Biochemical, Immunological and Toxicological Characteristics of the Crystal Proteins of *Bacillus thuringiensis* subsp. *medellin*

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Characterization of the insecticidal and hemolytic activity of solubilized crystal proteins of Bacillus thuringiensis (Bt) *subsp.* medellin (Btmed) *was performed and compared to solubilized crystal proteins of isolates 1884 of* B. thuringiensis *subsp.* israelensis (Bti) *and isolate PG-14 of* B. thuringiensis *subsp.* morrisoni (Btm). *In general, at acid pH values solubilization of the* Bt *crystalline parasporal inclusions (CPI) was lower than at alkaline pH. The larvicidal activity demonstrated by the CPI of* Btmed *indicated that optimal solubilization of CPI takes place at a pH value of 11.3, in* Bti *at pH values from 5.03 to 11.3 and in* Btm *at pH values from 9.05 to 11.3. Hemolytic activity against sheep red blood cells was mainly found following extraction at pH 11.3 in all* Bt *strains tested. Polyacrylamide gel electrophoresis under denaturing conditions revealed that optimal solubilization of the CPI in all* Bt *strains takes place at the alkaline pH values from 9.05 to 11.3. An enriched preparation of* Btmed *crystals was obtained, solubilized and crystal proteins were separated on a size exclusion column (Sephacryl S-200). Three main protein peaks were observed on the chromatogram. The first peak had two main proteins that migrate between 90 to 100 kDa. These proteins are apparently not common to other* Bt *strains isolated to date. The second and third peaks obtained from the size exclusion column yielded polypeptides of 68 and 28-30 kDa, respectively. Each peak independently, showed toxicity against 1st instar* Culex quinquefasciatus *larvae. Interestingly, combinations of the fractions corresponding to the 68 and 30 kDa protein showed an increased toxicity. These results suggest that the 94 kDa protein is an important component of the* Btmed *toxins with the highest potency to kill mosquito larvae. When crystal proteins of* Bti *were probed with antisera raised independently against the three main protein fractions of* Btmed, *the only crystal protein that showed cross reaction was the 28 kDa protein. These data suggest that* Btmed *could be an alternative bacterium for mosquito control programs in case mosquito larval resistance emerges to* Bti *toxic proteins.*

Key words: *Bacillus thuringiensis* - crystal proteins - pH-mediated solubilization - mosquito larval toxicity

Bacillus thuringiensis (Bt), an ubiquitous gram positive rod has been used in agriculture during the last 30 years to control insects. Since the discovery of the first mosquito active strain in 1977 by Goldberg and Margalit, it has been implemented worldwide in mosquito control programs. In recent years, an increased and extensive search for new strains of *Bt* have been performed in order to discover new or increased activities. *Bt* strains are toxic to either lepidopteran, dipteran, or coleopteran insects and some to nematodes (Höfte & Whiteley 1989, Feitelson et al. 1992).

This bacterium produces crystalline parasporal inclusions (CPI) usually composed of one or several polypeptide subunits, which are toxic when ingested by susceptible insects. These CPI contain proteins that exhibit a variety of biological actions including cytolytic, hemolytic and entomocidal activities (Aronson et al. 1986, Höfte & Whiteley 1989).

Numerous natural variations in the primary structure of the crystal proteins exist and are responsible for differences in susceptible host range of each toxin (Höfte & Whiteley 1989). Many physicochemical properties of the crystal inclusions have been reviewed (Huber & Lüthy 1981, Tyrrel et al. 1981, Pfannenstiel et al. 1986, Koller et al. 1992, Du et al. 1994) including the pH required for solubilization of the crystal, an important parameter, since it is an essential step for toxicity in susceptible insects. In this paper we describe the solubility, immunological, and toxicological properties of crystal proteins produced by *B. thuringiensis* subsp. *medellin* described by Orduz et al. (1992, 1994) and investigate the role of the individual proteins and their mixtures in toxicity to *Culex quinquefasciatus* larvae.

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MATERIALS AND METHODS

Bacillus thuringiensis *strains and mosquitoes* - Strains 1884 of *Bt* subsp. *israelensis* (*Bti*) and PG-14 of *Bt* subsp. *morrisoni* (*Btm*) were obtained from the Unite de Bacteries Entomopathogenes, Institut Pasteur, Paris. Strain 163-131 of *Bt* subsp. *medellin* (*Btmed*) was isolated from Colombia (Orduz et al. 1992). Bacteria were grown on LB agar plates (tryptone 10 g, NaCl 10 g, yeast extract 5 g, agar 20 g per liter of distilled water), and incubated for 24 hr at 30ºC. Tubes containing 5 ml of liquid M-one medium (Proflo 1 g, peptone 5 g, glucose 3 g, K_2HPO_4 6 g, chloride salts 10 ml, containing 0.02 g of each one of the following $MgCl_2.6H_2O$, $MnCl_2.4H_2O$, $FeCl_3.6H_2O$, $ZnCl_2$, and $CaCl_2.2H_2O$, phosphate buffer 200 ml, in 1 liter of distilled water and pH adjusted to 7.2) were inoculated and incubated for 8 hr at 30ºC and 200 rpm. Bacterial cultures were transferred to 200 ml of M-one, and incubated at 30ºC, 250 rpm in an environmental incubator shaker for two days. The final whole culture (FWC) was collected by centrifugation at 4ºC, 9000 x g for 30 min. The resulting pellet was treated for 30 min with 1M NaCl, washed twice in distilled water, resuspended in 1/20 of the original volume with distilled water and stored at -20ºC until needed. *C. quinquefasciatus* used in these experiments were field-collected in the vicinity of Medellin, and were maintained under laboratory conditions at 30±2ºC under a 12:12 (light:dark) photoperiod.

pH-mediated solubilization of crystals - For solubilization assay, a universal buffer with constant ionic strength at several pH values was prepared as described by Koller et al. (1992). One hundred microliters of *Btmed* FWC was treated with 400 µl of each one of the pH solutions of the buffer system, ranging from 2.55 to 11.22, during 24 hr, 30ºC at 200 rpm. The solubilized proteins were dialyzed against phosphate buffer saline (PBS), (0.32 g NaH_2PO_4, 1.18 g Na_2HPO_4, 8.58 g NaCl, per liter, pH 7.2). The solid material was collected by centrifugation, and its protein concentration was measured by Bradford's method (Bradford 1976). Aliquots of 400 µl of the supernatant were acetone-precipitated, and proteins were separated on 10% sodium dodecyl sulfate polyacrilamide gel by electrophoresis (SDS-PAGE acrylamide-bis-*N-N'* acrylamide) (Laemli 1970).

Gel filtration - A sample of 12.5 mg of the solubilized preparation of *Btmed* crystals purified by sucrose gradient centrifugation was loaded on a Sephacryl S-200 column (1 x 0.05 m.). Fraction elution was performed in a buffer system as described by Thiéry (1987), collected in 4 ml samples and absorbance measured at OD_{280} nm. Fractions from the Sephacryl column, were dialyzed against PBS pH 7.2. Samples of 400 µl of the fractions were acetone-precipitated, and proteins separated on a 10% SDS-PAGE as described above.

Hemolytic activity assay - Sheep red blood cells were collected and washed three times in 0.85% saline solution (SS), and brought to a final concentration of 2.8% in SS. One hundred microliters aliquots of a fraction eluted from the Sephacryl column were mixed with an equal volume of a 2.8% suspension of sheep red blood cells in U-shaped bottom microtiter plates. Plates were incubated at 37ºC with 5% CO_2 for 2 hr, then centrifuged at 500 x g for 10 min and absorbance of the supernatants was measured at 540 nm in a Multiskan MCC/340 ELISA reader.

Toxicity on mosquito larvae - Solubilized crystal proteins of *Btmed* separated in the Sephacryl column and the proteins extracted with the buffer system were tested for toxicity towards first instar *C. quinquefasciatus* larvae. Five, first instar *C. quinquefasciatus* larvae were placed in each one of a 24 well plate with 1 ml of deionized water. Treatments were given to determine the LC_{50} of each one of the gel filtration fractions, and their mixtures. Each dose or pH extract was assayed twice, and experiments were conducted in four different days. Interactions between the different proteins of *Btmed* separated in the Sephacryl S-200 column were evaluated according to the formula described by Tabashnick (1992).

Preparation of antisera against Btmed *crystal proteins and Western blot* - Polyclonal antisera against individual *Btmed* crystal proteins were prepared in mice by weekly intraperitoneal injections of protein fractions separated in 10% PAGE-SDS. First injection was given in Freund´s complete adjuvant, and other three injections in Freund´s incomplet adjuvant. Crystal proteins of *Btmed*, and *Bti* were separated in a 10% SDS-PAGE and transferred to nitrocelulose paper. Western blot with antisera was performed by probing the membranes with antibodies raised against *Btmed* 94, 68, and 30 kDa toxic proteins, diluted 1:500 and incubated at 4ºC overnight. Other incubations were performed at room temperature in TBS pH 7.4, using 3% gelatin as blocking agent, and 0.05% Tween-20. For immunodetection, alkaline-phosphatase conjugated to Protein A was followed by a substrate/color system composed of naphtol phosphate/Fast Red (Sigma Chemical Co.)

RESULTS

As a general trend, the alkaline treatment of CPI of all three *Bt* subspecies evaluated in this study, extracted higher amounts of proteins than acid treatment. The solubilized CPI of *Bt* subspe-

cies evaluated in this study displayed hemolytic activity. The CPI of *Bti*, *Btm* and *Btmed* solubilized at pH 11.3 produced 100% hemolysis in sheep red blood cell, while at the pH below this value, hemolysis was found between 0% and 30%. Untreated FWC of *Bti*, *Btm* and *Btmed* caused 20%, 15% and 10% hemolysis respectively (Table I).

On SDS-PAGE, CPI from FWC of *Btmed* showed polypeptides of 90-100 kDa, multiple bands at 80, 75, 67,65, 40, and 28-30 kDa (Fig. 2A, lane 2). CPI from FWC of *Bti* and *Btm* showed mainly protein bands of 144, 135, 125, 67, and 28 kDa when solubilized at pH values between 9.98 and 11.3 (Figs 2B, 2C, lane 2). The optimal solubilization of *Btmed* CPI takes place at pH 11.3 (Fig. 2A, lane 12), but crystal proteins could be observed also at pH values from 4.1 to 9,98 (Fig. 2A, lanes 5 to 11). When tested on a bioassay challenging 1st instar *C. quinquefasciatus* larvae, samples corresponding to lanes 6 to 11 (Fig. 2A), caused less than 62% mortality. Similarly to *Btmed*, crystals from *Btm* show solubilized proteins at pH values between 4.1 and 11.3 (Fig. 2B, lanes 5 to 12); however, solubilized crystal proteins from *Btm* caused less than 50% mortality at pH values below 6.01 and more than 80% at pH values higher than 7.04. Larval mortality was 100% only when crystals were solubilized at pH values from 9.05 to 11.3 (Fig. 1). In *Bti*, solubilized crystal proteins were observed mainly at pH values between 9.05 and 11.3 (Fig. 2C, lanes 10 to 12), however more than 90% mortality of *C. quinquefasciatus* larvae was observed in pH values between 5.03 and 11.3 (Fig. 1). In *Bti* and *Btm*, the 68 kDa crystal protein was also observed when solubilization of the CPI took place at pH values of 5.03 and 6.01 respectively (Figs 2B and 2C, lanes 6 and 7, respectively).

Fractionation in Sephacryl S-200 of crystal proteins solubilized at alkaline pH showed three main peaks (Fig. 3A). Fraction 23 showed two protein bands when analyzed in SDS-PAGE (Fig. 3B, lane 3), which migrated between 90 to 100 kDa, with the most abundant protein component at approximately 94 kDa. For the larvicidal studies these fractions will be referred to as the 94 kDa protein. The main polypeptide present in fractions 24 through 30 (lanes 4 to 10) migrated with a molecular weight

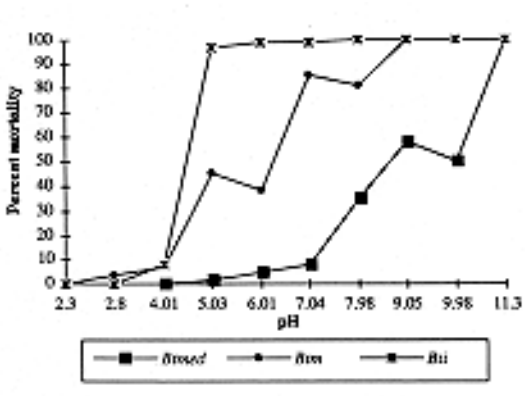

Fig. 1: mortality of first instar *Culex quinquefasciatus* larvae treated with the solubilized proteins at different pH values. *Btmed:* strain 163-131 of *Bacillus thuringiensis* subsp. *medellin*; *Btm:* strain PG-14 of *Bt* subsp. *morrisoni*; *Bti:* strain 1884 of *Bt* subsp. *israelensis*.

TABLE I

Protein concentration and hemolytic activity assay of *Bacillus thuringiensis* subsp. *medellin*, *B. thuringiensis* subsp. *morrisoni* and *B. thuringiensis* subsp. *israelensis* at different pH values

	Protein concentration[a] (mg/ml)			Hemolytic activity (%)[b]		
pH[c]	*Btmed*	*Btm*	*Bti*	*Btmed*	*Btm*	*Bti*
2,3	<0,025	<0,025	<0,025	0	5	5
2,8	<0,025	<0,025	<0,025	5	5	5
4,01	<0,025	<0,025	<0,025	5	5	5
5,03	<0,025	<0,025	<0,025	5	5	5
6,01	<0,025	<0,025	0,06±0.02	5	5	10
7,04	<0,025	0,06±0.02	0,08±0.01	5	5	15
7,98	0,06±0.02	0,05±0.02	0,08±0.02	5	10	10
9,05	0,10±0.02	0,05±0.02	0,08±0.01	5	10	10
9,98	0,09±0.01	0,08±0.04	0,10±0.01	10	30	10
11,3	0,30±0.06	0,29±0.10	0,36±0.10	100	100	100
FWC	0,31±0.17	0,24±0.08	0,40±0.02	10	15	20

a: protein concentration measured by Bradford's method; *b*: solubilized proteins tested by duplicate and repeated in two different days; *c*: pH at which the CPI were solubilized. pH was adjusted to 7.2 before hemolysis tests were set; *Btmed:* strain 163-131 of *B. thuringiensis* subsp. *medellin*, *Btm:* strain PG-14 of *B. thuringiensis* subsp. *morrisoni*, *Bti:* strain 1884 of *B. thuringiensis* subsp. *israelensis*; FWC: final whole culture.

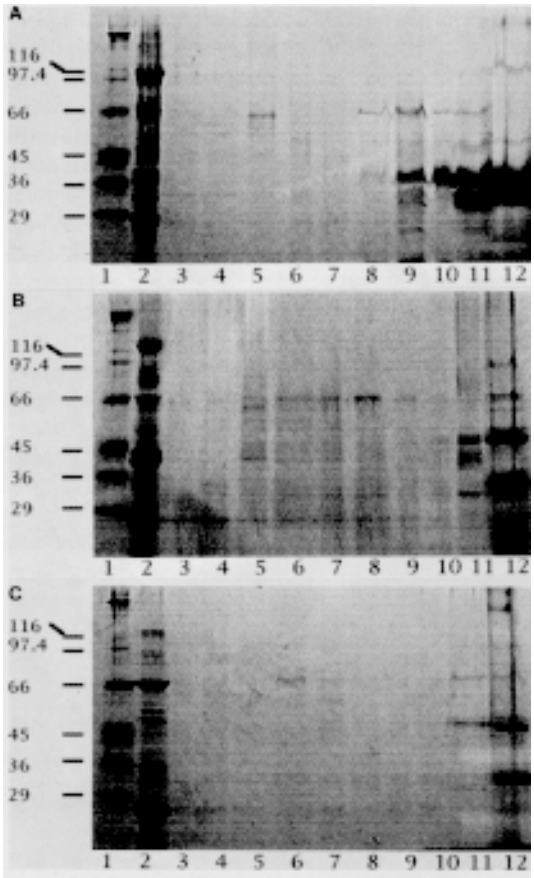

Fig. 2: SDS-10% PAGE, Coomassie blue stained gels of (A) *Bacillus thuringiensis* subsp. *medellin*, (B) *Bt* subsp. *morrisoni*, (C) *Bt* subsp. *israelensis* final whole cultures treated at different pH values. Lanes 1, molecular weight markers indicated in kDa, lanes 2 crystal proteins from (A), *B. thuringiensis* subsp. *medellin*, (B), *Bt* subsp. *morrisoni*, and (C), *Bt* subsp. *israelensis*, lanes 3 pH 2.3; lanes 4, pH 3.8, lanes 5, pH 4.1; lanes 6, pH 5.03; lanes 7, pH 6.01; lanes 8, pH 7.04; lanes 9, pH 7.98; lanes 10, pH 9.05; lanes 11, pH 9.98; lanes 12, pH 11.3.

between 66 and 68 kDa. Fractions 32 and 33 (lanes 12 and 13) showed two main protein bands at approximately 28 and 30 kDa. Hemolytic activity was mainly found in fractions containing the 28-30 kDa proteins (Table II).

The mosquito larvae mortality results obtained in the treatments with the fractions collected from the Sephacryl S-200 column indicate that the 94 kDa protein is perhaps the most important component of the *Btmed* toxins with an LC_{50} of 82.6 ng of protein/ml (Table III). Proteins of 68 and 30 kDa of *Btmed* have $LC_{50's}$ of 1256 and 1948.5 ng/ml respectively in *C. quinquefasciatus* first instar larvae. The effect of crystal protein mixtures of *Btmed* was also evaluated in bioassays with mosquito larvae, and results analyzed according to the formula described by Tabashnick (1992). Results indicate

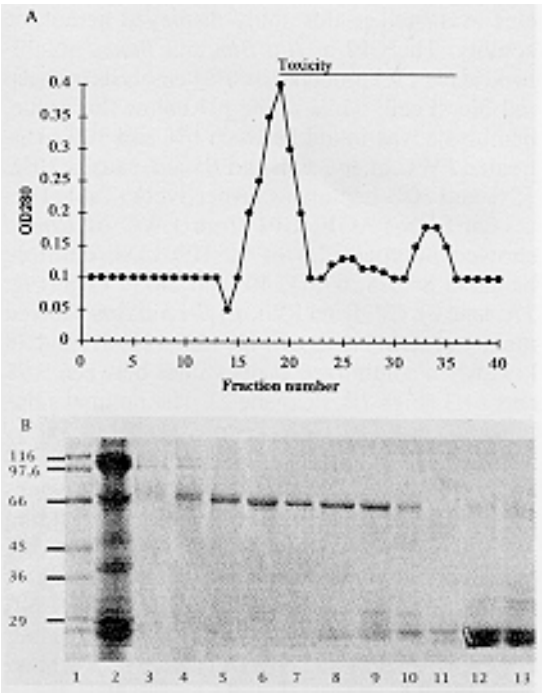

Fig. 3A: elution profile of the alkali solubilized *Bacillus thuringiensis* subsp. *medellin* crystal proteins from a Sephacryl S-200 column. Fig. 3B. SDS-10% PAGE, stained with Coomasie Blue of fractions of *Btmed* crystal proteins eluted from a Sephacryl S-200. Lane 1, molecular weight markers indicated in kDa; lane 2 final whole culture of *Btmed* lanes 3 through lane 12, fractions 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, and 33 respectively. Fractions causing mosquito larval mortality are indicated by a line.

TABLE II

Percent hemolysis of sheep red blood cells caused by different *Bacillus thuringiensis* subsp. *medellin* crystal proteins separated in a Sephacryl S-200 column

Fraction number	Size of protein in kDa	Percent hemolysis[a]
20	94	0
21	94	0
22	94	0
23	94	0
24	94	0
25	94	0
26	68	35
27	68	35
28	68	60
29	68	100
30	28-30	100
31	28-30	100
32	28-30	100
33	28-30	100
34	28-30	100
35	28-30	100

a: solubilized proteins tested by duplicate and repeated in two different days.

that the mixture of the 94 and 68, and 94 and 30 kDa proteins did not interact synergistically; however the 68 and 30 kDa proteins interact synergistically. The mixture of the fractions containing the 94, 68 and 28-30 kDa proteins produced a slight increase in toxicity compared to the expected, but this could not be considered a synergistic effect (Table III).

Crystal proteins of *Btmed*, and *Bti* were probed by Western blot with each one of the antisera raised in mice independently against the three main protein fraction of *Btmed* (94, 68, and 30 kDa proteins). The anti 94 kDa and the anti 68 kDa protein did not recognize any of the crystal proteins of *Bti*, while the anti 30 kDa recognized the 28 kDa protein of *Bti* (data not shown).

TABLE III

Observed and expected 24 hr mortality of first instar *Culex quinquefasciatus* larvae treated with *Bacillus thuringiensis* subsp. *medellin* enriched fractions

Toxin(s) (proportions)	LC_{50} ng of protein/ml (proportions) observed	expected[a]
94	82.6	
68	1256	
30	1948.5	
94+68 (0.2:0.8)	430.4 (86.1:344.3)	326.9
94+30 (0.18:0.82)	373.8(67.3:306.5)	384.6
68+30 (0.42:0.58)	857.8(360.3:497.5)	1582.1
94+68+30(0.1:0.42:0.48)	351.8(35.3:147.7:168.8)	358.2

a: calculated according to the formula described by Tabashnick (1992).

DISCUSSION

The *C. quinquefasciatus* larval mortality and sheep red blood cell hemolytic activity of the *Bt* CPI solubilized under different pH conditions correlates with the amount of protein extracted at each particular pH value. We also observed a constant tendency in which larvicidal and hemolytic activity of *Bti* crystal proteins were displayed at lower pH values than in *Btm* and *Btmed.*

The ability of the solubilized crystal proteins from *Btmed* to cause hemolysis suggests that most of the mosquitocidal strains of *Bt* identified to date contain cytolytic proteins (Thomas & Ellar 1983, Gill et al. 1987). Furthermore, at least in *Bti*, *Btm*, and *Btmed*, this biological activity is caused by proteins of low molecular weight (20-30 kDa). In all the *Bt* subspecies that are active against mosquito at the ng/ml level, there is an immunological cross reaction of the low molecular weight proteins. As in the case of *Bti* and *Btm*, it has been shown that the hemolytic proteins of *Btmed* are the low molecular weight components of the CPI. In particular, it has been shown that in *Btmed*, the 28-30 kDa protein is responsible for the hemolytic activity, and that the various degrees of hemolysis caused by the 68 kDa protein fractions could be due to contamination with small amounts of the 28-30 kDa protein during the gel filtration procedures. In the pH mediated solubilization experiments with all *Bt* strains tested, mortality and hemolytic activity increased with pH increase in the treatments. This indicate that most of the crystal proteins were solubilized at alkaline pH values.

Contrary to the data obtained by Koller et al. (1992) when working with CPI of *Bt* var *san diego*, we were unable to detect proteins in the acid extraction treatments under pH of 4.01 in any of the *Bt* strains tested. Gringorten et al. (1992) have demonstrated that solubilized CPI from *Bt* subsp. *kurstaki* required an alkaline environment in order to retain its full activity, however in our toxicity experiments, neutralization of the alkaline extraction treatments did not prevent expression of toxic activity as shown in Fig. 1.

The role of the four major polypeptides (CryIVA, CryIVB, CryIVD and CytA) of *Bti* in the mosquitocidal activity has been controversial (Wu & Chang 1985, Ibarra & Federici 1986, Pfannenstiel et al. 1986, Visser et al. 1986, Thiéry 1987). More recently, it has been demonstrated that the CytA protein is not essential for larvicidal activity on *Culex* and *Aedes* larvae (Delécluse et al. 1991) and that the CryIVA and CryIVB are responsible, for a major part of the toxicity to *C. pipiens* larvae in particular (Delécluse et al. 1991). Synergism between CryIVA and CryIVB has been shown by Angsuthanasombat et al. (1992), Delécluse et al. (1993). However, analysis of the data from Chilcott and Ellar (1988) evaluated by the method of Tabashnick (1992), demonstrated that the 27 kDa protein of *Bti* interacts synergistically with the proteins of 68 and 130 kDa. The effect of crystal protein mixtures of *Btmed* was also evaluated in bioassays with mosquito larvae, and the results analyzed according to the formula described by Tabashnick (1992). It became clear that the only mixture that interact synergistically was that of 68 and 30 kDa proteins (Table III). The mixture of the fractions containing the 94, 68 and 28-30 kDa proteins produced a slight increase in toxicity.

We have previously shown that differences in biological activity of *Btmed*, *Btm*, and *Bti* against *C. quinquefasciatus*, *Anopheles albimanus*, and *Aedes aegypti*, could be due to the absence of the 125-135 kDa proteins in *Btmed*, the presence of the 94 kDa protein in *Btmed*, the lack of immuno-

logical similarity with the strains PG-14 of *Btm* and 1884 of *Bti* or a combination of these factors (Orduz et al. 1992, 1994). The 94 kDa protein is the most important component of the *Btmed* toxins with an LC_{50} of 82.6 ng of protein/ml. This is in contrast to the observation made by Chilcott and Ellar (1988) who found that the most toxic component of the *Bti* crystal was the 68 kDa protein with an LC_{50} of 4 ng/ml.

The total immunological relationship of the crystal proteins of *Bti* and *Btm* has been demonstrated (Gill et al. 1987, Thiery 1987) and the 27 kDa crystal protein from these two subspecies differ only by one base and an amino acid change (Earp & Ellar 1987, Galjart et al. 1987). In this study the main inmunological cross reaction was observed between the 28-30 kDa protein of *Btmed* and CytA of *Bti*, in agreement with the results of Orduz et al. (1994). In other mosquito-active strains immunological similarities between crystal proteins of *Bti* and crystal proteins of *Bt* subsp. *fukuokaensis* (Yu et al. 1987), and *Bt* subsp. *darmstadiensis* (Dobroniewski & Ellar 1989) have not been observed. However, antibodies prepared against the 135 and 68 kDa crystal proteins of *Bti*, recognized 70 and 26 kDa crystal proteins from *Bt* subsp. *kyushuensis* (Held et al. 1990). In the opposite way, antibodies prepared against the crystal proteins of *Bt* subsp. *kyushuensis* recognizes crystal proteins of *Bt* subsp. *israelensis*, isolate PG-14 of *Bt* subsp. *morrisoni*, and *Bt* subsp. *darmstadiensis* (Ishii & Ohba 1992). In the case reported by Held et al. (1990), monoclonal antibodies prepared against the crystal proteins of *Bti* showed no cross-reactivity. These data suggest that, in some way, the mosquitocidal strains of *Bt* share common epitopes, which could be suggestive of common structures.

The role of the 94 kDa protein in the toxicity of the *Btmed* crystal which does not react with any of the three *Bti* antisera raised against CytA, CryIVD and CryIVA+B has been demonstrated. This study also provides pertinent information on the characteristics of the CPI of *Btmed*. The biological activity in this *Bt* subspecies is derived from proteins that are immunologically distinct from the previously known *Bt* mosquitocidal proteins. Furthermore, the hemolytic activity of the 28-30 kDa protein of *Btmed* is in agreement with those of similar size and immunologically related toxins produced by *Bti* and *Btm*. Further studies with recombinant strains with *Btmed* and *Bti* genes will provide important information on the mode of action and relationship of these biologically important molecules, that could lead to implement *Btmed* or a recombinant bacterium with the *Btmed* toxin genes in mosquito control programs worldwide.

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