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ISOLATION OF A PROTEIN FROM THE PARASPORAL CRYSTAL OF BACILLUS THURINGIENSIS VAR. KURSTAKI TOXIC TO THE MOSQUITO LARVA, AEDES TAENIORHYNCHUS[1/]

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SUMMARY: The parasporal crystal produced by a strain of *Bacillus thuringiensis* var. *kurstaki* (HD-1) contains two serologically distinct proteins. These proteins were isolated from a preparation of the parasporal crystal by Sephacryl S-300 column chromatography. Their molecular weights were estimated as 135,000 and 65,000. Both proteins were toxic to the cabbage looper, *Trichoplusia ni*, but only the 65,000-dalton protein was toxic to larvae of the mosquito, *Aedes taeniorhynchus*. Biochemical comparisons based on isoelectric focusing and peptide mapping by two-dimensional gel electrophoresis indicated that the two toxins were distinctly different.

Bacillus thuringiensis var. *kurstaki*, like most isolates of *B. thuringiensis*, produces a parasporal crystalline inclusion body that is toxic to many insect pests. When cells of this organism lyse, the crystals and spores are released into a culture fluid. The parasporal crystals can be isolated by isopycnic centrifugation in a density gradient medium such as NaBr (1). The crystals are made of at least one protein subunit with a mol. wt. of about 130,000 (2,3).

Krywienczyk et al. (4) discovered that crystal antigens of *B. thuringiensis* var. *kurstaki* could be divided into two types by double immunodiffusion. The two crystal types were designated as *k-1* and *k-73*. Both crystal types, *k-1*

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and *k-73*, were highly toxic to lepidopteran species, but only *k-1*-type crystals were toxic to mosquito larvae (5).

In this communication, we report that a protein, that is toxic to mosquito larvae, has been isolated from *k-1*-type crystals and partially characterized by biochemical analyses.

MATERIALS AND METHODS

B. thuringiensis var. *kurstaki* (HD-1) isolated by Dulmage (6) was cultured in tryptose phosphate broth until they lysed. The mixture of crystals and spores was washed in 1 M NaCl at least three times by means of centrifugation in order to remove the proteinase produced by *B. thuringiensis*. The crystals were then purified by centrifugation in a linear 1.30-1.34 (sp. gr.) NaBr density gradient that was repeated three times. Crystals banded at the bouyant density of 1.32 were collected, dialyzed in water, lyophilized, and stored at -20°C.

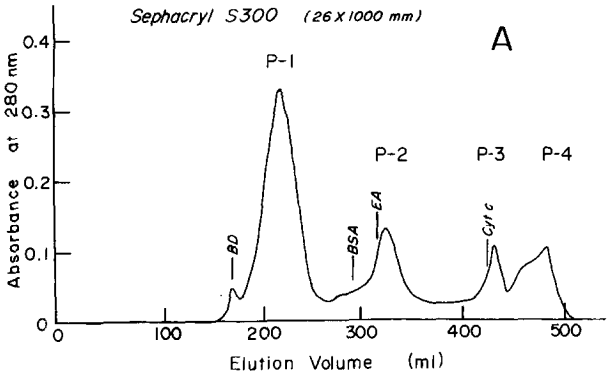
The crystals (50 mg) were dissolved in 3 ml of 2% 2-mercaptoethanol-water solution that was adjusted to pH 10 with 2 N NaOH. After centrifugation at 100,000 g for 30 min, the supernatant was chromatographed with a 2.5 X 100 cm column of Sephacryl S-300 (Pharmacia). The eluate was monitored by absorbance at 280 nm and by fused-rocket immunoelectrophoresis using anti-solubilized-crystal-serum. The details of the immunoelectrophoresis have been described by Yamamoto et al. (7).

Isoelectric points of the toxins were determined by electrofocusing in 10-40% sucrose density gradient containing 2% carrier ampholytes (LKB Ampholine pH 3.5-10) and 0.1% Triton X-100. A micro isoelectric focusing column (5 ml capacity) was constructed (a drawing of the column can be obtained from one of the authors, T. Y.). After focusing at 1,000 V for 8 hr, the solution in the column was pumped through a flow cell (100 µl) where the pH was measured. The column eluate from the flow cell was fractionated into 150-µl aliquots and analyzed for the crystal proteins by fused-rocket immunoelectrophoresis.

Peptide maps of the toxin were produced by controlled proteolysis in a sodium dodecyl sulfate (SDS)-containing gel by using *Staphylococcus aureus* V8 proteinase (Miles). The method of the mapping described by Bordier and Crettol-Järvinen (8) was essentially followed. A vertical slab gel (14 X 11 X 0.1 cm) was used for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with a uniform buffer system (0.1 M Tris-acetate, pH 8.2, containing 0.1% SDS).

Gut juice was collected from fifth-instar cabbage looper larvae (*Trichoplusia ni*), and the proteinase in the gut juice was partly purified by ammonium sulfate fractionation (15-80%, saturated). The proteinase fraction was immobilized on CNBr-activated Sepharose 4B (Pharmacia). The toxins were exposed to the proteinase-immobilized Sepharose gel in 50 nM CAPS-NaOH (cyclohexylaminopropane sulfic acid) pH 10.2, and then digested toxins (fragments) were analyzed by SDS-PAGE and peptide mapping.

Activities of the toxins were bioassayed with first-instar cabbage looper larvae, *Trichoplusia ni*, and second-instar mosquito larvae, *Aedes taeniorhynchus*. The toxin was diluted in water, and the seven dilutions were made around a tentative LC_{50} that was estimated by preliminary bioassay with a series of one-tenth dilutions. In bioassays against cabbage looper larvae, the toxin was added to an artificial diet, and 0.5 ml of the diet was fed to each larva. Twenty-five larvae were used for each treatment. In the case of mosquito larvae, the toxin was deployed in 4 ml of well water containing 30 mosquito larvae and 0.06% tropical fish food (Tetramin). LC_{50} values were determined by a log concentration-probit analysis.



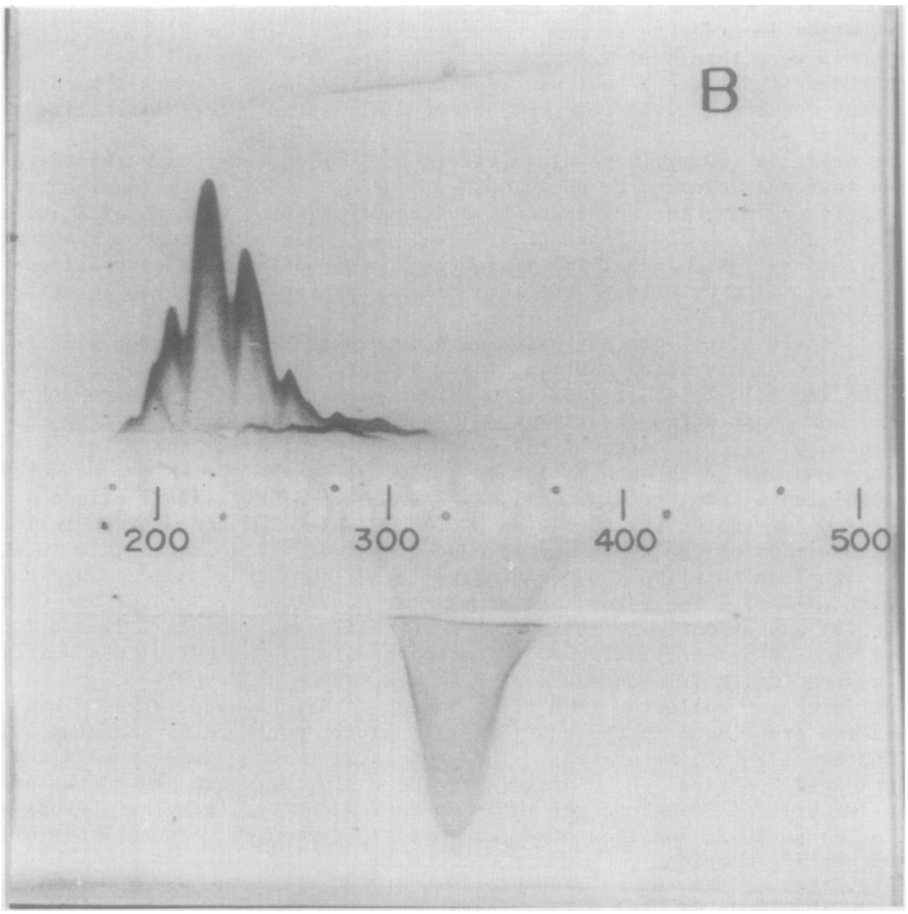


Fig. 1. Elution pattern of the solubilized crystal. Crystals were solubilized in 2% 2-mercaptoethanol-NaOH, pH 10, and chromatographed in a column of Sephacryl S-300 equilibrated with a mixture of 50 mM Tris-HCl, ph 8, 0.1% 2-mercaptoethanol, and 1 mM EDTA. A, monitored by absorbance at 280 nm; B, by fused-rocket immunoelectrophoresis. The column was calibrated by BD, blue dextran; BSA, bovine serum albumin; EA, egg albumin; Cyt c, cytochrome c. Numbers appearing in B indicate the elution volume in ml.

Table 1. Toxic activities of protein isolates from the parasporal crystal of *B. thuringiensis* var. *kurstaki*

Toxins	LC_{50} (μg/ml)[a/]	
	Cabbage looper[b/]	Mosquito[b/]
P-1	0.271 (0.268-0.286)[c/]	Not toxic[d/]
P-2	0.311 (0.307-0.315)	1.48 (0.94-2.01)

a/ Concentrations of the toxin in the diet (cabbage looper) or in the water (mosquito).
b/ The mortality was read at 7 days for cabbage looper and at 3 days for mosquito.
c/ 95% confidence limits are given in parentheses.
d/ No significant toxicity was found up to 500 μg/ml.

RESULTS

Four major peaks appeared during the gel filtration of the solubilized crystals (Fig. 1-A). Fused-rocket immunoelectrophoresis (Fig. 1-B) indicated that the first two peaks, P-1 and P-2, were antigenic. These two peaks were almost equally toxic to the cabbage looper larva (Table 1). Peaks, P-3 and P-4, appeared to be not proteins but nucleic acids by absorbance spectrum analysis and were not toxic to the insect.

The mol. wts. of P-1 and P-2 were determined as 135,000 and 65,000, respectively, by SDS-PAGE (Fig. 2). However, these mol. wts., in particular that of P-2, had not been expected by gel filtration. The results of the gel filtration can be explained by the hypothesis that P-1 formed a dimer, and P-2 had a weak affinity to the column medium in the buffer system used in the chromatography. The affinity of P-2 to the column medium was diminished by the addition of 0.1% SDS to the elution buffer. When this was done, P-2 appeared at the elution volume expected for proteins with a mol. wt. of 65,000.

As shown in Figs. 1-B and 2, the single chromatograph resulted in a good separation of P-1 and P-2. These two components were collected and assayed against mosquito larvae. Only P-2 had a significant activity against this insect (Table 1).

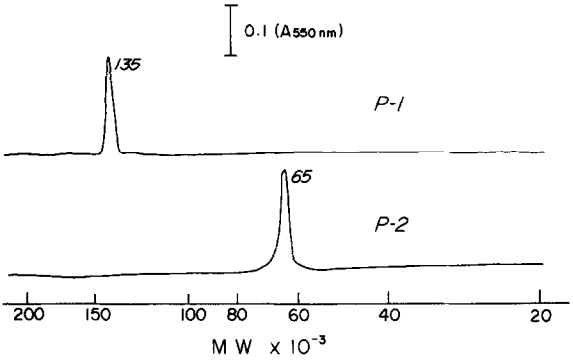


Fig. 2. SDS-PAGE of P-1 and P-2 isolated by Sephacryl column chromatography. Samples were treated in 2% SDS and 2% 2-mercaptoethanol at 100°C for 5 min and electrophoresed in 10% gel.

Since P-2 migrated toward the negative electrode in the immunoelectrophoresis procedure run at pH 8.2, P-2 was considered to be an alkaline protein. Isoelectric points (pI) of P-1 and P-2 were determined as 4.4 and 10.7, respectively, by isoelectric focusing (Fig. 3). To prevent precipitation of

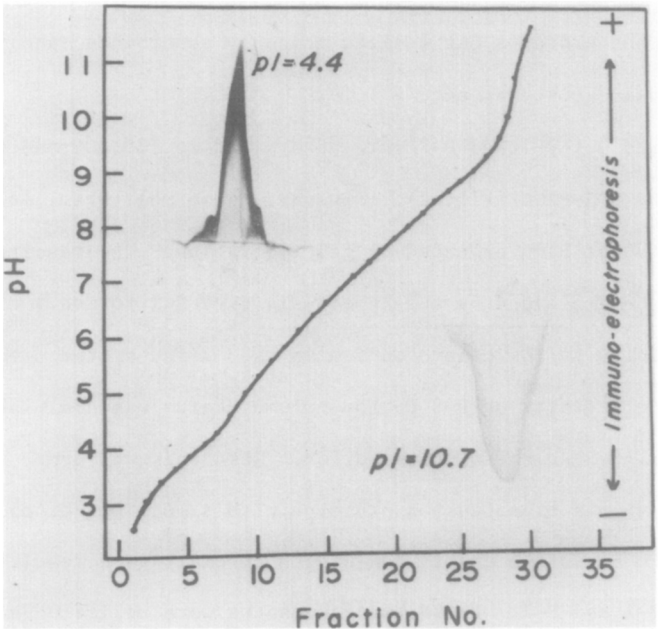


Fig. 3. Isoelectric focusing of the solubilized crystal. About 100 μg protein was focused in a 5-ml column. The column eluate was monitored by fused-rocket immunoelectrophoresis using anti-solubilized-crystal-serum.

the proteins that would have disturbed the column during the focusing, the amount of sample was greatly reduced (Fig. 3). About 100 µg of the sample was adequate because of the sensitivity of the immunoelectrophoresis.

The isolated P-1 was susceptible to proteinases. When P-1 was incubated with proteinase from cabbage looper larva gut juice that had been immobilized on Sepharose 4B, 135,000-dalton protein decreased in amount, and fragmented proteins appeared as distinct bands representing mol. wts. of 120,000, 100,000, 83,000, and 62,000 on SDS-PAGE (Fig. 4-B, 1st dimension). When the mixture was incubated for 40 min or longer, only the 62,000-dalton protein could be detected by SDS-PAGE. These fragments were subsequently analyzed by peptide mapping, and the patterns were compared with those of intact P-1 and P-2 (Fig. 4). There was no difference in the peptide pattern among the fragmented moeties of P-1, but peptide pattern of P-1 differed greatly from that of P-2.

On the other hand, proteinases such as trypsin, chymotrypsin, and cabbage looper larva gut juice constituents had no effect on P-2.

DISCUSSION

Several different buffer systems were tested in the gel filtration to obtain a better separation of P-1 and P-2. P-2 was apparently less soluble than P-1 at pH 8. P-2 appeared to be overlapped by P-1 due to an aggregation of P-2 when the concentration of the buffer was lowered, unless the pH was kept higher than 10. A reducing agent, 2-mercaptoethanol, was also essential for separation. Other workers (9) have reported toxic activities associated with small peptides isolated from the crystals by chromatography. In our chromatographic system, we could not detect these small peptides. However, we confirmed the report (10) that even after extensive NaCl-treatment, there was a trace of the *B. thuringiensis* proteinase. A partially digested P-1 can be seen on the chromatographs (Figs. 1-A and 1-B) at the elution volume of 280 ml. It was important to process the sample promptly and to run the chromatography at a low temperature (4°C) in order to separate P-1 and P-2.

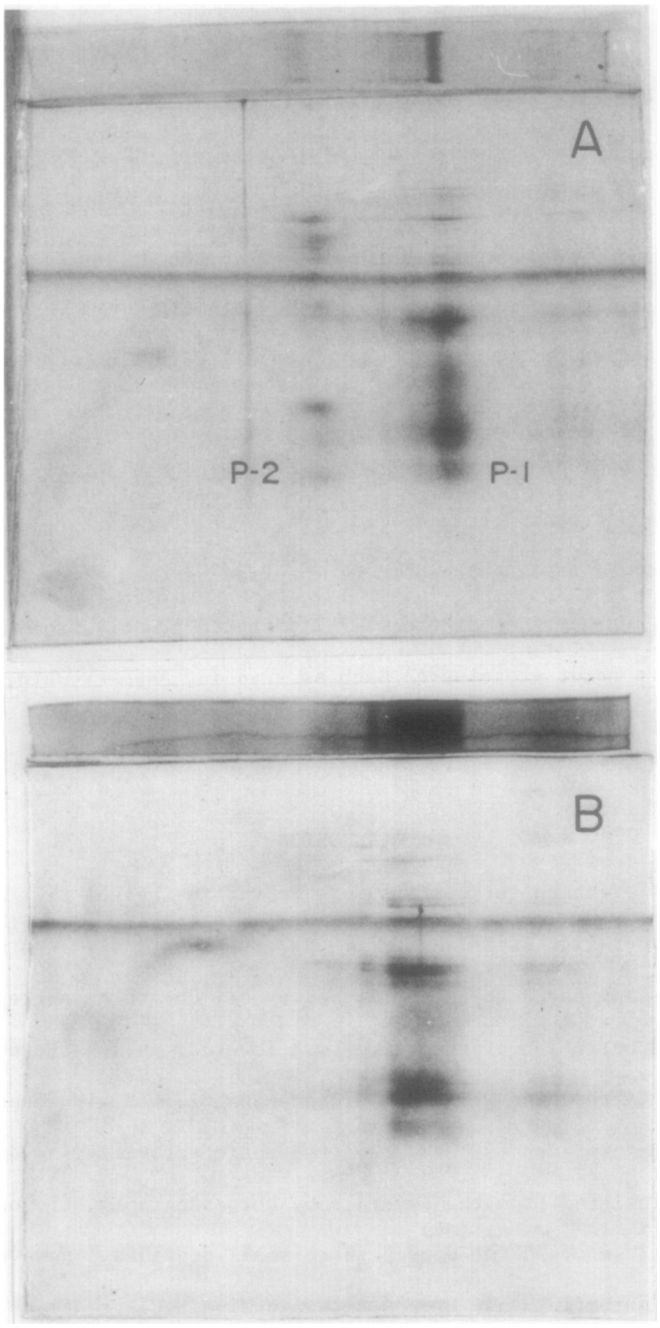


Fig. 4. Two-dimensional SDS-PAGE peptide mapping of toxins. In 1-D, 5% gel was used, and the sample (80 μg protein) migrated from right to left. In 2-D, 15% gel was used, and 20 μg of *S. aureus* V8 proteinase was overlayed on the top of the 1-D gel. The line across the 2-D-gel is the band of *S. aureus* proteinase. A, solubilized crystal; B, P-1 partly digested by the proteinase from cabbage looper gut juice.

From the enzymatic digestion of P-1, we conclude that P-1 consisted of two parts: the proteinase-resistant 62,000-dalton portion and the other proteinase-sensitive part with mol. wt. of 73,000. Since there was no difference in the peptide pattern among proteins derived from P-1, we speculated that the proteinase-sensitive fragments cleaved from the molecule of P-1 by the proteinase digestion were broken down further to small peptides which were not retained by the polyacrylamide gel during the fixation process. Although one could hypothesize that P-2 is a fragment of P-1 produced by proteinase digestion, the biochemical analyses, in particular peptide mapping, indicate that P-2 is an entirely different protein from P-1 or its proteinase-resistant portion.

Since only P-2 is toxic to the mosquito larvae, we propose to designate P-2 as mosquito factor. The mosquito factor can be detected easily by immunoelectrophoresis as an alkaline protein. This factor (P-2) has been detected only in *k-1*-type crystals produced by *B. thuringiensis* var. *kurstaki* and var. *thuringiensis* (Yamamoto, unpublished). We are continuing the study on the structure and the mode of action of the mosquito factor.

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