Isolation and activity of a *Bacillus thuringiensis* Toxins which is Toxic to the *Aedes eagypti*

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Abstract: A *Bacillus thuringiensis* isolates has been discovered which is toxic to the dengue fever vector, *Aedes eagypti* as well as other Diptera and Lepidoptera . Crystal &- endotoxins purified from this isolates killed 50% of *Aedes aegypti* larvae at the concentration of 10.2 ug/ml , and β-exotoxin was not detected . Sodium dodecyl polyacrylamide get electrophoresis of the purified crystals revealed three protein species which were related to Cry IA(b),CryIB and CryIIA toxins on the basis of immunoreactivity and amino-terminal sequence determination. southern bolt and DNA restriction analysis suggested that the strain has sequences related to one cryIA(b),one cryIIA, and two cryIIB genes .

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1. Introduction

During sporulation, strains of *Bacillus thuringiensis* (*Bt*) make crystalline cytoplasmic protein inclusions that have been used for over 30 years as highly specific insecticides against certain species of Lepidoptera and Dipter, Van Franken huyzen, (1443). Although these toxins have been remarkably successful, their high specificity has precluded their use as a general pesticide. A strain of *Bt* has been identified that has demonstrable activity against the larvae of the danque fever vector (*Aedes aegypti*) . Although in the past such toxicity has always been attributed to the secretion of B-exotoxins (Vankova, 1981), this doesn't appear to be the case for this isolate.

The production and application of Bt has been developed quickly .*Bt* toxins contain crystal proteins Cry1Aa, CryIAbl, CryAc, Cry2A and Cry 2B (Mohan and Gujar, 2001). These *Bt* toxins are effective against dipterous pests. A generally accepted mode of action for Cry toxins describes the sequential steps of protoxin, activation, specific binding and cell toxicity, Soberon *et al.,* 2009. Both the required activation and more importantly binding steps confer remarkable pest specificity to Cry proteins (Pigolt *et al.,* 2007). Ingested insecticidal crystal proteins are activated to a toxic form by proteinases from the digestive insect gut fluids. After crossing the pertrophic matrix, activated toxins bind to specific receptor proteins on the mid gut microvilli(Hura *et al.,* 2003).

This paper describes the toxicity spectrum of this strain , its crystal proteins , and its gene content on the basis of restriction fragment profiles.

2. Material and Methods

Bacterial strains and growth conditions:

Bt strain YBT – 226 was identified in *Aedes aegypti* screen and is the property of E.I Dupont de Nemours, *Bt* subsp. *Kurstaki* HD-1 was obtained from H.D. Burges, Institute for horticultural Research , Little Hampton , UK . The conditions for growth and sporulation on CCY medium were as described for B.megaterium KM, Stewart *et al.* (1981).

Purification of protein inclusions:

Protein inclusions were purified from spore / crystal mixtures by centrifugation through discontinuous sucrose gradients, **Thomas and Ellar** (1983). Protein yield was determined by the method of Lowry *et al.* (1951).

Differential solubilization and activation of crystal proteins:

Protein inclusions were incubated at 37˚c for 60 min. at the concentration of 2 mg/ml in 50 mM Na_2Co_3.Hcl buffer at pH 4.5, 10.5 H.5 and in the presence or absence of 10 mM dithiathreitol. Insoluble material was pelleted by centrifugation at 10000 xg for 10 min. Soluble proteins were precipitated by adding 12% (w/v) critic acid until the solution reached pH 4.5 then incubating at – 20 ˚c for at least 3 hrs. these precipitates were centrifuged at 10000 x g for 15 min and the pellets washed in 2 mM sodium citrate pH 4.8 before resuspension in water. proteolytic activations of toxins by *Pieris* gut extracts chymotrypsin , and trypsin were as described else where (Nicholls *et al.* , 1984) .

4. Bioassays:

a. Aedes aegypti

For each test assay, larval feed consisted of 3g wheat bran and 0.4 g yeast extract thoroughly mixed and auto cleaved and 1.3 ml *Bt* Crystal suspension were mixed thoroughly into the feed, then added to 250 ml distilled water, and 20 one-day-old larvae added. Dead larvae were counted after 5 – days, during which time normal healthy larvae grow and pupate the concentrations at which 50% of larvae were killed (LC50) were determined by measuring in triplicate the death rate at different toxin concentrations.

b. *Pieris brassica* and *Musca domestica* .

These bioassays were carried essentially as described in Casse *et al.* (1979). *Pieris* larvae were examined after 8 and 72 hrs and *Musca* larvae at daily intervals up to one week, with mortalities being evaluated at the last time point. Equivalent quantities of complete and fractionated crystal proteins were estimated in each instance.

β– Exotoxin:

Two aliquots of about 500 mg purified crystals and four 5 ml aliquots of post – sporulation YBT Culture (in CCY medium) were taken and treated as follows: two of the latter aliquots were spun at 2700 xg for 15 min to pellet the spores , protein inclusions and cell debris after which only the supernatants were retained. One aliquot each of purified crystals, complete culture and culture supernatant were boiled for 10 min, and the various samples used in a β– exotoxin assay (Levinson *et al.*, 1990), using *Spodoptera littoralis* as the target organism . Distilled water, which is boiled by definition, was also used as a water control.

DNA preparation and analysis:

Bt plasmid DNA was prepared by the method of Casse *et al.* (1979).DNA restricition , southern blotting , probe preparation by *in vitro* transcription and hybridization were carried out as described by Sambrook *et al.* (1989). Hybridization was carried out overnight at 42˚C and the filters washed in 0.1xssc, 0.1% (w/v) SDS Several times for 15 min each at 50˚C. End- labeled lambda- Hind in size markers were prepared by incubating the restricted DNA in the presence of Klenow polymerase and α35 S – dATP. for cryL related sequence , the Eco RIF fragment of plasmide PESI (Kronstad *et al.*,1983) was cloned into the Eco RI site of PSELECT- 1. For Cryll - related sequences, the Accl-Hind III fragment encompassing the CryIIA gene (Donovan *et al.*, 1988 and Widner *et al.*, 1989) was end repaired with Klenow polymerase and blunt end ligated into the smaII site of PSELECT – 1. After linearising the CryI and CryII plasmids respectively with SSTI and Hinc II (which cuts within the coding sequence), messenger – sense α^{32} _ P _ labeled transcripts were made using T7 RNA polymerase.

4. Results and Discussion:

Crystal protein characterization:

After sporulation on CCY plates, microscopic examination of YBT-226 colonies revealed the presence of large crystals in the cells, generally bipyramidal with two other smaller inclusions at the same end. Experiments showed that the CryII - related species remained insoluble in bicarbonate buffer PH 9.5 in the presence of DTT while the larger proteins were dissolved these enabled the CryII toxin to be studied separately in bioassays the CryI-related proteins always dissolved under these conditions and were not separable by this technique. N- terminal sequencing of the proteins before and after proteolytic digestion yielded the date in table (1) . All the date on the 63 KDA band suggest that this toxin is very closely related, if not identical to Cry IIA. The 120 KDa and 130 KDa bands appear to be related respectively to CryIA and CryIB toxins, **Nicholls** *et al.* (1989).

Bio assays:

Certain *Bt* strains are known to produce β. exotoxins these are small, heat-stable adenine or uridine analogues, excreted from long phase cells which are through to inhibit DNA- dependent RNA polymerase and consequently have an indiscriminate toxicity spectrum. The ∂ - endotoxins, however, are heat sensitive and highly specific, Levinson *et al.* (1990).

The results in table (2) suggest that YBT- 226 does not produce any β - exotoxin that could account for observed toxicity, since boiling abolished the activity from complete culture and the centrifugation pellet (Wilton and Klowden, 1985).

Other bio assays results are summarized in table (3). Whole purified crystal had an LC_{50} of 10.2 mg/ml for *Aedes aegypti*. Even though the dual - specific Cry IIA _ related protein was toxins to *Musca*, the other dipteran species, it had no effect on *Aedes aegypti*. The CryI protein mixture had the expected toxicity to the Lepidopteran but not to the dipteran species - CryIA proteins have been widely studied and only one has been shown to possess dipteran toxicity (Haider and Ellar, 1987), namely to *Aedes*. Since the CryI protein precipitate had no effect on that insect, it seems unlikely that the Cry -IA protein possesses *Musca* toxicity. CryIB has always been considered to be Lepidopteran-specific, though preliminary work suggests it may also be toxic to Coleoptera (Bradley, *et al.*, 1992).

Furthermore, it shares 62% amino acid identity with the Cryv endotoxin. Tailor *et al.* (1992), so designated because it is toxic to Coleoptera and Lepidoptera.Thus, it is not clear whether the observed *Aedes aegypti* toxicity is due to an individual toxin or to some synergism between the toxins(Filha *et al.*,1999,Regis *et al.*,2001,Yuan *et al.*, 2001, Yuan *et al.*, 2003 and Wirth *et al.*, 2004.

DNA analysis:

Restriction digests of YBT-226 plasmid preparations were probed with CryI and CryII gene sequences. The former detected a fragment of about 5.2 kbp, while the latter illuminated fragments of about 5.2, 6.5 and 9 kbp. Washing filters at 60˚C removed the probe from the 6.5 and 9 kbp, species indicating their weaker sequence similarity to the CryIIA gene, while washing at 70˚C stripped the filters completely. The 5.2 kbp fragment detected by the CryI probe was cloned from a size - selected Hind III - fragment library and had the Eco RI restriction profile of the CryIA (b) gene. The 5.2 and 6.5 kbp fragments detected by the CryA probe were also cloned from size -selected fragment libraries. In restriction analysis, the former resembled the HD-1 CryIIA gene in every respect, while the latter was shown to be a CryIIB gene(Hodgman *et al.*,1990& 1993) almost identical to that described by other groups (Donovan *et al.*,1988 and Widner *et al.*,1989). The latter is encoded on a 9 kbp Hind III fragment on the same plasmid as the CryIIA gene(Donovan *et al.*,1988)and its hybridization stringency relative to CryIIA matches that of the YBT-226 9 kbp fragment , suggesting that YBT has this CryIIB gene as well. Since CryIIA was the only CryIIA gene product detected in the protein crystals, the identify of the 9 kbp fragment was not pursued further, many authors hoped that similar researches could be developed for *Bti* use against diseases vectors such as *Aedes aegypti* (Sihuincha *et al.*,2005, Arredondo *et al.*, 2006 , Seccacini *et al.*, 2008 , Ponlawat *et al.*, 2009 and Ritchie *et al.* , 2010) . Generally, *Bti* subspecies HD-I and HD-I3 induced toxicity against *Aedes aegypti* when administrated at the high Concentration of 500 mg / ml , by activating these toxins they killed larvae or inhibit pupation and reduce the life span of the target pest .

Table 1: Amino terminal sequences of YBT-226 Proteins

Protein band	Sequence obtained	related sequence
130 KDa	TSN?KNENEL ? NAV	cryIB protoxin
120 KDa	MDNNPNINE? IPYN	cryIA(a. b or c) protoxin
120 KDa after trypsin activation	IETGYT? IDIS	cryIA(a, b)activated toxin
63 KDa	MNNVLNSGRTTI?D	cryIIA protoxin
63 kDa after pieris gut extract treatment	VAPVVGTVS	cryIIA activated
63 kDa after pieris gut extract and Chymotrypsin treatment	ITSSVNTMQQLFLN	cryIIA activated

Table 2: Effect of YBT-226 culture fractions on the vitality of *Spodoptera littoralis*

Fraction	No. of dead spodoptera larvae (out of 5)	
	Untreated	Boiled
Complete culture	**3**	**0**
Pellet	**2**	**0**
Supernatant	**0**	**1**
Water control	**n.d.**	**1**
n.d. = not determined		

Table 3: Bioassays of YBT-226 crystals and fractions

=Fraction	Mortality induced in		
	M. domestica	*P. brassicae*	*A . aegypti*
Whole purified crystal	+	+	+
CryI precipitate	**n.d.**	+	-
Insoluble cryII protein	-	+	+
Water control	-	-	-
In these assays, + and – refer to 100% and 0% mortality respectively. The LC_{50} value for the positive result in the Musca assay was 10.2 ug/ml. n.d. = not done.			

Fig 1: YBT-226 plasmid preparation probed with Cry and Cry11 gene sequences.

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