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Cyt2Ba of *Bacillus thuringiensis israelensis*: Activation by putative endogenous protease

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Dedicated to the memory of our dear friend and colleague, Dr. Yossef Sofer, who untimely passed away on January 31, 2006.

Abstract

The gene *cyt2Ba* of *Bacillus thuringiensis* subsp. *israelensis* was cloned for expression, together with *p20*, in an acrystalliferous strain. The large hexagonal crystals formed were composed of Cyt2Ba, which facilitated its purification. Crystal solubilization in the presence of endogenous proteases (with spores and cell debris) enabled quick and simple procedure to obtain rather pure and active toxin species by cleavage between amino acid residues 34 and 35, most likely by a camelysin-like protease that was discovered in association with activated Cyt2Ba. The product of this cleavage displayed haemolytic activity comparable to that of exogenously activated Cyt2Ba. The sequence of this putative protease shares high homology with the cell envelope-bound metalloprotease (camelysin) of the closely related species *Bacillus cereus*.

Keywords: *Bacillus thuringiensis* subsp. *israelensis*; Cyt2Ba; Camelysin

Bacillus thuringiensis is a gram-positive soil bacterium considered as the most promising biological control agent against insect larvae [1]. During sporulation, this entomopathogenic bacterium produces parasporal crystalline proteins (δ-endotoxins), which possess larvicidal and cytolytic activities, as well as endo-proteases [2]. The crystal in subsp. *israelensis* is composed of four main proteins, Cry4Aa, Cry4Ba, Cry11Aa, and Cyt1Aa. The genes for these and other Cry and Cyt proteins are located on the large plasmid (128 kb) pBtoxis of *B. thuringiensis* subsp. *israelensis* [3]. Analysis of the plasmid sequence revealed, in addition to *cyt1Aa*, genes for two other Cyt proteins, *cyt2Ba* [4] and *cyt1Ca* [3].

Cyt1Aa was the first cytolytic toxin to be isolated and comprehensively characterized [5]. Other cytolytic proteins have been detected later in different strains of *B. thuringiensis*, some related to Cyt1Aa whereas others have been classified into other groups based on immunological properties and sequence homologies [6]. Purified Cyt1Aa is cytolytic to cells of Dipteran origin, and at several fold higher concentrations, it is also toxic to a broad range of cells from both vertebrate and invertebrate origins [7]. Maximal Cyt1Aa toxicity occurs upon alkali solubilization and proteolysis from both N- and C-termini, converting the pro-toxin of 27 kDa to an activated form (22–25 kDa) [8].

Cyt2 toxins from various mosquito larvicidal *B. thuringiensis* subspecies have been identified and partially characterized: Cyt2Aa from *darmstadtiensis* and *kyushensis* [9,10], Cyt2Ba, Cyt2Bb, and Cyt2Bc from *israelensis* [4], *jegathesan* [11], and *medellin* [12], respectively. Cyt2Aa

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shows 39% identity and 70% similarity in amino acid sequence with Cyt1Aa [4], and 67% similarity with Cyt2Ba. The presence of *cyt2* genes in various subspecies of *B. thuringiensis* was correlated with their anti-dipteran activities [6].

A mixture of *B. sphaericus* with either Cyt2Ba or Cyt1Ab was synergistic towards larvae of *Aedes aegypti* and *Culex quinquefasciatus* [13]. Cyt2Bc was about half as active against susceptible mosquitoes as Cyt2Ba, and less toxic than Cyt1Aa by a factor of 4–12 [6]. Cyt2Bb and Cyt1Aa were comparably haemolytic. Haemolysis by Cyt2Aa displayed a sigmoidal dose–response curve, suggesting cooperativity between its monomers [10].

The proteolytic enzymes of *B. thuringiensis* (belonging to the cysteine, metallo, and serine families) can process or degrade protoxins [2]. One of its zinc-requiring metalloproteases, for example, InhA can specifically degrade both the inducible antibacterial peptides and the collagen-containing substrate in various lepidopteran and dipteran larvae [14]. It may thus be implicated in the mechanism by which *B. thuringiensis* invade the haemocoel and interfere with the immune system of the host. InhA2 metalloprotease is not the primary virulence factor but provides synergism of *B. thuringiensis* spores to the toxicity of Cry1C against *Galleria mellonella* following infection via the oral route [15].

Here, *cyt2Ba* was cloned, with or without the *p20* ''helper'' gene, in acrystalliferous strain of *B. thuringiensis*. Purified Cyt2Ba crystals were solubilized and activated by endogenous and exogenous proteases to explore haemolytic and larvicidal activities of the obtained toxin. A new putative endogenous protease camelysin seems to activate Cyt2Ba protoxin.

Materials and methods

Bacterial strains and plasmids. Strain 4Q2-72 of *B. thuringiensis* subsp. *israelensis*, which bears pBtoxis as its only plasmid [3], was kindly supplied by D.R. Zeigler (*Bacillus* Genetics Stock Center, Colombus, Ohio). The acrystalliferous plasmid-less derivative strain IPS78/11 was kindly obtained from D. Ellar (Cambridge, UK). *Escherichia coli* XL1-Blue MRF' (Stratagene, La Jolla, CA) was used as a host for sub-cloning in the *E. coli–B. thuringiensis* 6.5 kb shuttle vector pHT-315 [16].

DNA isolation and PCR amplification. pBtoxis was obtained from exponentially growing cells of strain 4Q2-72 as described before [17].

The following oligonucleotide primers were used for PCR amplifications: For the *cyt1Aa* promoter: 5′-GGGATTTAGAgCATGcTTAAGT AGAATAGACG-3′ (Pr-*cyt1Aa-Sph*I-D) and 5′-CAATGATTTAAATT TTCCATAtgTAAACAACTCC-3′ (Pr-*cyt1Aa-Nde*I-R). For *cyt2Ba*: 5′-G GGGGATTATTAAcATATGCACC-3′ (*cyt2Ba-Nde*I-D) and 5′-ATCT CTCTAgAGTACTATGGCTATTTC-3′ (*cyt2Ba-Xba*I-R). For *p20*: 5′-C CGTTtCTAgAGTAGAAGTCATGTTAGC-3′ (*p20-Xba*I-D) and 5′-CA AATTCATCTGAGcTcTTATATCGATTCAC-3′ (*p20-Sac*I-R). The small letters in the primers sequence represent bases modified from the original sequence to encode an appropriate enzyme restriction site.

Amplification was carried out with the high fidelity Vent DNA polymerase (New England Biolabs) in a DNA MiniCycler (Biometra), under the following conditions: 4 min at 95 °C initial denaturation, followed by 30 cycles of 40 s at 95 °C, 45 s at 54 °C, 1–2 min at 72 °C, and additional step at 72 °C for 10 min. Samples from each PCR were electrophoresed on 0.7% agarose gels and DNA visualized by ethidium bromide.

Cloning cyt2Ba into pHT315 and sequencing. The amplicons *cyt1Aa* promoter (552 bp), *cyt2Ba* with downstream sequence (858 bp), and *p20* (1024 bp) were purified from agarose gel and digested by the appropriate restriction enzymes. The first two were *Nde*I-ligated into *Sph*I–*Xba*I-digested pHT315, and the latter was subsequently ligated into the *Xba*I–*Sac*I sites. DNA was introduced into *E. coli* XL1-Blue MRF' by electroporation (using a Bio-Rad mini apparatus set). The plasmid was isolated, sequenced, and electroporated into the acrystalliferous strain IPS78/11 of *B. thuringiensis* subsp. *israelensis*. Screening for transformants was performedon LB plates containing 20 μg ml^{-1} erythromycin at 30 °C. Crystals were seen under a phase-contrast microscope. DNA sequences were verified by ABI PRISM® 3100 Genetic Analyzer.

Crystal protein preparation. Bacillus thuringiensis subsp. *israelensis* strain IPS78/11 harboring *cyt2Ba*, with or without *p20*, was grown in PGSM medium [18] at 30 °C for 4 days with erythromycin (10 μg ml^{-1}). Cultures were centrifuged (15,000*g* for 10 min), the Sediment (E) containing crystals, spores, and cell debris was washed twice with cold water (Fig. 1). The crystals were separated from the other components by biphasic separation (1% sodium sulfate, water, dichloroethane 1:1.2:1) for 20 min. The water phase containing the crystals (Sediment N) was centrifuged (45 min at 15,000*g*) and washed in cold water. Protein concentrations were determined by the method of Bradford using BSA as a standard.

Electron microscopy. Cultures were grown for 48 h in CCY sporulation medium [19], and samples were fixed, dehydrated, embedded, cut, and stained as described before [20]. The sections were dried and viewed by JEOL JEM1230 EM transmission electron microscope at an accelerating voltage of 80 kV.

Solubilization and activation of crystal protein. Crystals were solubilized, in both, presence (Sediment E) or absence (Sediment N) of cell debris and spores, by incubation at 37 °C (1 h in 50 mM Na_2CO_3, 10 mM DTT) at the pH range of 8–12, followed by centrifugation. The supernatants containing solubilized Cyt2Ba were adjusted to pH 8 with 1 M Tris–HCl, pH 6.5. Solubilized Cyt2Ba from Sediment N was activated with 10% w/w of trypsin, chymotrypsin or proteinase K.

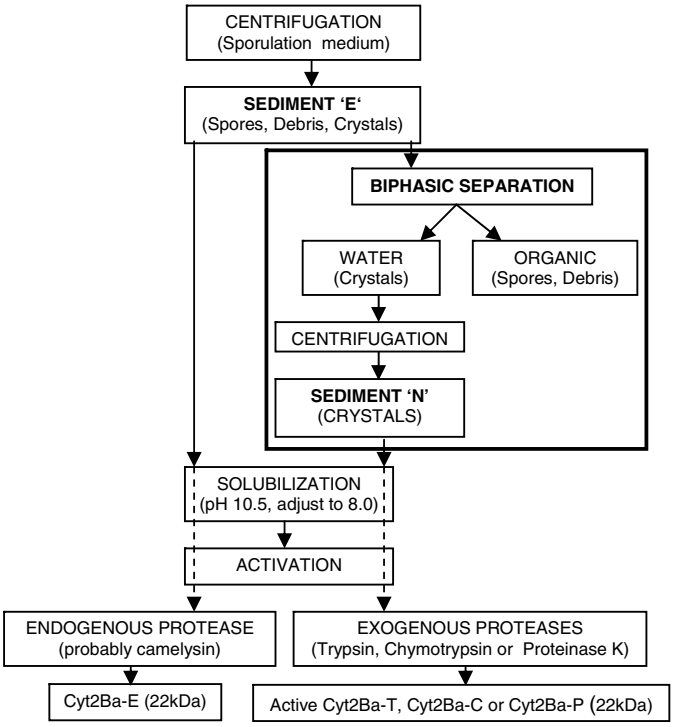

Fig. 1. Flow diagram of Cyt2Ba separation.

Sediment N was also solubilized in the presence of the following protease inhibitors: 5 mM of 1,10-phenanthroline (OP), 40 mM EDTA or 1 mM phenylmethyl-sulfonyl fluoride (PMSF).

Purification of Cyt2Ba on DEAE–cellulose. Activated Cyt2Ba was purified by anion-exchange chromatography using FPLC (Pharmacia) supplied with DEAE–cellulose column, pre-equilibrated with 50 mM Tris–HCl buffer, pH 8.5. Cyt2Ba was eluted with a gradient of 0–0.6 M NaCl in the same buffer at a flow rate of 1.5 ml min^{-1}. Fractions were collected and monitored at 280 nm.

SDS–PAGE. Protein homogeneity and molecular weight were determined by SDS–PAGE in 4% stacking and 12.5% separating polyacrylamide gels, using Dalton Mark VII-L mixture (Sigma) as standards.

Haemolysis assay. Rabbit red blood cells (RBS) were washed with 0.1 M PBS, pH 6.5, and suspended to 0.1–1% v/v in the same buffer. Aliquots of solubilized or activated Cyt2Ba were mixed with 1 ml of suspended RBS and incubated at 37 °C for 3 or 24 h. The absorbance of the released hemoglobin in the supernatant was monitored at 540 nm (A_{540}). Percent haemolysis was calculated by: $\frac{A_{540}-A_{540}^{0\%}}{A_{540}^{100\%}-A_{540}^{0\%}} \times 100\%$, where $A_{540}^{100\%}$ was obtained by incubating the RBC with water leading to 100% lysis, and $A_{540}^{0\%}$ by incubating the cells in PBS.

Mosquito larvicidity assay. Mixtures of Cyt2Ba crystals and spores were added to 10 third instar *A. aegypti* larvae in 100 ml sterile tap water. Larval viability was counted after 24 h at 28 °C as described previously [21]. Values of LC_{50} and LC_{95} (concentrations of cells that kill 50% and 95% of the exposed populations, respectively) were determined by probit analysis with eight doses [22].

Azocasein assay. Samples of solubilized Cyt2Ba were added to 1 ml of 0.3% azocasein (Sigma) in 50 mM Tris–HCl buffer, pH 8, and incubated at 37 °C for 1 h as described previously [23]. One unit of activity is the amount of enzyme that causes an increase in A_{400} of 1 OD min^{-1}.

N-terminal protein sequencing. Cyt2Ba-N and Cyt2Ba-E (Cyt2Ba, with and without biphasic separation, respectively; Fig. 1) were separated on 12.5% SDS–PAGE and electro-transferred to polyvinylidene difluoride (PVDF) membrane [24]. The blotted membrane was stained with Coomassie blue-R250, Cyt2Ba bands were excised from the membrane, and N-terminal sequences were determined by automated Edman degradation on an Applied Biosystems PROCISE-491.

Proteolysis and mass spectrometric analysis. Coomassie blue-stained Cyt2Ba-E band was in-gel reduced with 10 mM DTT, incubated at 60 °C for 30 min, alkylated with 10 mM iodoacetamide at room temperature for 30 min and proteolyzed overnight at 37 °C by modified trypsin (Promega) at a 1:100 enzyme-to-substrate ratio. The tryptic digest peptides were resolved by reverse-phase chromatography on 0.1× 300-mm fused silica capillaries (J&W, 100 μm ID) packed with POROS R2-10 reversed-phase material (Applied Biosystems) and eluted using 80 min linear gradient of 5–95% acetonitrile with 0.1% formic acid in water at a flow rate of about 0.3 ml min^{-1}. Mass spectrometry (DecaXP, Thermo, San Jose, CA) was performed in the positive mode using repetition of full MS scan followed by fragmentation of the three most dominant ions selected from the first full MS scan. The mass spectrometry data were compared to simulated proteolysis and CID of the proteins in the NR-NCBI database using the Sequest software (J. Eng and J. Yates, University of Washington and Finnigan, San Jose) and the Pep-Miner software (I. Beer, IBM).

Results and discussion

Separation, solubilization, and activation of Cyt2Ba from parasporal crystals

The concentration of Cyt2Ba in parasporal crystals of *B. thuringiensis* subsp. *israelensis* is very low and can only be revealed by immunoblot analysis or PCR screening [4]. Therefore, *cyt2Ba* was cloned, with or without *p20* encoding the "helper" P20 [25], in the acrystalliferous strain IPS78/11 named IPS (*cyt2Ba/p20*). Cyt2Ba was assembled to a hexagonal crystal (Fig. 2A), which facilitated its purification (Fig. 1). The size of the hexagonal-shaped crystals produced ranged between 0.4 and 0.6 μm (Fig. 2A); in the absence of *p20* (in clone IPS (*cyt2Ba*)), the crystals were about half the size (data not shown). Likewise, expression of *cyt1Aa* alone in acrystalliferous strains of *B. thuringiensis* is poor and no obvious inclusions are observed, whereas relatively large ovoidal, lemon-shaped inclusions of Cyt1Aa are produced in the presence of *p20* as well [26]. Furthermore, expression of *p20* raises the rate of production of nascent Cry1Ac [27] and heterologous truncated Cry1Ca [28] in acrystalliferous *B. thuringiensis* subsp. *kurstaki*, apparently due to protection from endogenous proteases. Indeed, highest endoprotease activity in vivo occurs concurrent with expression of toxins.

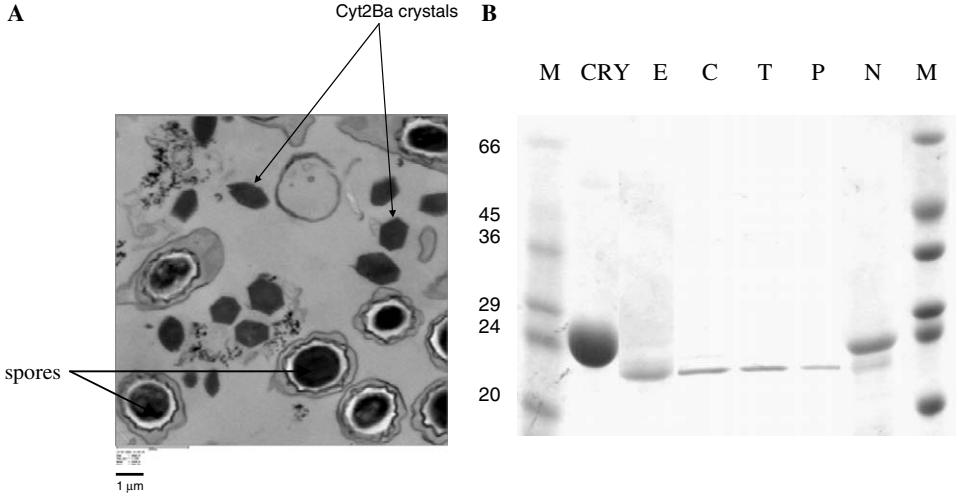

Fig. 2. Electron micrograph (A) and analysis (B) of solubilized and activated Cyt2Ba crystals on SDS–PAGE (12.5%). (B) Samples (4–8 μg of protein) of Cyt2Ba-N (solubilized at pH 10.5) were activated with exogenous proteases (10% w/w, 37 °C, 1 h). Lanes: M, molecular mass markers; lane CRY, Cyt2Ba crystals (approximately 30 μg); lane E, solubilized Cyt2Ba without biphasic separation (Cyt2Ba-E); lane C, activated with chymotrypsin (Cyt2Ba-C); lane T, activated with trypsin (Cyt2Ba-T); lane P, activated with proteinase K (Cyt2Ba-P); lane N, solubilized Cyt2Ba after biphasic separation (Cyt2Ba-N).

Sediment E, obtained from strain IPS (*cyt2Ba/p20*) (crystal protein preparation), including crystals, spores, and debris, was divided into two portions for further purification. The first (Fig. 1, left-hand side) was solubilized and centrifuged, and the supernatant was adjusted to pH 8. The supernatant consisted of a predominant protein band with a molecular mass of 22 kDa (Fig. 2B, lane E), which was designated Cyt2Ba-E for endogenous bacterial proteases that seem to digest the intact Cyt2Ba (see below).

The second portion of Sediment E (Fig. 1, right-hand side) was partially purified by biphasic separation. The crystals from the water phase were centrifuged and washed in cold water. This Sediment (designated N for Native Cyt2Ba) was solubilized, centrifuged, and the supernatant pH adjusted to 8. The protein fraction contained a main band on SDS–PAGE, with molecular mass of about 24 kDa (Fig. 2B, lane N).

The crystal purification method described here is based on a biphasic separation using dichloroethane as an organic phase (Fig. 1). The crystals thus obtained were purified only partially and contained relatively high percent of spores and cell debris, but the endogenous proteases contaminating this fraction were removed or inactivated by the organic phase. Thereby, the obtained soluble protein fraction contained only unprocessed, native Cyt2Ba form (Cyt2Ba-N). The haemolytic activity of Cyt2Ba-N was negligible, confirming again the non-processed character of Cyt2Ba (see below).

Soluble Cyt2Ba-N was incubated with each of the following proteases, chymotrypsin, proteinase K and trypsin, and the proteolytic products were marked Cyt2Ba-C, Cyt2Ba-P, and Cyt2Ba-T, respectively. All three products yielded a major band of 22 kDa (Fig. 2B, lanes: C, P, T), with the same size as Cyt2Ba-E. An additional treatment of Cyt2Ba-E with the above-mentioned proteases did not affect its molecular weight (data not shown). Crystals of several Cyt2, previously purified by ultracentrifugation on discontinuous sucrose or NaBr gradients, are contaminated by low amounts of spores and debris [11,12,20]. In those cases, however, the associated proteases are still active, and Cyt2 thus obtained is partially degraded. Solubilizing the crystals on ice rather than at 37 °C only partially prevents the activity of endogenous proteases [20]. The endogenous protease activity is not entirely abolished even upon adding EDTA to Cyt2Bb inclusions before purification on NaBr gradient [11].

The effect of pH on the efficiency of Sediment N solubilization (Fig. 1) was examined by estimating Cyt2Ba-N concentration and SDS–PAGE analyses. Sediment N was solubilized upon varying pH of the buffer from 8 to 12. In the lower basic buffers, the quantity of solubilized Cyt2Ba-N was negligible, but strong basic conditions led to rather high concentrations, from 0.8 mg ml^{-1} at pH 10.5 to 1.5 mg ml^{-1} at pH 12 (data not shown). A significant Cyt2Ba-N band at 24 kDa was only found at the strong basic conditions. However, after processing of these species by exogenous proteases, the toxin obtained at pH 10.5 was most haemolytic. The low activity of Cyt2Ba solubilized at pH 12 can be explained by changes of the toxin configuration.

Endogenous protease activity in Sediment E

To examine the possibility that endogenous proteases are responsible for the proteolytic activation of Cyt2Ba-E, Sediment E, containing crystals, spores, and cell debris, was incubated with BSA at 37 °C for 1 h. Sediment E cleaved native BSA (66 kDa) to smaller polypeptides (50 and 46 kDa), which could not have been derived from Sediment E itself. Sediment E contained a single protein band with a molecular mass of about 24 kDa (data not shown). It can therefore be concluded that Sediment E contains endogenous protease(s) that degrade native Cyt2Ba (24 kDa) to Cyt2Ba-E (22 kDa), while in the biphasic separation method these proteases are removed or inactivated by the organic phase. Most proteolytic activities in subsp. *tenebrionis* and *kurstaki* stem of endogenous metalloproteases, while in subsp. *israelensis*, 50% activity is contributed by serine proteases [29].

Amino acid fragments of Cyt2Ba-N, Cyt2Ba-E, and the endogenous protease

Samples of Cyt2Ba-N and Cyt2Ba-E, excised from PVDF membrane, were subjected to Edman degradation. The N-terminal sequence of Cyt2Ba-N, MHLNN, is identical to that of Cyt2Ba [5], indicated that it was not degraded during purification and solubilization. The N-terminal sequence of Cyt2Ba-E was however indefinite. To clarify the reason for this ambiguity, Cyt2Ba-E (cut from gel) was subjected to total tryptic digestion, and the fragments were resolved by reverse-phase HPLC followed by mass spectrometric analysis. Two peptides that belong to the Cyt2Ba sequence were identified: TVPSSDITNFNEI FYVEPQYIAQAIR and LTNTFQGAIDPLTLNFNF EK. The former was identical to the original amino acid residues 35–60 of Cyt2Ba [5], but not overlapping the tryptic digest product (cleaved between L (Leu) 34 and T (Thr) 35), as the N-terminus of Cyt2Ba-E, having Threonine-35 as the first residue; it resembles the N-terminus of the previously observed Cyt2Aa [20]. The latter peptide (amino acid residues 61–80) indeed matched the tryptic cleavage site after arginine (between R (Arg) 60 and L (Leu) 61).

The tryptic digest of Cyt2Ba-E surprisingly included 16 additional peptides, nine of which are included in the major seven (Table 1A). Blast analysis discovered that all 7 (totaling 120 amino acids) are identical to parts of the 199 amino acid-long protease camelysin from *Bacillus cereus* [30], a species closely related genetically to *B. thuringiensis* [31]. Linking the seven peptides by sequence alignment yielded four larger fragments (and three gaps) found between amino acids 30 and 173 of the latter (Table 1B). The putative

Table 1A
Peptide sequence and mass fingerprint of major additional fragments

Sequence	Mol mass	No.
FLWNWDKQSEPVYETTLADLQK	2711.3	1
FFSDKEVSNNTFAAGTLDLTLNPK	2629.3	2
GGLAAGTEDYLWVQFEFVDDGK	2417.1	3
TLVDIKDLKPGDSVK	1627.9	4
KEFLLQNSGSLTIK	1577.9	5
DAKGDNAGEDFGK	1323.6	6
DIFAPEWGEK	1191.6	7

Table 1B
Linked peptides (from A), based on sequence alignment with camelysin (displayed in C)

FFSDKEVSNNTFAAGTLDLTLNPK^2TLVDIKDLKPGDSVK^4
KEFLLQNSGSLTIK^5----------DAKGDNAGEDFGK^6-----
FLWNWDKQSEPVYETTLADLQK^1--------DIFAPEWGEK^7
GGLAAGTEDYLWVQFEFVDDGK^3

- indicate amino acid gaps between #: 83–93; 107–111; 134–141, when aligned to the cell envelope-bound metalloprotease (camelysin) of *B. cereus* ATCC 14579, Accession No.: NP_831063 [30].

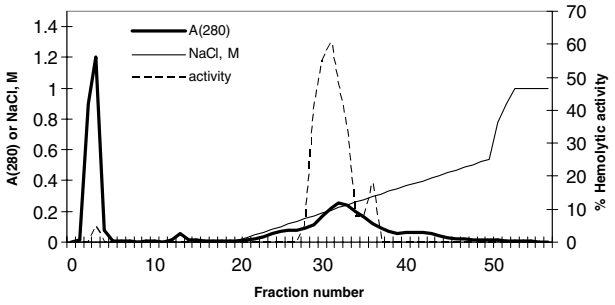

Fig. 3. Chromatogram of Cyt2Ba-E separation on DEAE-cellulose column (1 × 26 cm, 0–0.6 M NaCl gradient in 0.05 Tris–HCl buffer, pH 8.5).

camelysin found here displays 96.6% identity to a metalloprotease of *B. thuringiensis* subsp. *konkukian* (Accession No. YP_035508) and 52.9% identity (and 73.9% similarity) to the cell envelope of *B. thuringiensis* subsp. *israelensis* (ZP_00738775) (Table 1C). It also resembles putative spore coat-associated proteins, one (with 95.8% identity) of *Bacillus anthracis* (NP_843760; [32]), another (with 55.4% identity and 75.6% similarity) of pBtoxis from *B. thuringiensis* subsp. *israelensis* (CAD30177; [3]).

The proteolytic activity of camelysin is preferentially directed towards the peptide bonds when aliphatic hydrophobic or hydrophilic amino acid residues are at C side of the peptide bond [33]. The fact that threonine is located at the N-terminus of Cyt2Ba-E suits the concept that camelysin from debris or spore envelope present in Sediment E is responsible for endogenous degradation of Cyt2Ba.

The hypothesis that the metalloprotease camelysin is responsible for the activation of Cyt2Ba in Sediment E was tested by solubilizing the crystals in the presence of protease inhibitors. With a metalloprotease inhibitor (OP or EDTA), the solubilized protein remained at 24 kDa (as Cyt2Ba-N in Fig. 2B) and demonstrated a low haemolytic and typical camelysin activity of 5% and 8%, respectively. However, the main product obtained by solubilization in the presence of the serine proteases inhibitor PMSF had a MW of 22 kDa, as in the absence of any protease inhibitor. These fractions possessed haemolytic activity (65% at 3 h), typical for Cyt2Ba-E, and a rather high proteolytic activity of camelysin towards azocasein (0.006 U). Cyt2Ba-E, purified on DEAE–cellulose column as a single peak at 0.2–0.3 M NaCl (Fig. 3), did not possess a typical camelysin proteolytic activity on azocasein; activity of 0.03 U ml^{-1} was detected before purification.

Bacillus thuringiensis crystal polypeptides are activated in vivo by proteolysis in the insect gut [34,35] and by several endogenous proteases during sporulation [2,36]. Under denaturing/reducing conditions, for instance, such endogenous metalloprotease of subsp. *kurstaki* mediates proteolytic processing of 132 kDa protoxin to an active 66 kDa toxin [37], highly active against the cotton leafworm, *Spodoptera littoralis*, a species insensitive to native *kurstaki* crystals or toxins generated by exogenous proteases [38].

Biological activities

Haemolysis

Haemolytic activities against 0.2% RBC were determined for solubilized and activated Cyt2Ba (20 μg ml^{-1}). Cyt2Ba-N was only slightly haemolytic (less than 1%).

Table 1C
Alignment of the peptide sequences (from A) to the *B. cereus* camelysin [30]

Putative	1	FFSDKEVSNNTFAAGTLDLTLNPKTLVDIKDLKPGDSVKKEFLLQNSGSLTIK--------
		FFSDKEVSNNTFAAGTLDLTLNPKTLVDIKDLKPGDSVKKEFLLQNSGSLTIK
Camelysin	30	FFSDKEVSNNTFAAGTLDLTLNPKTLVDIKDLKPGDSVKKEFLLQNSGSLTIKDVKLATK
Putative		----DAKGDNAGEDFGK-----FLWNWDKQSEPVYETTLADLQK--------DIFAPEWG
		DAKGDNAGEDFGK FLWNWDKQSEPVYETTLADLQK DIFAPEWG
Camelysin	90	YTVKDAKGDNAGEDFGKHVKVKFLWNWDKQSEPVYETTLADLQKVDPDLLAKDIFAPEWG
Putative		EKGGLAAGTEDYLWVQFEFVDDGK 120
		EKGGLAAGTEDYLWVQFEFVDDGK
Camelysin	150	EKGGLAAGTEDYLWVQFEFVDDGK 173

Numbers of amino acids in each of the sequences are shown.

Trypsin-activated Cyt2Ba-N (Cyt2Ba-T) was 20% and 50% haemolytic within 3 h after solubilization at pH 12 and 10.5, respectively. Processing by chymotrypsin (Cyt2Ba-C) or proteinase K (Cyt2Ba-K) caused 27% and 34% haemolysis, respectively, after solubilization at pH 10.5. On the other hand, the haemolytic activity of Cyt2Ba-E (56% haemolysis) was not affected by the exogenous proteases. Incubation of varying Cyt2Ba-E concentrations with 0.2% RBC for 3 h yielded an HC_{50} value for haemolysis of 17.6 μg ml^{-1}. Inverted correlation was found between RBC concentration and haemolysis in assays performed during 3 h (data not shown): less than 20% at 1% RBC, reaching 70% at 0.1% RBC. When the assay was performed during 20 h, haemolysis was more than 80% at all RBC concentrations. This inverse correlation can be explained by demand for a certain number of toxin molecules needed for haemolysis of each red blood cell on its membrane [39,40]. At 3 h incubation, 100% haemolysis was not achieved even at low RBC concentration, perhaps due to slow aggregation of Cyt2Ba molecules needed for the cell membrane damage.

Mosquito larvicidity

The activity against *A. aegypti* larvae of Cyt2Ba crystals in Sediment E was determined in the concentration range of 16–800 μg ml^{-1} by larval mortality after 24 h: values of LC_{95} and LC_{50} reached 150 and 33 μg ml^{-1}, respectively. The previously found 4-fold higher activity of Cyt2Ba against *A. aegypti* [13] can be explained by different proportion of crystals and spores in the Sediment used.

Some *B. thuringiensis* proteases, synthesized during sporulation, are metalloproteases with possible physiological functions. They can either degrade or activate δ-endotoxins and affect toxicity against the target insect, for example. In neutral protease A-deficient strains, Cry3Bb and Cry1Bb were not degraded as they were in the wild-type strain [29,41,42]. Results of such studies may enable to raise efficacy of entomopathogenic bacteria for Integrated Pest Management.

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