CytA inclusions. The CytA inclusions were purified as described by Thomas & Ellar (1983). Briefly, the spore-crystal mixture was initially washed with ice-cold deionized water to remove the cell debris. The pellet was then resuspended in Tris/KCl buffer (50 mM Tris/HCl, 10 mM KCl, pH 7.5) and layered onto a discontinuous sucrose density gradient of 67, 72 and 79 % (w/v) sucrose made up in Tris/KCl buffer. Gradients were centrifuged in a SW28 rotor at 80000 *g* for 16–20 h at 4 °C in a Beckman ultracentrifuge. The inclusions sedimented as a distinct band at the interface between 67 and 72 % sucrose. The band containing the inclusions was harvested from the gradients using a 10 ml syringe and washed three times in ice-cold deionized water. The purity of the inclusions was confirmed by phase-contrast microscopy and the purified inclusions were stored at –70 °C in deionized water.

CytA inclusions were solubilized at 5–8 mg ml^{-1} by incubation at 37 °C for 1 h in the presence of 10 mM DTT in either 50 mM Na_2CO_3 (pH 10.3) or deionized water adjusted to pH 12 with NaOH. Solubilized CytA was then separated from insoluble material by centrifugation at 12000 *g* for 15 min.

Proteolytic processing of the solubilized CytA. The soluble CytA in 50 mM Na_2CO_3 /10 mM DTT (pH 10.3) was incubated at 37 °C with 5 % (w/w) trypsin (Sigma) or chymotrypsin (Sigma) or 0.5 % (w/w) proteinase K (Sigma) overnight, or with 1 unit of *Culex* or *Anopheles* gut extract (Angsuthanasombat *et al.*, 1992) per 25 µg of soluble CytA for 2 h. CytA was also treated with 10 % (w/w) insoluble proteinase K (proteinase K attached to agarose beads; Sigma). In this activation regime and prior to incubation with soluble CytA, the insoluble proteinase K was always washed by centrifugation in the solubilization buffer until no soluble protein was detected by measurement of

A_{280} in the supernatant. The incubation was carried out at 37 °C overnight with continuous gentle mixing. Following the activation, insoluble proteinase K was removed from the activated CytA by centrifugation at low speed (1000 *g*) and by filtration through a 0.20 µm membrane filter (Sartorius). Activated CytA was stored at 4 °C or –20 °C until use.

Determination of protein concentrations. CytA concentration was determined by A_{280} measurement based on the method of Cantor & Schimmel (1980). For this assay it was estimated that an absorbance of 1.0 is equivalent to 1.0 mg ml^{-1} , 1.1 mg ml^{-1} and 1.2 mg ml^{-1} for soluble CytA, CytA activated with trypsin, and CytA activated with proteinase K, respectively. These values were compared with CytA concentrations determined by amino acid analysis and found to be within ± 10 % of the above values.

Purification of CytA activated with trypsin or proteinase K by fast protein liquid chromatography (FPLC). CytA activated with trypsin or proteinase K was purified by anion-exchange chromatography using a Mono Q column (Pharmacia) connected to a Waters 650E-FPLC system (Millipore). The Mono Q column was equilibrated with 10 mM NH_4HCO_3 (pH 8.5 adjusted with ammonia solution) and eluted at a flow rate of 1.0 $ml\ min^{-1}$ with a gradient of 10–600 mM NH_4HCO_3 (pH 8.5). Activated CytA and proteinase K samples were filtered through 0.20 µm membranes prior to loading on the columns. Column effluents were monitored at 280 nm and 1.0 ml fractions collected. Samples of protein peaks were analysed by SDS-PAGE.

SDS-PAGE. SDS-PAGE was carried out using the discontinuous Tris/glycine buffer system (Laemmli, 1970) as modified by Thomas & Ellar (1983). Gels comprised a stacking gel of 6 % (w/v) acrylamide and a separating gel of 13 % (w/v) acrylamide.

Protein samples in SDS sample buffer [50 mM Tris/HCl (pH 7.5), 1 mM EDTA, 0.5 % (w/v) SDS, 5 % (v/v) glycerol, 0.05 % (w/v) bromophenol blue, 25 mM DTT] were boiled for 5 min at 100 °C prior to loading onto the gels. Gels were stained for 1–2 h or overnight with Coomassie blue staining solution [0.1 % (w/v) Coomassie blue, 10 % (v/v) methanol, 10 % (v/v) acetic acid] and destained in 10 % (v/v) methanol/10 % (v/v) acetic acid overnight at room temperature. Alternatively, gels were silver-stained using the method of Wray *et al.* (1981).

Protein molecular masses were estimated by comparison of log molecular mass to the relative mobility of protein molecular mass standards: *E. coli* β -galactosidase (116 kDa), bovine serum albumin (67 kDa), hen ovalbumin (45 kDa), rabbit muscle glyceraldehyde phosphate dehydrogenase (36 kDa subunit), bovine erythrocyte carbonic anhydrase (29 kDa), trypsinogen (24 kDa), soyabean trypsin inhibitor (20 kDa), bovine milk α -lactalbumin (14 kDa).

N-terminal protein sequencing. Proteins were subjected to SDS-PAGE with 0.015 % (v/v) thioglycolic acid in the electrophoresis buffer and then transferred onto Problot membranes (Applied Biosystems) using a semi-dry blot apparatus at a constant current. The transfer buffer consisted of 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid pH 6.5, 1 mM DTT and 10 % methanol.

The blotted membrane was stained for 1–2 min with Coomassie staining solution and then destained in 50 % (v/v) methanol/10 % (v/v) acetic acid for 10 min. The membrane was briefly rinsed in deionized water and air dried. Stained bands were excised from the membrane and the proteins sequenced in an ABI 477A (Applied Biosystems) automated pulsed liquid-phase sequencer. Sequence analysis was performed using

standard RUN 477-1 software under the control of a 900A data/controller module. The amino acid phenylthiohydantoin derivatives were analysed on line using a 120A PTH-amino acid analyser.

Amino acid analysis. Approximately 0.5–1 nmol protein was lyophilized and hydrolysed at 110 °C for 24 h in an argon atmosphere by the vapour from 6 M HCl containing 1% (v/v) thioglycolic acid. Hydrolysed samples were analysed on a Pharmacia LKB Alpha Plus II amino acid analyser (sodium ion-exchange system) using norleucine as an internal standard and ninhydrin complex detection.

In vitro haemolysis assays. Haemolysis assays were carried out as described by Thomas & Ellar (1983) except that a 5% (v/v) erythrocyte suspension in haemolysis buffer [50% (w/v) phosphate buffered saline (PBS; pH 7.4), 2.25% (w/v) glucose, 0.1% (w/v) gelatin] was used. Rat blood was collected in Alsevers solution (0.1 M trisodium citrate, 40 mM glucose) and stored at 4 °C until use. The erythrocytes were usually washed immediately before use with 50% (w/v) PBS/2.25% (w/v) glucose until all lysed cells were removed. The cells were then diluted in haemolysis buffer to make up the 5% (v/v) erythrocyte suspension. The end-point of haemolysis was assessed after 3–12 h as the last well in which colouration of the liquid was still visible.

In vitro cytotoxicity assays against *Aedes aegypti* cells. The mosquito *Aedes aegypti* cell line used and its growth and maintenance conditions have been described previously (Knowles & Ellar, 1986). *Aedes aegypti* cells (at $1\text{--}2 \times 10^6$ cells ml^{-1} in tissue culture medium) were incubated at room temperature for 30, 60, and 120 min with $1 \mu\text{g ml}^{-1}$ of purified CytA activated with trypsin or proteinase K. Controls containing equal volumes of buffer in which the test samples were originally suspended were also incubated with *A. aegypti* cells. Cell mortality was assessed by vital staining with 0.1% (w/v) Trypan blue in PBS buffer (Thomas & Ellar, 1983). Cells were counted in a haemocytometer counting chamber.

RESULTS

Solubilization and activation of cloned CytA crystals

The crystals were solubilized at $5\text{--}8 \text{ mg ml}^{-1}$ in 50 mM $\text{Na}_2\text{CO}_3/\text{HCl}$ (pH 10.3) with 10 mM DTT at 37 °C for 1 h and gave two bands with molecular masses of 23.5 and 27 kDa on SDS-PAGE (Fig. 1, lane 3). In the absence of DTT, however, crystals were only about 20% soluble at pH 10.3. Crystals were also soluble in deionized water with 10 mM DTT brought to pH 12 by adding NaOH. Unlike solubilization in sodium carbonate, this regime produced a single 27 kDa band on SDS-PAGE (Fig. 1, lane 1), possibly as a result of inhibition of endogenous proteases by the higher pH. When crystals were solubilized in SDS-sample buffer a predominant 24 kDa band was seen (Fig. 1, lane 2), which suggested that the protein might have undergone N-terminal processing during sample preparation. However, when this band was sequenced it had N-terminal Met 1 while the alkali-solubilized CytA (Fig. 1, lane 3, lower band) had Val 26 as the N-terminal amino acid. This difference suggests that the SDS-solubilized crystals undergo C-terminal processing and/or that SDS solubilization causes the protein to migrate abnormally, as has been observed with CytB crystal protein (Koni & Ellar, 1993).

Fig. 1. Coomassie-Blue-stained SDS/13% PAGE showing CytA solubilization under various conditions. Twenty micrograms of each sample in SDS sample buffer containing 25 mM DTT, 5 mM phenylmethylsulfonyl fluoride (PMSF) dissolved in 2-propanol, and 1 mM EDTA were loaded per lane. Lane 1, CytA crystals solubilized in water/10 mM DTT adjusted to pH 12.0 with NaOH; lane 2, CytA crystals solubilized in SDS sample buffer; lane 3, CytA crystals solubilized in 50 mM Na_2CO_3 /10 mM DTT, pH 10.3; lane 4, molecular mass markers.

The alkali-soluble CytA was digested with a range of proteases to determine the pattern of activation. Chymotrypsin, trypsin, proteinase K, and *Culex* and *Anopheles* gut extracts were tested to find the enzyme producing the most effective cleavage of CytA with maximum retention of toxicity. SDS-PAGE revealed that CytA was processed to 24.5–22 kDa protease-resistant products by these regimes (Fig. 2). The products were sequenced from the N-terminus to determine the site of cleavage and showed the pattern depicted in Table 1.

Purification of trypsin- and proteinase-K-activated CytA products

The soluble CytA activated with proteinase K was subjected to ion-exchange chromatography and compared with CytA activated with trypsin according to the method of Chilcott & Ellar (1988). CytA activated with 0.5% (w/w) proteinase K was applied to a Mono Q column equilibrated with 10 mM NH_4HCO_3 (pH 8.5) and eluted with a 10–600 mM NH_4HCO_3 (pH 8.5) gradient. The A_{280} trace (Fig. 3a, solid line) typically showed four peaks; the breakthrough (unretarded) peak, designated peak I, and three peaks in the middle of the gradient,

Fig. 2. Coomassie-Blue-stained SDS/13% PAGE showing the pattern of CytA activation with various proteases. Soluble CytA at 2.0 mg ml^{-1} in $50 \text{ mM Na}_2\text{CO}_3/10 \text{ mM DTT}$, pH 10.3, was activated with 5% (w/w) trypsin or chymotrypsin or 0.5% (w/w) proteinase K at 37°C overnight, and with 1.0 unit of *Culex* or *Anopheles* gut extracts per $25 \mu\text{g}$ of CytA for 2 h at 37°C . Five microlitres of activated samples were mixed with $5 \mu\text{l}$ of $2 \times$ SDS sample buffer containing 25 mM DTT , 5 mM PMSF dissolved in 2-propanol, and 1 mM EDTA . Samples were boiled for 5 min and $5 \mu\text{l}$ was loaded per lane. Lanes 1–5, CytA treated with proteases: lane 1, *Culex* gut extract; lane 2, *Anopheles* gut extract; lane 3, proteinase K; lane 4, chymotrypsin; lane 5, trypsin. Lane 6, CytA solubilized in $50 \text{ mM Na}_2\text{CO}_3/10 \text{ mM DTT}$, pH 10.3, at 4°C ; lane 7, CytA solubilized in $50 \text{ mM Na}_2\text{CO}_3/10 \text{ mM DTT}$, pH 10.3, at 37°C ; lane 8, molecular mass markers.

Table 1. N-terminal residue of CytA activated with various proteases

CytA activated with	N-terminal residue	Approx. size (kDa)	Figure reference
No activation	Met 1	27	1, lane 1
Endogenous proteases	Val 26, Ile 27 or Arg 30	23.5	1, lane 3, lower band
Chymotrypsin	Lys 18	24.5	2, lane 4
Trypsin	Val 26	23.5	2, lane 5
Proteinase K	Arg 30 or Val 31	22	2, lane 3
<i>Anopheles</i> gut extract	Arg 30 or Val 31	22.5	2, lane 2
<i>Culex</i> gut extract	Arg 30 or Val 31	22.5	2, lane 1

designated peaks II–IV (Fig. 3a). SDS-PAGE of fractions from these peaks showed that the activated CytA was in peaks III and IV, whilst peaks I and II contained no detectable protein (Fig. 3b). However, fraction 21 (F21) from peak III had an additional band at the dye front which was due to the presence of co-eluting proteinase K. (Fig. 3a, dashed line). The purity of CytA activated with

Fig. 3. (a) Purification of CytA activated with proteinase K (solid line) and a control run containing only proteinase K (dashed line) by FPLC using a Mono Q column equilibrated with $10 \text{ mM NH}_4\text{HCO}_3$, pH 8.5, and eluted with a $10\text{--}600 \text{ mM NH}_4\text{HCO}_3$, pH 8.5, linear gradient. Soluble CytA at 8.0 mg ml^{-1} in $50 \text{ mM Na}_2\text{CO}_3/10 \text{ mM DTT}$, pH 10.3, was activated with 0.5% (w/w) proteinase K at 37°C for 6 h. The sample was then adjusted to $10 \text{ mM NH}_4\text{HCO}_3$, pH 8.5, before loading on the Mono Q column. Two milligrams of proteinase K was dissolved in $10 \text{ mM NH}_4\text{HCO}_3$, pH 8.5, and loaded on the Mono Q column. Peaks are numbered with Roman numerals; flow rate 1.0 ml min^{-1} ; fraction volume 1.0 ml . (b) Coomassie-Blue-stained SDS/13% PAGE analysis of fractions from Mono Q column chromatography of CytA activated with proteinase K (a, solid line). Twenty-five microlitres of each fraction were mixed with an equal volume of $2 \times$ SDS sample buffer and boiled for 5 min before loading. Lane 1, molecular mass markers; lane 2, fraction 5; lane 3, fraction 10; lane 4, fraction 16; lane 5, fraction 20; lane 6, fraction 21; lane 7, fraction 24.

proteinase K was greatly improved when insoluble proteinase K (10% w/w, 37°C , overnight) was used for the activation and the Mono Q column eluted with a step gradient as shown in Fig. 4(a). A silver stained SDS-PAGE of fractions from this step gradient run is shown in Fig. 5 (lanes 6–9).

Although activated CytA was resolved into three separable peaks on the column, SDS-PAGE showed that fractions 24 and 25 (F24 and F25) from peak III and F37 from peak V had the same size band at about 22 kDa, while F30 from peak IV had a band of approximately

Fig. 4. Purification of CytA activated with proteinase K (a) or trypsin (b) by FPLC using a Mono Q column, equilibrated with 10 mM NH_4HCO_3 , pH 8.5, and eluted with a 10–600 mM NH_4HCO_3 , pH 8.5, step gradient. Soluble CytA at 5.0 mg ml⁻¹ in 50 mM Na_2CO_3 /10 mM DTT, pH 10.3, was activated with 10% (w/w) insoluble proteinase K or with 1% (w/w) trypsin at 37 °C, overnight. The samples were adjusted with equilibration buffer before loading on the Mono Q column. Peaks are numbered with Roman numerals; flow rate 1.0 ml min⁻¹; fraction volume 1.0 ml.

Fig. 5. Silver-stained SDS/13% PAGE analysis of fractions from Mono Q column chromatography of CytA activated with proteinase K and trypsin (Fig. 4a, b). One microgram of sample in SDS sample buffer containing 25 mM DTT was loaded per lane. Lane 1, soluble CytA activated with 1% (w/w) trypsin at 37 °C, overnight; lanes 2 and 3, samples from fractions 24 and 28, respectively, of the trypsin run (Fig. 4b); lane 4, molecular mass markers; lane 5, soluble CytA activated with 10% (w/w) insoluble proteinase K at 37 °C, overnight; lanes 6–9, samples from fractions 24, 25, 30 and 37, respectively, of the proteinase K run (Fig. 4a); lane 10, soluble CytA in 50 mM Na_2CO_3 /10 mM DTT, pH 10.3.

Table 2. Haemolytic assay of different forms of CytA

Sample	N-terminal residue	Approx. size (kDa)	Lane in Fig. 5	Approx. concn ($\mu\text{g ml}^{-1}$) at end-point haemolysis
100 mM NH_4HCO_3 , pH 8.5 or 50 mM Na_2CO_3 /10 mM DTT, pH 10.3	–	–	–	No haemolysis
Soluble CytA	Met 1 or Val 26	27–23.5	10	2.3
F28 of trypsin run	Val 26	23.5	3	2.3–1.2
F24 of proteinase K run	Arg 30	22	6	0.6
F28 of trypsin run mixed with F24 of proteinase K run				0.6
F30 of proteinase K run	Arg 30	23	8	2.3–1.2
F37 of proteinase K run	Val 31	22	9	0.6

Fig. 6. Toxicity assay against the mosquito cell line *Aedes aegypti*, using CytA activated with trypsin and proteinase K and purified by Mono Q column chromatography (Figs 4 and 5). To 47.5 μ l of cell suspension ($1.0\text{--}1.5 \times 10^6$ cells ml^{-1}), 2.5 μ l of toxin solution ($20 \mu\text{g ml}^{-1}$) was added to give a final concentration of $1.0 \mu\text{g ml}^{-1}$. Cells were incubated with the toxin at room temperature for 30 (a), 60 (b), and 120 (c) min before being counted using Trypan blue staining. Error bars (mean $\pm$ SEM, $n = 3$) are based on three separate experiments. The mortality in control cells incubated with equal volumes of buffer rarely exceeded 10–15%; these values have been subtracted in the Figure.

23.5 kDa. The N-terminal sequence of these fractions revealed that the N-terminal residue of F24, F25 and F30 was Arg 30 while F37 had Val 31 as its N-terminal amino acid. Since F30 had a higher molecular mass band than F24, F25 and F37, this suggests that the product in F24, F25 and F37 was probably processed from both termini (for further details see Discussion).

Comparison of the elution profile of CytA activated with proteinase K with that of trypsin-activated CytA under the same chromatography conditions showed that the trypsin product was resolved into two peaks and trypsin was eluted in an earlier fraction than proteinase K (Fig. 4b). Silver-stained SDS-PAGE revealed that the lower band of trypsin fraction 24 (F24) ran at about the same

Fig. 7. Coomassie-Blue-stained SDS/13% PAGE showing the pattern after a second round of activation of the purified fractions from the trypsin and proteinase K runs from the Mono Q column (Fig. 4). Approximately 20 μ g of each sample in SDS sample buffer containing 25 mM DTT, 5 mM PMSF and 1 mM EDTA was loaded per lane. Lane 1, molecular mass markers; lane 2, fraction 24 of the proteinase K run; lanes 3–5, fraction 24 of the proteinase K run treated respectively with 0.5% (w/w) proteinase K, 5% (w/w) chymotrypsin, 5% (w/w) trypsin at 37 °C, overnight; lanes 6–8, fraction 28 of the trypsin run treated respectively with 0.5% (w/w) proteinase K, 5% (w/w) chymotrypsin, 5% (w/w) trypsin at 37 °C, overnight; lane 9, fraction 28 of the trypsin run.

position as F24 of proteinase K (Fig. 5, lanes 2 and 6, respectively) while trypsin F28 ran at higher molecular mass than proteinase K F24 (Fig. 5, lanes 3 and 6, respectively). However, both F24 and F28 of the trypsin product had Val 26 as their N-terminal amino acid. It is probable that the F24 toxin (Fig. 5, lane 2, lower band) from trypsin-activated CytA, like the proteinase K-activated F24 toxin, was also processed from both termini (see Discussion).

Haemolytic and cytolytic assays

The enzymic cleavage products of CytA were further characterized by comparing their toxicity. CytA is haemolytic and a haemolysis assay is a simple and convenient means of testing the products. Samples of alkali-soluble CytA and CytA processed with various proteases were assayed against rat erythrocytes as described in Methods. The haemolytic activity decreased in the following order: proteinase K product > trypsin product > chymotrypsin product = soluble CytA. This pattern was confirmed when fractions obtained from the Mono Q column were assayed. The F24 and F37 from the proteinase K run were the most haemolytic followed by trypsin F28, while soluble CytA was the least haemolytic (Table 2). It is interesting to note that the most haemolytic of the samples tested was also the smallest.

The F24 and F37 from proteinase K activation and F28 from trypsin activation runs were also assayed against an *Aedes aegypti* cell line. The results indicated that the proteinase K F24 and F37 were more toxic than trypsin

F28. In fact, the proteinase K F24 and F37 were twice as toxic as trypsin F28 after 30 min incubation (Fig. 6a). The difference in toxicity, however, became progressively less as the time of incubation increased (Fig. 6b, c). This was possibly due to lysis of cells before being counted and/or the smaller CytA product being able to form a pore at a faster rate.

DISCUSSION

Thomas & Ellar (1983) found that CytA toxin could be selectively dissolved from native Bti crystals without DTT. By contrast, when the *cytA* gene was expressed with the 20 kDa 'helper' protein gene in Bti IPS 78/11 the large lemon-shaped inclusions that were formed (Crickmore *et al.*, 1990) were only soluble at pH 10.3–10.5 with the addition of 10 mM DTT as a reducing agent. It is difficult to explain this difference in CytA solubility between the native and cloned CytA inclusions. CytA contains two cysteine residues (Ward & Ellar, 1986) and it is possible that intermolecular disulfide bonds can form when CytA is synthesized and packed into inclusions in the absence of the other Bti toxins. The 20 kDa 'helper' protein has four cysteine residues (Adams *et al.*, 1989) and could possibly also be involved in disulfide bonding with CytA. However, the 20 kDa 'helper' protein could only be detected immunologically at very high gel loading in native Bti inclusions (Adams *et al.*, 1989) and could not be detected in the cloned CytA inclusions by Coomassie staining (S. Al-yahyaee, unpublished result). Nevertheless, some role for the 20 kDa 'helper' protein in the formation of disulfide bridges in the cloned CytA inclusion is suggested by the observation that the cloned CytA inclusions produced in *B. subtilis* (Ward *et al.*, 1986; Armstrong, 1992) without the 20 kDa 'helper' protein, are soluble in the absence of DTT.

During solubilization, CytA is processed by endogenous proteases to 24.5–22 kDa products (data not shown). This processing can be mimicked by incubating soluble CytA with proteases such as trypsin and proteinase K. Trypsin cleaves CytA at Arg 25, which is in agreement with the report by Chilcott & Ellar (1988). However, trypsin-activated products purified by Mono Q ion-exchange chromatography were resolved into two peaks (Fig. 4b) and when checked on SDS-PAGE, two products were seen which differed by about 2.0 kDa, although both had Val 26 as their N-terminal residue (Fig. 5, lanes 2 and 3). Similarly, SDS-PAGE analysis of proteinase K products purified by the Mono Q column showed two different molecular mass products (Fig. 5, lanes 7 and 8), both of which had Arg 30 as their N-terminal amino acid. These data suggest that trypsin and proteinase K each process CytA from both termini. Position 225 of CytA seems to be a potential cleavage site for proteinase K and trypsin at the C-terminus. This site has the sequence Leu 224–Lys 225–Phe 226–, which is structurally equivalent to the proteinase K cleavage site Leu 29–Arg 30–Val 31– at the N-terminus. Furthermore, in the former sequence Lys 225 can serve as a potential cleavage site for trypsin. In fact, when trypsin-treated F28 was treated with proteinase K and chymotrypsin (with Phe 226 as a potential cleavage

site) (Fig. 7, lanes 6 and 7, respectively) it produced a band on SDS-PAGE equivalent to that of trypsin F24 (Fig. 5, lane 2, lower band), while proteinase-K-treated F24 showed no difference in size after being treated with trypsin and chymotrypsin (Fig. 7, lanes 4 and 5). If both trypsin and proteinase K process CytA at the same position from the C-terminus, then the difference in molecular mass between trypsin-treated F24 and proteinase-K-treated F30 (Fig. 5, lanes 3 and 8, respectively), both of which were only N-terminally processed, can be attributed to the 5 amino acid difference at the N-terminus. Furthermore, the difference in molecular mass between the whole CytA (27.4 kDa) and proteinase-K-treated F24 (22 kDa) can be accounted for by the removal of a total of about 55 amino acids from both termini. In summary, the data above provide experimental evidence to support the argument that CytA, like CytB (Knowles *et al.*, 1992; Koni & Ellar, 1994) and Cry toxins (Hofte & Whiteley, 1989) is processed from both termini.

Soluble CytA, unlike CytB (Koni & Ellar, 1993) and Cry toxins (Hofte & Whiteley, 1989), is probably active without prior processing. However, the high susceptibility of the CytA N-terminus to endogenous proteases hindered previous attempts to assay the unprocessed CytA. The N-terminal processing can be minimized if CytA crystals are solubilized at pH 12 with 10 mM DTT, as shown in Fig. 1, lane 1. When this sample was assayed against rat erythrocytes it was found to be as haemolytic as processed soluble CytA in Na₂CO₃, pH 10.3 (data not shown). While this result may imply that N-terminal processing is not necessary for toxicity, it is possible that the minor processed product seen in front of the main 27 kDa band was responsible for toxicity. In fact when this sample was applied to a Mono Q column equilibrated with 10 mM NH₄HCO₃ (pH 8.5) and eluted with a 10–600 mM NH₄HCO₃ (pH 8.5) linear gradient, the protein was collected in a single broad peak (data not shown), which suggested that the sample was heterogeneous in composition, possibly as a result of differentially processed components.

A comparative toxicity study of CytA processed with various proteases established that the 22 kDa proteinase K product was the most active both against rat erythrocytes (Table 2) and *Aedes aegypti* cells (Fig. 6). A similar observation was reported by Gill *et al.* (1987), whose 24 kDa product was more active than the 25 kDa product. Their 24 kDa product is probably equivalent to the 22 kDa product reported here since both had the same N-terminus, but they used different molecular mass markers to estimate the size. If the 22 kDa proteinase K product was processed from both termini, it is feasible that the enhanced toxicity was as a result of either differential N-terminal and/or C-terminal processing. For instance, the difference in toxicity between soluble CytA and the N-terminally processed trypsin-treated F28 (see Table 2) can be attributed to the N-terminal processing, based on the assumption that soluble CytA was only partially processed (Fig. 1, lane 3). However, the following observations support the hypothesis that C-terminal processing was responsible for the further

increase in toxicity. First, when the proteinase-K-treated products F24 and F30 with the same N-terminus, but different molecular masses, were assayed against rat erythrocytes it was found that F24 was at least twofold more haemolytic than F30 (Table 2). Second, the same assay showed that the 22 kDa F24 and F37 were equally haemolytic even though they had different N-termini (Table 2). Third, the 22 kDa F24 from trypsin digestion was more cytolytic to the *Aedes aegypti* cell line than the 24 kDa F28, although both products had the same N-terminus (data not shown). In the light of the results above it is clear that processing at both termini contributes to the increase in toxicity. Unfortunately since it was not possible to produce a CytA derivative processed only from the C-terminus, the relative contribution of the C-terminal processing of this toxin cannot be assessed. Although the results discussed above were from *in vitro* experiments, a CytA product with a similar size to the 22 kDa proteinase K product and same N-terminus was obtained when mosquito gut extract was used to activate soluble CytA (Fig. 2, lanes 1 and 2). This suggested that the extent of processing is probably the same in the mosquito gut and hence the 22 kDa protein is the most active. Therefore, it appears that CytA, like other Bt δ -endotoxins, is processed to a well-defined protease-resistant product and this processing enhances the toxicity both *in vitro* and *in vivo*.

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