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Lack of Mosquitocidal Activity by the Cytolytic Protein of the *Bacillus thuringiensis* subsp. *israelensis* Parasporal Crystal

Peter Y.K. Cheung, Dan Buster, and Bruce D. Hammock

Departments of Entomology and Environmental Toxicology, University of California, Davis, California, USA

Abstract. A 25,000-dalton cytolytic protein was isolated from the parasporal crystal of *Bacillus thuringiensis* subsp. *israelensis*. Hemolytic activity of this protein decreased with increasing pHs and was totally inhibited at pH 10.0. No mosquito larvacidal activity was observed with this protein either in the solubilized form or when the protein was adsorbed to latex beads.

Bacillus thuringiensis subsp. *israelensis* (Bti) is an entomopathogen for mosquito and blackfly larvae [7, 14]. A proteinaceous crystal body is produced by the bacterium during sporulation. This crystal body (delta-endotoxin) is made up of a number of protein components [2, 13], one of which has been isolated and demonstrated to possess potent cytolytic activity [1, 6, 12]. Although some mosquito larvacidal activities have been reported with this cytolytic protein, there is a significant reduction in specific toxicity when compared to the crystals or the unfractionated alkaline extract. This decrease in toxicity has been attributed to the fact that mosquito larvae selectively concentrate particulate matter. Thus, when solubilized toxin was adsorbed onto latex beads or encapsulated in lipid droplets and presented to mosquito larvae, specific toxicity similar to those of the crystal form was reported [4, 11].

In this study, the cytolytic protein of the crystal toxin was isolated by anion exchange and further purified by disulfide exchange chromatographies. Cytolytic activities of this protein were assayed against rabbit red blood cells between pHs 7 and 10. This protein was also bioassayed against mosquito larvae in the soluble and particulate forms.

Materials and Methods

Bacillus thuringiensis subsp. *israelensis* HD-567 was obtained from Dr. B.C. Carlton (then of the University of Georgia). Cells for experimental use were cultured in a modified glucose–yeast extract–salt medium [10] and harvested as described previously [5]. A discontinuous sucrose density-gradient of 50%, 63%, and 73% sucrose (wt/vol) in 0.03% Triton X-100 was used to separate the crystals and the spores [3]. Purified crystals were then washed with distilled water (3×) to remove sucrose and Triton X-100.

Toxin was solubilized by suspending the crystal in 0.5% sodium carbonate and 0.02% sodium azide (pH 11.0) for 3 h with sonication at 30-min intervals. Nonsoluble matter was removed by centrifugation at 10,000 rpm/15 min (SS-34 rotor, Sorvall). The alkaline-solubilized proteins were fractionated by anion exchange chromatography on a DEAE–Sephacel column as described in a previous publication [3]. Fractions with hemolytic activity were pooled and added to thiopropyl-Sepharose 6B gel (Pharmacia) to remove sulfhydryl-containing contaminants. After end-over-end mixing at room temperature for 30 min, the slurry was passed through a small column and the supernatant was collected.

Both the unfractionated alkaline extract and the isolated cytolytic protein were adsorbed to 0.8-μm latex beads (Sigma) as described by Schnell et al. [11]. Toxin concentration was first adjusted to 50 μg/ml in a 0.1 *M* Tris–HCl solution (pH 7.5) and 100 μl of the latex bead suspension was added. The suspension was incubated at room temperature for 1 h. Beads were then removed by centrifugation and excess binding sites were covered by suspension in 0.01% polyvinylpyrrolidone. Efficiency of adsorption was estimated by measuring the protein concentration of the supernatant before and after addition of latex beads using the method described by Lowry et al. [9]. As described previously [5], mosquito toxicities were assayed against early 4th instar larvae of *Aedes aegypti* and hemolytic assays were performed on rabbit red blood cells (RBC).

Results and Discussion

SDS–PAGE. The protein compositions of the Bti whole crystal, alkaline extract of the crystal, and a 25,000-dalton cytolytic protein isolated from the crystal were compared by sodium–dodecyl-sulfate polyacrylamide gel electrophoresis (SDS–PAGE, 12.5%, as described by Laemmli [8]) and the results are shown in Fig. 1. The whole crystals were dis-

Address reprint requests to: Dr. Peter Y.K. Cheung, Department of Entomology, University of California, Davis, CA 95616, USA.

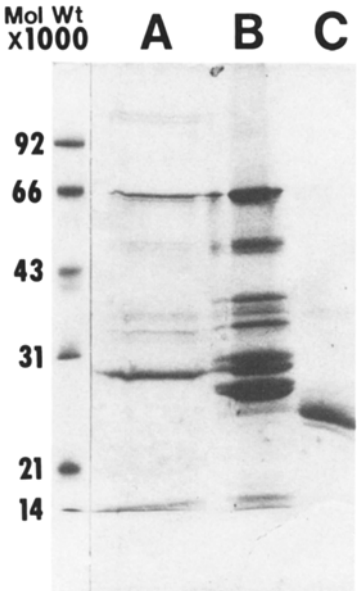

Fig. 1. Protein compositions of the (A) whole crystals, 60 μg; (B) alkaline-solubilized toxin, 50 μg; and (C) 25,000-dalton cytolytic protein, 10 μg.

solved in the SDS–PAGE sample buffer which contains SDS and 2-mercaptoethanol. A 28,000-dalton protein and a 60,000-dalton protein were the two major components of the Bti crystals. Alkaline extraction of the crystal gave a host of proteins with molecular weight ranging from 10- to 60,000-daltons. The protein profile of the alkaline extract became increasingly complex as extraction time increased. Endogenous protease(s) associated with the Bti crystal are believed to be responsible for this observation.

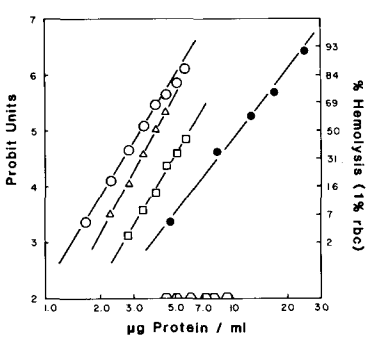

Fig. 2. Hemolysis assay of Bti toxin preparations. Percent hemolysis is defined as OD_{500} of sample supernatant divided by OD_{500} of total lysis (by SDS) of a 1% rabbit RBC suspension after 15 min incubation at room temperature: (--●--) alkaline extract at pH 7.2, (--○--) 25,000-dalton cytolytic protein at pH 7.2, (--△--) cytolytic protein at pH 8.1, (--□--) cytolytic protein at pH 9.1, and (--◇--) cytolytic protein at pH 10.1.

Table 1. Toxicities of *Bacillus thuringiensis* subsp. *israelensis* toxin preparations to 4th instar larvae of *Aedes aegypti*

Toxin preparation	LC_{50}[a] (ng/ml)
Whole crystals	5.81 (5.98–5.64, 4.23)
Alkaline-solubilized toxin	6889 (7126–6060, 3.46)
25,000-dalton protein	>40,000[b]
Solubilized toxin adsorbed to latex beads	196 (206–186, 6.78)
25,000-dalton protein adsorbed to latex beads	>1000[b]

[a] LC_{50}, median lethal concentration. The 95% confidence intervals, and slope of probit analysis are in parentheses. Probit analyses were performed on at least four toxin concentrations giving 10%–90% mortality at 24 h.

[b] No mosquito toxicity up to the concentration shown.

Hemolysis vs pH. Hemolytic activities of the 25,000-dalton cytolytic protein and the alkaline extract of the crystal are shown in Fig. 2. At physiological pH, specific hemolytic activity of the purified 25,000-dalton protein was approximately threefold over the alkaline extract. Hemolytic activities of the 25,000-dalton protein decreased with increasing pH. At pH 10.1, with a fivefold increase in toxin concentration, no hemolysis was observed within the 15-min assay time. Subsequent incubation of the same RBC at pH 7.2 did not lead to hemolysis. To determine whether hemolysis was affected by high pH, RBC suspensions at pHs 7.2–10.1 were subjected to lysis by SDS (40 μg/ml) and distilled water. Results showed that hemolysis was not affected at high pH by either SDS or distilled water. Furthermore, preincubation of the cytolytic protein at pH 10.1 did not interfere with its hemolytic activity at pH 7.2. Therefore, it appears that binding of the cytolytic protein to RBC was impaired at high pH. Whether this same pH effect will extend to cultured mosquito cell lines will be extremely interesting to investigate.

Larvacidal activities. Results of the mosquito larvacidal activities are summarized in Table 1. The toxicities of the intact crystal and the solubilized toxin were in the expected ranges with an 1187-fold decrease in toxicity when toxin was presented to the mosquito larvae in the solubilized form. No toxicity was observed with the purified cytolytic protein up to 40 μg/ml. This represents a greater than fivefold

decrease in toxicity from the alkaline extract. When the alkaline extract was adsorbed to latex beads and presented to mosquito larvae, LC_{50} was reduced from 6889 to 196 ng/ml, a 35-fold increase in specific toxicity. On the other hand, no increase in toxicity was detected with the cytolytic protein-coated latex beads. Similar results were observed when the two toxin preparations were encapsulated in microlipid droplets. [4].

In conclusion, the data presented in this report strongly suggest that the cytolytic component of the Bti crystal is not insecticidal. Biological assays of the 25,000-dalton protein suggested that its pH optimum for RBC lysis is at physiological rather than at high alkaline pHs. In addition, the low larval toxicities of the cytolytic protein in either the soluble form or particulate form further support the conclusion that it does not act alone as the insecticidal component of the Bti delta-endotoxin.

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