Proteolytic Processing of the Cyt1Ab1 Toxin Produced by *Bacillus thuringiensis* subsp. *medellin*

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Bacillus thuringiensis produces δ-endotoxins that require proteolytic processing to become active. The activation of the *B. thuringiensis* subsp. *medellin* 28 kDa (Cyt1Ab1) cytolytic toxin by trypsin, chymotrypsin and gut extract from *Culex quinquefasciatus* larvae was analyzed. The Cyt1Ab1 toxin of *B. thuringiensis* subsp. *medellin* was processed by all proteases tested to fragments between 23 and 25 kDa, while processing of the Cyt1Aa1 toxin produce fragments between 22.5 and 24.5 kDa. The Cyt1Ab1 toxin was preferentially processed at the alkaline pH of 12. The *in vitro* proteolytic processing of the Cyt1Ab1 toxin by *C. quinquefasciatus* larvae midgut extract showed a 25 kDa fragment; a similar result was observed when the activation was performed in the *in vivo* experiments. The solubilized Cyt1Ab1 toxin and the protease resistant cores generated by *in vitro* processing showed hemolytic activity but not mosquitocidal activity. Amino terminal sequence of the *C. quinquefasciatus* gut extract resistant fragment indicated that the cutting site was located between Lys^{31} and Asp^{32}, with a sequence DDPNEKNNHNS; while for the trypsin-resistant fragment the cutting site was determined between Leu^{29} and Arg^{30}, and for the chymotrypsin-resistant fragment between Arg^{30} and Lys^{31}.

Key words: *Bacillus thuringiensis* - Cyt1Ab1- cytolytic - hemolytic - proteolytic processing - midgut proteases

The *Bacillus thuringiensis* parasporal inclusions are composed of polypeptides, grouped in two types Cry and Cyt toxins (Crickmore et al. 1998, Schnepf et al. 1998). The Cry type of proteins are toxic to different orders of insect such as Lepidoptera, Diptera and Coleoptera. The Cyt proteins with molecular weight between 25 and 30 kDa display cytolytic and mosquitocidal activity (Chilcott & Ellar 1988, Koni & Ellar 1994, Orduz et al. 1996); however, the mosquitocidal activity is lower than the Cry toxins (Chang et al. 1993, Crickmore et al. 1995, Orduz et al. 1996).

Several reports have demonstrated the *in vivo* and *in vitro* activation of the Cry-type of toxins (Carroll & Ellar 1989, Pfannenstiel et al. 1990, Ogiwara et al. 1992, van Frankenhuyzen et al. 1993, Dai & Gill 1993), and the Cyt-type of toxins (Al-yahyaee & Ellar 1995, Li et al. 1996), and some of them when activated by proteases showed an enhanced cytolytic and larvicidal activity (Chilcott & Ellar 1988, Knowles et al. 1992, Koni & Ellar 1994).

B. thuringiensis subsp. *medellin* is a mosquitocidal strain described by Orduz et al. (1992). Its parasporal inclusion contains three main proteins of 94 kDa (Cry11Bb1), 68 kDa, and a 28 kDa (Cyt1Ab1) (Orduz et al. 1994, 1998, Thiéry et al. 1997). It has been suggested that Cyt toxins act sinergistically with the Cry toxins to produce mosquitocidal activity (Chang et al. 1993, Crickmore et al. 1995). The protein Cyt1Ab1 of 28 kDa produced by *B. thuringiensis* subsp. *medellin* acts synergically with the 68 kDa (Orduz et al. 1996), and the Cyt1Ab1 toxin shares 86% aminoacid identity with the toxin Cyt1Aa1 of *B. thuringiensis* subsp. *israelensis*. The *cyt1Ab1* gene encodes a polypeptide of 251 amino acids with a predicted molecular mass of 27.5 kDa (Thiéry et al. 1997). It has been demonstrated that the Cyt1Aa1 protein is also toxic to coleopteran insects, and that it can reduce the resistance ratio of *Chrysomela scripta* to the Cry3Aa toxin (Federici & Bauer 1998). At the same time, Thiery et al. (1998) demonstrated that the Cyt1Ab1 protein can also reduce the resistance of *Culex quinquefasciatus* to the binary toxin of *B. sphaericus.*

In this work, we evaluated the *in vitro* activity of standard and midgut proteases on the Cyt1Ab1

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toxin and analyzed the resulting products. We also studied the effect of the pH on the *in vitro* proteolysis of the Cyt1Ab1 protein, determined the *in vivo* processing of the toxin, and assayed the hemolytic and mosquitocidal activity of the *in vitro* processed Cyt1Ab1 protein.

MATERIALS AND METHODS

Bacterial strains, mosquitoes, antibodies and proteases - The Cyt1Ab1 toxin was obtained from a recombinant strain SPL407, a crystal negative strain of *B. thuringiensis* subsp. *thuringiensis* carrying the *cyt1Ab1* gene cloned from *B. thuringiensis* subsp. *medellin* (Thiéry et al. 1997). The *B. thuringiensis* recombinant strain carrying the plasmid pWF45 encoding the Cyt1Aa1 protein, was kindly provided by Dr Brian A Federici, University of California. The bacteria were grown in Luria-Bertani (LB) liquid medium or on LB agar plates (Sambrook et al. 1989) supplemented with erythromycin (25 µg/ml). *C. quinquefasciatus* were maintained under laboratory conditions (30°C±2°C and a 12:12 light:dark photoperiod). Antibodies anti-Cyt1Ab1 were raised in mice according to Sambrook et al. (1989). Trypsin and chymotrypsin were purchased from Sigma Chemical Co. (St. Louis, Mo).

Gut extract preparation - The gut extract was prepared from third instar *C. quinquefasciatus* larvae according to Garczynski et al. (1991) with modifications. Mosquito larvae were chilled on ice for 30 min. The guts were excised and collected in MET buffer (Mannitol 300 mM, EDTA 5 mM, Tris 20 mM, pH 7.2). Then, they were disrupted and centrifuged. The supernatant with protease activity was recovered and the protein content was determined by Bradford microassay protocol (Bio-Rad, Hercules, CA, USA), using bovine serum albumin (BSA) as standard.

Crystal purification - Recombinant strains containing the *cyt1Aa1* and *cyt1Ab1* genes were incubated in 5 ml of M-one liquid medium (Restrepo et al. 1997) supplemented with erythromycin (25 µg/ml), for 12 h at 30°C and 200 rpm. Cultures were transferred to 200 ml of M-one liquid medium and incubated for 120 h at 30°C and 200 rpm until sporulation. The presence of crystals and spores was checked by light microscopy. The medium was removed by centrifugation at 9,000 rpm, at 4°C for 15 min, the pellet (crystal-spore complex) was resuspended in 1/20th of the original volume in 1 M NaCl and shaken for 2 h, at 37°C and 150 rpm to neutralize protease activity, and then washed twice in ice-cold distilled water supplemented with 1 mM phenyl-methyl-sulfonyl-fluoride (PMSF) and 10 mM EDTA (Thiéry et al. 1997). Crystals were separated on a discontinuous sucrose gradient 67-79% (w/v) (Thiéry et al. 1998) at 25,000 rpm for 2 h at 4°C. The pure crystals were collected, washed four times with sterile ice-cold distilled water and concentrated by centrifugation at 4°C, 12,000 rpm during 10 min. Finally, the crystals were resuspended in 2 ml of sterile water, aliquoted, and stored at -20°C until use. The purity of crystals was determined by light-microscopy and the crystal composition was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and proteins were stained with Coomasie brilliant blue.

Solubilization of the Cyt1Ab1 crystals - The solubilization of Cyt1Ab1 crystals was performed at different pH with the buffer system listed in Table I. For the solubilization assay, 10 µl of crystals were incubated with 15 µl of each buffer at 37°C, 200 rpm during 2 h. Unsoluble material was removed by centrifugation at 12,000 rpm and soluble protein concentration was measured by Bradford as above.

In vitro proteolytic processing of the Cyt1Ab1 toxin - The Cyt1Ab1 toxin was treated with trypsin (10 units per mg of protein), chymotrypsin (40-60 units per mg of protein) and *C. quinquefasciatus* gut extracts in a 1:10 ratio (w/w, enzyme/ toxin) in a final volume of 10 µl and incubated at 30°C for 14 h in buffers of different pH. The resulting digests were examined by SDS-PAGE 10%, stained with Coomasie brilliant blue and analyzed by the NIH program v. 1.61 (Seebacher & Bade 1996).

The processing of the Cyt1Ab1 toxin in time was studied with trypsin, chymotrypsin and mosquito larvae gut extracts in a 1:2.5 ratio (w/w enzyme/toxin) using carbonate buffer pH 11. Ten microliters of each reaction were collected at different times (1, 5, 10, 30 min, 1, 2, 3, 14, and 24 h), and mixed with 15 µl of Laemmli buffer, 3 µl of PMSF 100 mM, and 3 µl EDTA 100 mM (pH 8.5). Samples were boiled for 5 min, and then subjected to SDS-PAGE 10%. Gels were stained with Coomasie brilliant blue and analyzed as above.

To compare the *in vitro* proteolytic processing of Cyt1Ab1 and Cyt1Aa1 crystals, both toxins were treated with trypsin, chymotrypsin and *C. quinquefasciatus* gut extract larvae in a 1:2.5 ratio (w/w enzyme/toxin) using carbonate buffer pH 11 during 24 h, subjected to SDS-PAGE 10% and analyzed as above.

In vivo proteolysis assays of the Cyt1Ab1 crystals - These assays were conducted in microplates containing five *C. quinquefasciatus* third instar larvae in each well and 1 ml of distilled water. Three different Cyt1Ab1 crystal concentrations were tested (25 µg/ml, 12.5 µg/ml and 6 µg/ml), and bioassays were incubated at room temperature

(26±2°C) until the first signs of larval intoxication were observed (1 to 2 h later). Larval guts were dissected and collected in a tube containing 15 µl of Laemmli buffer, 3 µl of PMSF 100 mM, and 3 µl EDTA 100 mM (pH 8.5). Samples were boiled for 5 min and subjected to SDS-PAGE 10%. Proteins were electro-transferred to nitrocellulose membranes at a constant current of 26 mA for 2 h. Non-specific binding sites were blocked with 3% gelatin in Tris buffer saline, TBS (Tris 20 mM, NaCl 500 mM, pH 7.5) for 1 h, then washed three times with TTBS (TBS, 0.05% Tween 20). Subsequently, the membrane was incubated with polyclonal anti-Cyt1Ab1 antibody (1:1000 working dilution) in 1% gelatin in TTBS for 1 h and washed three times in TTBS. Goat anti-mouse IgG (H+L)-alkaline phosphatase conjugate (1:1000 working dilution) was used as secondary antibody and incubated for 1 h, and then washed with TTBS. Color was developed by using a chromogenic reaction with nitroblue tetrazolium chloride (1 mg/ml) and 5-bromo-4-cloro-3-indolyl-phosphate (5 mg/ml) in carbonate buffer pH 9.85.

Identification of protease cleavage sites - The *in vitro*-processed Cyt1Ab toxin with trypsin chymotrypsin and *C. quinquefasciatus* gut extracts were separated by SDS-PAGE and transferred to PVDF membrane as described by Mozdzanowski and Speicher (1992). Individual bands were excised with a sterile blade and applied to a sample cartridge of an automated sequencer (Applied Biosystems model 477A). The analysis of aminoacid-phenylthiohydantoin derivatives was performed on line using an HPLC system (Applied Biosystems model 120A).

In vitro hemolysis assays - This assay was carried out as described by Thiéry et al. (1997) with a few modifications. Five ml of sheep blood were washed twice in 15 mM Tris-HCl 0.17 M NaCl, pH 8.0 and resuspended to a final concentration of 4.5% in the same buffer. The Cyt1Ab1 toxin was solubilized in NaOH 50 mM, EDTA 10 mM (pH 12) during 45 min at 37°C, and treated with trypsin, chymotrypsin and gut extracts from *C. quinquefasciatus* larvae as above. Five hundred µl of each concentration of the soluble and protease treated toxin were added to 500 µl of the erythrocyte suspension. Each treatment was carefully mixed and incubated at 37°C for 45 min and then centrifuged at 12,000 rpm for 2 min. The amount of hemoglobin released was estimated in a spectrophotometer at 535 nm. The half hemolytic dose (HD_{50}) defined as the amount of toxin needed to release half the hemoglobin from the erythrocytes was calculated by probit analysis using a computer program (Raymond 1995). Each assay was performed by triplicate in three different days.

Mosquitocidal activity assay - The toxicity of ten dilutions of crystals, solubilized and activated Cyt1Ab1 toxin were tested against *C. quinquefasciatus* first instar larvae in 24 well plates. Five mosquito larvae were set by well in 1 ml of sterile distilled water according to Orduz et al. (1996). Each toxin concentration was tested by duplicate, and bioassays were conducted in three different days. Larval mortality was scored 24 h later, the half lethal concentration (LC_{50}) was calculated as above.

RESULTS

Solubilization of Cyt1Ab1 parasporal inclusions - Solubilization of the Cyt1Ab1 crystals was directly proportional to the increase in pH. In general, the Cyt1Ab1 toxin was preferentially solubilized in higher amounts in buffers of basic pH. The acidic buffers (pH 5.0 and 6.0) and neutral buffers (pH 7.0) showed the lowest solubilization. In the basic buffers (pH 8.0 to 10.0), a gradual increase of soluble toxin was observed, obtaining the higher toxin concentration in the buffers with pH values between 10.6, and 12.0, with a maximal toxin concentration obtained of 2.33 µg/ml (Table I).

Effect of pH on the proteolytic processing of Cyt1Ab1 crystals - The proteolytic activity of the

TABLE I

Solubilization of *Bacillus thuringiensis* subsp. *medellin* Cyt1Ab1 toxin in buffers of different pH

Buffer	pH	Concentration of solubilized Cyt1Ab1 toxin (µg/ml)
Na_2HPO_4 50 mM, KH_2PO_4 50 mM	5.0	0.3
Na_2HPO_4 50 mM, KH_2PO_4 50 mM	6.0	0.3
Na_2HPO_4 50 mM, KH_2PO_4 50 mM	7.0	0.3
Tris HCl 50 mM	8.0	0.4
Na_2CO_3 50 mM, $NaHCO_3$ 50 mM	9.0	0.5
Na_2CO_3 50 mM, $NaHCO_3$ 50 mM	10.0	0.7
Na_2CO_3 50 mM, $NaHCO_3$ 50 mM	10.6	1.2
Na_2CO_3 50 mM, $NaHCO_3$ 50 mM	11.0	1.5
NaOH 50 mM, EDTA 10 mM	12.0	2.3

C. quinquefasciatus gut extract was only observed in the alkaline buffers after 14 h of incubation (pH 10.6 and above). In the buffers of pH 10.6 and 11, the proteolytic product of the Cyt1Ab1 was seen as a weak band and the complete proteolysis was indicated by the formation of a 25 kDa fragment, observed at the pH of 12 (Fig. 1A, lane 11). The activation of the Cyt1Ab1 toxin with trypsin and chymotrypsin was slightly different at the same pH conditions and produced fragments of 26 kDa and 25 kDa, respectively (Figs 1B, 1C, lanes 11).

Kinetics of the Cyt1Ab1 proteolysis - The appearance of intermediaries and protease resistant fragments of the Cyt1Ab1 toxin was observed at different time intervals after treatment with proteases. The action of *C. quinquefasciatus* gut extract in the processing of Cyt1Ab1 was observed 1 min after treatment, being most evident 10 min later. Between 3 and 14 h, the Cyt1Ab1 toxin was converted to a 25 kDa fragment (Fig. 2A). Proteolysis with trypsin did not generate intermediaries and produced a 26 kDa fragment 14 h after treatment (Fig. 2B, lane 10). Processing of the Cyt1Ab1 toxin with chymotrypsin clearly showed the generation of 25 and 26 kDa fragments as early as 1 min after the proteolysis had started, and generated a 24.5 kDa protease resistant core 21 h later (Fig. 2C, lane 11).

Comparative proteolytic processing - In vitro processing of Cyt1Ab1 and Cyt1Aa1 toxins with proteases showed slight differences in the size of the generated fragments. After 24 h of treatment with *C. quinquefasciatus* gut extract, Cyt1Ab1 and Cyt1Aa1 generated fragments of 23 and 22.5 kDa, respectively (Fig. 3, lanes 4, 5); treatment with trypsin generated fragments of 25 and 24 kDa (Fig. 3, lanes 6, 7), and treatment with chymotrypsin produced fragments of 25 and 24.5 kDa, respectively (Fig. 3, lanes 8, 9).

In vivo proteolysis of Cyt1Ab1 inclusion - The processing of the parasporal inclusion containing

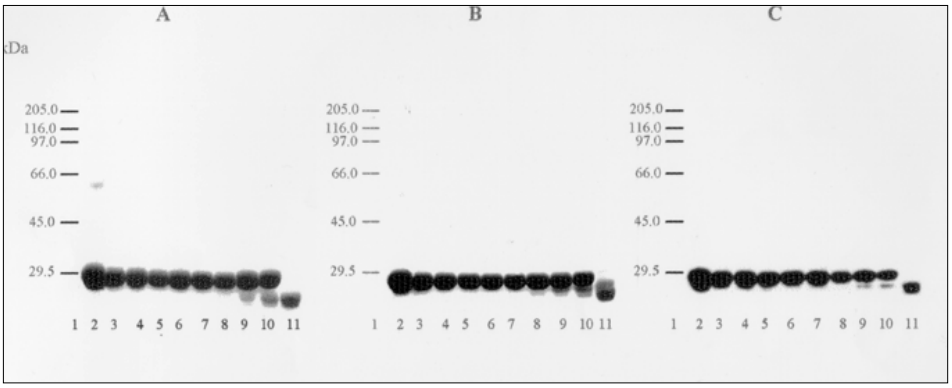

Fig. 1: effect of pH on the proteolysis of the Cyt1Ab1 crystal inclusions. A: proteolysis by *Culex quinquefasciatus* gut extract; B: proteolysis by trypsin; C: proteolyis by chymotrypsin. Lanes 1: molecular weight marker; lanes 2: Cyt1Ab1 crystal inclusion; lanes 3: proteolysis of soluble Cyt1Ab1 toxin at pH 5; lanes 4: at pH 6; lanes 5: at pH 7; lanes 6: at pH 8; lanes 7: at pH 9; lanes 8: at pH 10; lanes 9: at pH 10.6; lanes 10: at pH 11; lanes 11: at pH 12

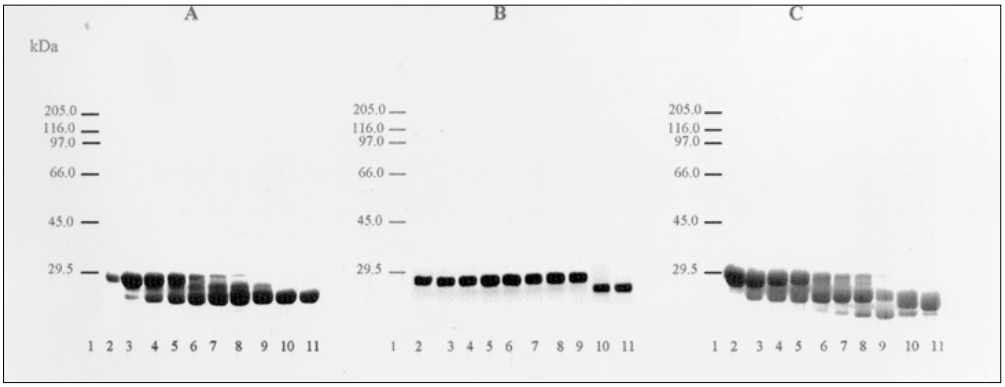

Fig. 2: time course processing of Cyt1Ab1 toxin with different proteases. A: proteolysis by *Culex quinquefasciatus* gut extract; B: proteolysis by trypsin; C: proteolysis by chymotrypsin. Lanes 1: molecular weight marker; lanes 2: Cyt1Ab1 crystal inclusion; lanes 3: after 1 min; lanes 4: after 5 min; lanes 5: after 10 min; lanes 6: after 30 min; lanes 7: after 1h, lanes 8: after 2h; lanes 9: after 3 h; lanes 10: after 12 h; lanes 11: after 21 h

the Cyt1Ab1 toxin by third instar *C. quinquefasciatus* larvae was analyzed at different concentrations of the toxin (25 µg, 12.5 µg and 6 µg of toxin inclusions, and soluble toxin at 25 µg/ml). *In vivo* processing of the crystals generated a weak 25 kDa fragments, similar in size to those observed during the *in vitro* proteolysis (compare in Fig. 4, lanes 3-5 to lane 7).

In vitro hemolysis on sheep red blood cells with soluble and processed Cyt1Ab1 toxin - The alkali-solubilized Cyt1Ab1 toxin showed an HD_{50} of 4.5 µg/ml in sheep red blood cells (Table II) and 3.6 µg/ml for human erythrocytes (data not shown). Cyt1Ab1 toxin processed *in vitro* with mosquito larvae gut extract, trypsin and chymotrypsin showed similar HD_{50}, and this hemolytic activity was higher than the activity of the solubilized toxin (Table II).

Mosquitocidal activity assay - The mosquitocidal activity of the Cyt1Ab1 crystal inclusions against third instar *C. quinquefasciatus* larvae showed an LC_{50} of 1.3 µg/ml for Cyt1Ab1 toxin and 0.74 µg/ml for Cyt1Aa1 toxin. Even though we used 25 µg/ml of soluble Cyt1Ab1 toxin, or processed toxin by trypsin, chymotrypsin and *C. quinquefasciatus* gut extract, no toxicity could be detected in the bioassays (Table II).

Identification of protease cleavage sites - Protease-resistant fragments separated by SDS-PAGE

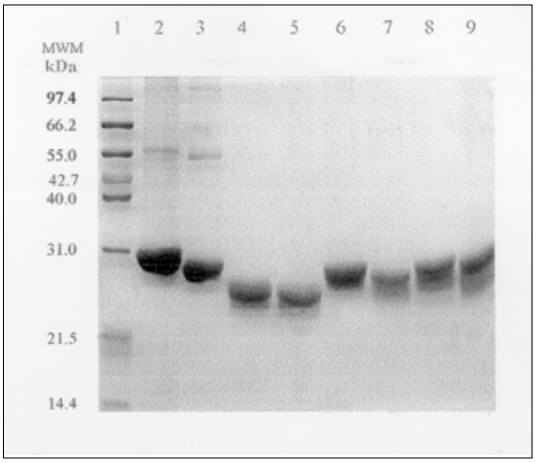

Fig. 3: comparative analysis of the *in vitro* proteolytic processing of the Cyt1Ab1 toxin of *Bacillus thuringiensis* subsp. *medelin* and Cyt1Aa1 toxin of *B. thuringiensis* subsp. *israelensis* with different proteases. Lane 1: molecular weight marker; lane 2: Cyt1Ab1; lane 3: Cyt1Aa1; lanes 4 and 5: Cyt1Ab1 and Cyt1Aa1 processed by *Culex quinquefasciatus* gut extract; lanes 6 and 7: Cyt1Ab1 and Cyt1Aa1 processed by trypsin; lanes 8 and 9: Cyt1Ab1 and Cyt1Aa1 processed by chymotrypsin.

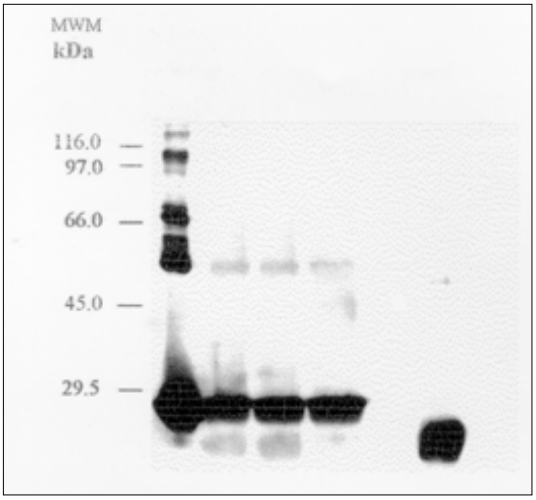

Fig. 4: *in vitro* processing of the Cyt1Ab1 soluble toxin by third instar *Culex quinquefasciatus* larvae. Larvae were incubated with 25 µg (lane 6) of soluble toxin. Lane 1: molecular weight markers; lane 2: Cyt1Ab1 toxin inclusions; lane 7: 10 µg of Cyt1Ab1 toxin *in vitro* digested with *C. quinquefasciatus* gut extract; lane 8: *C. quinquefasciatus* guts.

TABLE II

Mosquitocidal and hemolytic activities of inclusions, solubilized and proteolytically processed Cyt1Ab1 toxin of *Bacillus thuringiensis* subsp. *medellin* against *Culex quinquefasciatus* third instar larvae and sheep red blood cells

Toxin	Half lethal concentration LC_{50} (µg/ml)	Half hemolytic concentration HD_{50} (µg/ml)
Cyt1Aa1 crystals	0.74 (0.5-0.9) [a]	ND
Cyt1Ab1 crystals	1.3 (0.9-1.7)	NH
Cyt1Ab1 solubilized	NT	4.5 (3.4-6.1)
Cyt1Ab1 activated by *C. quinquefasciatus* gut extracts	NT	1. 4 (0.4-2.0)
Cyt1Ab1 activated by trypsin	NT	2.2 (1.6-3.3)
Cyt1Ab1 activated by chymotrypsin	NT	1.9 (1.5-2.6)

a : values in parenthesis represent the 95% confidence limits, determined by Probit analysis, ND: not determined; NH: not hemolytic; NT: not toxic

were transferred to PVDF membranes. Eleven aminoacids from the N-terminal part were sequenced and compared to the deduced aminoacid sequence (Thiery et al. 1997). The N-terminal sequence of the fragment generated by treatment with *C. quinquefasciatus* gut extract corresponded to DDPNEKNNHNS, with the cutting site located between residues Ile^{31} and Asp^{32} (Table III). The cutting site generated by trypsin was Leu^{29} and Arg^{30} and for chymotrypsin Arg^{30}-Lys^{31} indicating a shift of one and two aminoacids compared to the protease-resistant fragment generated by treatment of the Cyt1Ab1 toxin with mosquito larvae gut extract.

DISCUSSION

The midgut of mosquito larvae has an alkaline pH (Dadd 1975) and contains trypsin, chymotrypsin (Yang & Davies 1971, Kunz 1978) and thermolysin-like proteases which are active at this pH (Dai & Gill 1993). It is accepted that the *B. thuringiensis* crystals are solubilized in the alkaline environment of the insect midgut, and then cleaved by midgut proteases to produce the toxic fragments (Tojo & Aizawa 1983, Aronson et al. 1986). Dai and Gill (1993) showed that the Cry11Aa1 toxin of *B. thuringiensis* subsp. *israelensis* is processed to fragments of 30 and 28.5 kDa by trypsin and chymotrypsin-like proteases, and to a 28 kDa fragment by thermolysin-like proteases present in *C. quinquefasciatus* larval midguts.

The recombinant Cyt1Ab1 toxin was poorly solubilized in buffers with pH ranging from 5 to 10; while toxin solubilization superior to 1 µg/µl was only obtained in buffers of pH 10.6 and above (Table I). Reduced protease activity on the Cyt1Ab1 protein was observed in buffers of pH bellow 11 (Figs 1A, B, C), while fully processed Cyt1Ab1 toxin was only observed in the buffer of pH 12 (lanes 11, in Figs 1A, 1B, 1C). A clear dependence between Cyt1Ab1 crystal protein solubilization, pH, and proteolytic activity was observed, results that agree with the observations of Fast and Milne (1979) and Dai and Gill (1993), who showed that the pH is an important factor for *B. thuringiensis* crystal solubilization and that an alkaline pH is required for efficient toxin proteolytic activity on mosquito larvae midguts.

The proteolytical activity over the Cyt toxins has been described (Chilcott & Ellar 1988, Knowles et al. 1992, Koni & Ellar 1994, Al-yahyaee & Ellar 1995). The time course study of the Cyt1Ab1 proteolysis revealed that treatments with chymotrypsin and *C. quinquefasciatus* gut extract produced a 25 kDa fragment, and the proteolytic activity of trypsin generated a 26 kDa fragment. It is possible that activity similar to trypsin and chymotrypsin could be present in *C. quinquefasciatus* gut extract, and probably other proteases could participate in this process, as has been demonstrated previously for the activation of the Cry11Aa toxin (Dai & Gill 1993).

Longer incubation time (24 h) of the Cyt1Ab1 toxin with *C. quinquefasciatus* gut extract produced a fragment of 23 kDa (Fig. 3, lane 4); while the treatment with chymotrypsin generated a 25 kDa fragment (Fig. 3, lane 8), similar in size to the one observed after 21 h of treatment.

The treatment of the Cyt1Aa1 toxin with trypsin generated a 23.5 kDa fragment (Al-yahyaee & Ellar 1995), while treatment of the Cyt1Ab1 toxin with the same protease generated a 26 kDa fragment 21 after treatment (Fig. 2B, lane 11), and 3 h later, a 25 kDa fragment was observed (Fig. 3, lane 6). Although Cyt1Aa1 and Cyt1Ab1 toxins share 86% of aminoacid identity (Thiéry et al. 1997), the difference in size of the enzyme digested fragments suggests that the toxins could have different protease susceptibility.

TABLE III

N-terminal sequence of Cyt1Ab1 toxin of *Bacillus thuringiensis* subsp. *medellin* processed by different proteases

Toxin	N-terminal sequence	Cutting site	Size (kDa)
Cyt1Ab1	Met-Glu-Asp-Pro-Asn-His-Cys-Pro-Leu		28
Cyt1Ab1 toxin processed by *Culex quinquefasciatus* gut extract	Asp-Asp-Pro-Asn-Glu-Lys-Asn-Asn-Asn	Ile^{31}-Asp^{32}	23
Cyt1Ab1 toxin processed by trypsin	Arg-Lys-Asp-Asp-Pro-Asn-Glu-Lys-Asn	Leu^{29}-Arg^{30}	25
Cyt1Ab1 toxin processed by chymotrypsin	Lys-Asp-Asp-Pro-Asn-Glu-Lys-Asn-Asn	Arg^{30}-Lys^{31}	25

The results of the *in vitro* processing of the Cyt1Ab1 toxin with *C. quinquefasciatus* gut extract were similar to the *in vivo* processing by third instar *C. quinquefasciatus* larvae. However the 25 kDa fragment generated by the *in vivo* experiments was seen as a weak band (Fig. 4, lanes 3-5), indicating that the proteolytic processing of the Cyt1Ab1 toxin was not very efficient and/or poor crystal protein solubilization took place, as shown in the solubilization experiments (Table I) and as previously described by Orduz et al. (1996). The bands seen in this experiment are different to the strong band observed when the Cyt1Ab1 toxin was treated with *C. quinquefasciatus* gut extract at pH 12, a non physiological pH of the mosquito larvae midgut (Fig. 4, lane 7). It has been suggested that some Cry and Cyt toxins are active without prior proteolytic processing (Höfte & Whiteley 1989, Knowles et al. 1992, Koni & Ellar 1994). These observations could explain the larvicidal activity of Cyt1Ab1 crystal inclusions when they are ingested by *C. quinquefasciatus* larvae, despite the low processing within the larval gut (Fig. 4).

Thiéry et al. (1997) observed mortality in *C. pipiens* larvae treated with recombinant Cyt1Ab1 crystal inclusions (LC_{50} of 5.7 µg/ml). Although we also observed mortality of *C. quinquefascistus* larvae challanged with recombinant Cyt1Ab1 crystal inclusions (LC_{50} 1.3 µg/ml), no toxicity was observed when soluble and protease treated Cyt1Ab1 protein was used. Similar results were found by Schnell et al. (1984) where solubilized crystal proteins of *B. thuringiensis* subsp. *israelensis* were 7,000 times less active than crystals. Since Dadd (1975) demonstrated that mosquito larvae are mainly filter feeders, and do not ingest water or soluble compounds, it is possible that the mosquito larvae used in the bioassays with soluble and protease treated Cyt1Ab1 toxin did not ingest an adequate amount of toxin to cause larval mortality (Table II).

Even though the solubilized and processed Cyt1Ab1 toxin was not mosquitocidal, hemolytic activity was detected and produced a half hemolytic concentration (HD_{50}) of 4.5 µg/ml in sheep erythrocytes (Table II). The HD_{50} of solubilized Cyt1Ab1 toxin was similar in human and sheep erythrocytes with values of 4.5 µg/ml for sheep blood cells, and 3.6 µg/ml for human red blood cells (data not shown). Surprisingly, the *in vitro* processed Cyt1Ab1 toxin with gut extract, trypsin and chymotrypsin was more hemolytic to sheep erythrocytes than the untreated toxin form (Table II). This result indicates a possible role of the *in vitro* activation in potentiating the hemolytic activity of the toxin, as reported by Gill et al. (1987) and Al-yahyaee and Ellar (1995).

The N-terminal sequence analysis of the Cyt1Ab1 toxin processed by different proteases showed that the cutting sites in all treatments were very similar (Table III). *C. quinquefasciatus* gut extract cut at Ile^{31}-Asp^{32}, while Al-yahyaee and Ellar (1995) reported a cutting site between Arg^{30}-Val^{31} for the Cyt1Aa1 toxin treated with *Culex* sp. gut extract. For the Cyt1Ab1 toxin, trypsin and chymotrypsin cutting sites were identified between Leu^{29}-Arg^{30} and Arg^{30}-Lys^{31} respectively (Table III). Despite the almost 100% homology between the Cyt1Aa1 and Cyt1Ab1 toxins in the first 40 aminoacids of the N-terminal region, data from Al-yahyaee and Ellar (1995) indicates a shift of several aminoacids when Cyt1Aa1 toxin was treated with trypsin and chymotrypsin (cutting sites Val^{26} and Lys^{18} respectively).

The role of solubilization and proteolysis of the Cyt1Ab1 toxin in hemolytic activity against sheep erythrocytes was demonstrated, although the proteolytically cleaved Cyt1Ab1 toxin was not toxic against third instar *C. quinquefasciatus* larvae, the role of this processing in toxicity can not be excluded.

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