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Toxic Trypsin Digest Fragment from the *Bacillus thuringiensis* Parasporal Protein

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Enzymatic digestion in vitro of the *Bacillus thuringiensis* protoxin presumably releases and activates the toxin in a manner analogous to that which occurs when a *B. thuringiensis* sporulated fermentation preparation passes through the midgut of a lepidopteran larva. Therefore, a sporulated culture of *B. thuringiensis* subsp. *kurstaki* (serotype 3a3b) HD-263 was treated with trypsin to release an activated toxin soluble in bicarbonate buffer. A 63-kilodalton protein, toxic to cabbage looper larvae (*Trichoplusia ni*) and to lepidopteran cells in culture, was purified to homogeneity from this trypsin digest. The larvicide, a glycoprotein containing 5% carbohydrate (wt/wt), was purified from the soluble *B. thuringiensis* trypsin digest by using ammonium sulfate precipitation, anion-exchange chromatography, and hydrophobic-interaction chromatography. Its amino acid composition was high in nonpolar residues and unusually low in lysine and histidine. The isoelectric point was 6.5, and the amino acid on the N terminus was identified as isoleucine. The toxin was only slightly soluble in aqueous buffers unless the chaotropic agent potassium thiocyanate was added. Partial characterization of the toxin indicated that it corresponds well with reported sequences deduced from cloned genes.

Bacillus thuringiensis with the onset of sporulation produces intracellular crystalline protein which can kill lepidopteran larvae on ingestion (6, 20). The principal component of the crystals is a protein protoxin of M_r 130,000 to 155,000 which is partially digested in the caterpillar midgut to release the actual toxic peptide, probably of M_r 40,000 to 70,000 (8, 13, 22). The toxicity spectrum for susceptible caterpillar species varies for each *B. thuringiensis* isolate, although similar toxicity patterns are found within each of the 20 or so identified serotypes or subspecies (6). Commercial application of *Bt*, the unpurified insecticidal fermentation-sporulation residue, usually uses *B. thuringiensis* subsp. *kurstaki* HD-1. Recently, it has been suggested that *B. thuringiensis* subsp. *kurstaki* HD-263 has greater insecticidal potency for certain pests of economical importance (19).

We report a simple procedure for the isolation with good yield of a homogeneous trypsin digest fragment of M_r 63,000 from *B. thuringiensis* subsp. *kurstaki* HD-263.

MATERIALS AND METHODS

Organism. *B. thuringiensis* HD-263 is a *kurstaki* subspecies isolate, serotype 3a3b, crystal type k-1, obtained from Howard Dulmage, U.S. Department of Agriculture Cotton Insect Research Unit, Brownsville, Tex. Sporulated cultures that had been stored frozen were heated at 80°C for 15 min and inoculated into a citrate-salt growth medium (31) supplemented with (per liter) 0.5 mg each of $FeCl_3 \cdot H_2O$, $ZnSO_4 \cdot 7H_2O$, and $CaCl_2 \cdot 2H_2O$ and 1.0 mg of $MnSO_4 \cdot H_2O$. Glucose to 1.0 g/liter and casein amino acids to 0.4 g/liter were sterilized separately before addition to the medium. Growth and sporulation took place over 72 h at 30°C in flasks shaken at 200 rpm. The pelleted residue (spores, parasporal crystals, and cellular debris) obtained with a continuous-flow centrifuge was washed twice with 1 M NaCl and then with water and was freeze-dried.

Trypsin digestion and isolation of the toxic protein. The crude fermentation powder (10 mg/ml) was suspended with commercial bovine trypsin (T-8253; 0.25 mg/ml; Sigma Chemical Co.) in 20 mM $NaHCO_3$ (pH 9.0) and magnetically stirred at room temperature for 2 h. The supernatant was removed after centrifugation at 20,000 × *g* for 30 min at 4°C. Solid $(NH_4)_2SO_4$ was slowly added to the ice-chilled supernatant until 12% saturation was reached (27). A small precipitate was discarded after centrifugation at 4°C for 30 min. Additional $(NH_4)_2SO_4$ was added to the supernatant over a period of 1 h until 24% saturation was reached. After an incubation at 4°C for 30 min, the precipitate was removed by centrifugation, dissolved in a minimum volume of 20 mM $NaHCO_3$–0.1 M 2-mercaptoethanol (2-ME)–2 M KSCN (pH 9.5; buffer A), and then dialyzed at 4°C against 20 mM $NaHCO_3$ (pH 9.0) to remove the KSCN which was added to effect solution easily (9). The 2-ME concentration of the sample was adjusted to 0.1 M, and the pH was adjusted to 9.5; additional buffer (minus the KSCN; buffer B) was added, and the suspension was stirred for 7 h. Some insoluble residue was removed by centrifugation, and the supernatant was applied to a column of DEAE BioGel A resin (Bio-Rad Laboratories) previously equilibrated with buffer B. The column (diameter, 2.2 cm; bed volume, 40 m) was eluted with buffer B at 28 ml/h at 4°C. The unbound toxic fraction was dialyzed against water and freeze-dried. It was dissolved by stirring for 2 h at room temperature in 20 mM $NaHCO_3$–1 mM 2-ME–0.02% NaN_3 (pH 9.5; buffer C) containing 4 M NaCl. Insoluble material was removed by centrifugation, and the supernatant was applied to a column of phenyl-Sepharose CL-4B (Pharmacia Fine Chemicals; bed volume, 5.5 ml; column diameter, 1.6 cm). Unbound material was washed off with buffer C containing 4 M NaCl at room temperature at a flow rate of 21 ml/h. Buffer C without NaCl was used to remove weakly bound protein; buffer C containing 2 M KSCN was then used to elute the toxin. The fraction was then dialyzed against water and freeze-dried.

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Estimation of protein concentration. The protein concentration of solutions was estimated with either the Kalckar equation [milligrams per milliliter of protein = $(1.45 \times A_{280}) - (0.74 \times A_{260})$] (15) or the modified Lowry procedure of Hartree (12).

Electrophoresis, isoelectric focusing, and molecular weight estimation. Protein samples were analyzed after electrophoresis on 10% polyacrylamide slab gels containing sodium dodecyl sulfate (SDS-PAGE) by using a Tris-glycine discontinuous buffer system (24). Gels were stained for protein with Coomassie brilliant blue R and for glycoprotein (carbohydrate) with a basic fuchsin-Schiff reagent (7). Molecular weights of protein bands were estimated by comparing their mobilities with those of the following standard proteins run on the same gel: beta-galactosidase, 116 kilodaltons (kDa); phosphorylase *b*, 97.4 kDa; bovine serum albumin, 66 kDa; catalase, 60 kDa; and cross-linked hemoglobin, 16, 32, 48, and 64 kDa.

Isoelectric focusing was performed on horizontal slab gels containing 2.3% ampholytes (3/10 Bio-Lyte; Bio-Rad) and 6 M recrystallized urea (17). Reservoir buffers were 1 N NaOH and 1 N H_3PO_4. Proteins (10 to 100 μg) dissolved in 6 M urea were injected into a series of small cuts in the gel, along with bovine serum albumin (pI, 5.86 to 6.01) and sperm whale myglobin (pI, 6.90 to 7.73) (29). Gels were cooled at 10°C during the electrophoresis performed at 1 W of constant power for 14 to 24 h. The pH gradient was measured after incubating parallel gel slices in 1.5 ml of water and by using a pH electrode. Proteins were visualized after staining with Coomassie brilliant blue plus copper sulfate in methanol-acetic acid-water (18).

Immunochemistry. Antiserum was prepared by subcutaneously injecting into a male New Zealand albino rabbit 1 mg of the purified caterpillar toxin homogenized in 0.5 ml of 0.15 M phosphate-buffered saline (pH 7.2) plus 0.5 ml of Freund complete adjuvant. At 1 month later, a second injection was made, followed 10 days later by the collection of 15 ml of blood from an ear vein. The blood was allowed to clot for 2 h at room temperature and 4°C overnight before solid material was removed by three repeated centrifugations at 1,000 × *g*. Lipid was separated after centrifugation at 3,000 × *g* and 0°C. Antiserum samples were stored frozen. Antigen-antibody reactivity was determined by the Ouchterlony double-diffusion assay on microscope slides (23).

Caterpillar gut juice digestion of unpurified *B. thuringiensis*. *Trichoplusia ni* (cabbage looper) fourth- and fifth-instar larvae were caused to regurgitate by gently rubbing their underbellies and pinching their bodies with tweezers. The collected, pooled gut liquid was centrifuged at 17,000 × *g* at 4°C for 20 min. The gut juice supernatant had 100 mg of protein per ml. The proteolytic activity of the clarified supernatant was measured by using the Azocoll procedure (Calbiochem-Behring; procedure recommended by the manufacturer). The proteolytic activity of the gut juice supernatant was equivalent to 1.5 mg of trypsin per ml. The gut juice supernatant was diluted sixfold with 20 mM $NaHCO_3$ (pH 9.0) to bring its protease activity to the equivalent of 0.25 mg of trypsin per ml. Digestion of *B. thuringiensis* subsp. *kurstaki* fermentation residue at 10 mg per ml was then performed as previously described for trypsin, yielding the gut juice digest.

Amino acid and carbohydrate analysis. The purified toxin was hydrolyzed with 3 N mercaptoethanesulfonic acid (Sigma) at 100°C for 24 to 72 h (25). The amino acid analyses were done by using an amino acid analyzer (119 CL; Beckman Instruments, Inc.) with the standard three-buffer system. The amount of carbohydrate tightly bound to the purified toxin was estimated by using the phenol-sulfuric acid procedure (4) with a glucose standard curve.

End-group determination. The N-terminal amino acids were identified by the dansyl chloride procedure (10). The toxin (0.5 to 2.0 mg) was converted to the dansyl-peptide by reaction with 1-dimethylaminonaphthalene-5-sulfonyl chloride (dansyl chloride; Sigma) and then hydrolyzed with 0.5 ml at 5.7 N HCl at 110°C for 18 h. The hydrolysate was taken to dryness, taken up in water, and applied in a spot near one corner of a square of polyamide sheet (4 by 4 cm; Cheng Chin Trading Co.). Dansylated amino acid standards were applied on the opposite side of the sheet, and the sheet was developed with 1.35% (vol/vol) aqueous formic acid and then with benzene-acetic acid (9:1 [vol/vol]) in the perpendicular direction (32). Ultimately, positive identification was obtained by applying the labeled toxin hydrolysate and the suspected dansylated amino acid standards to the same spot to observe comigration.

Toxicity assay. *T. ni* larvae were obtained from our own colony. The larvae were fed a homogenized alfalfa meal-agar food mixture described by Dulmage (5). The caterpillar toxicity assays were performed with 4 to 6 ml of freshly made food poured into 5/8-oz (1 oz = 28.35 g) plastic soufflé cups. Sample solutions (0.15 ml) or water as a control was spread over the surface (6 cm^2) of the food and allowed to soak in and air dry. One second-instar larva was placed in each cup, which was then closed with a cardboard top. For each sample, three or four concentrations were tested with 10 cups at each concentration. Surviving larvae were counted at 5 days, and the data were plotted on logarithmic normal (probit) graph paper to obtain an estimate of the concentration of toxin which would kill 50% of the larvae (LC_{50}). Periodically, similar assays were performed with the international standard for *B. thuringiensis* subsp. *kurstaki* (HD-1-S-1980; obtained from H. T. Dulmage) so that results could be expressed in international units (5, 6). These assays did not use sufficient insects for a thorough statistical analysis, nor could they yield a true 50% lethal dose or LC_{50}. Nevertheless, they were adequate for comparative purposes.

RESULTS

The crude *B. thuringiensis* subsp. *kurstaki* fermentation examined under a phase-contrast light microscope (magnification, ×1,000) revealed a mixture of bipyramidal parasporal crystals, spores, and small unidentified particles which were possibly the cuboidal bodies described by Yamamoto and Iizuka (33). The fermentation was treated with the SDS-PAGE sample buffer (2% SDS, 5% 2-ME) (24) and heated for 2 min in a boiling water bath. The cloudy suspension was subjected to SDS-PAGE and revealed two major proteins; one was 66 kDa, and the other had a calculated size of 130 kDa. By staining specifically for glycoproteins, both major proteins were found to contain carbohydrate (Fig. 1).

The *B. thuringiensis* trypsin digest (TD) supernatants were found to contain a series of approximately five proteins with molecular sizes between 58 and 63 kDa. The two most prominent proteins were at 59 and 63 kDa, and they were both glycoproteins as revealed by staining (Fig. 1). Trypsin digestion performed 18 times as described in Materials and Methods produced a range of protein concentration in the supernatant of 2.3 to 7.5 mg/ml (mean, 4.6 mg/ml).

The LC_{50} of the TD supernatants against caterpillars varied from 0.2 to 5.0 μg of protein per cup for eight

measurements (mean, 1.4 μg per cup). This variation arose in part from differences in the size and vigor of the larvae, since a similar range was also measured for the LC_{50} of the 1980 *B. thuringiensis* standard preparation for five measurements (0.2 to 3.0 μg per cup; mean, 1.2 μg per cup).

The *B. thuringiensis* TD supernatant was also tested for toxicity against second-instar mosquito larvae (*Aedes aegypti*). Only 1 of 10 larvae was killed in a solution with a protein concentration of 340 μg/ml. For comparison, the mosquito toxicity of a sporulated fermentation of *B. thuringiensis* subsp. *israelensis* (isolate i-1069) had an LC_{50} of 5 ng/ml, and the extract of a *B. thuringiensis* subsp. *israelensis* fermentation solubilized in 50 mM Na_2CO_3 (pH 10.5) had an LC_{50} of 170 ng/ml.

The purification process for the caterpillar toxin is summarized in Table 1. Approximately 9% of the protein precipitated between 0 and 12% $(NH_4)_2SO_4$ saturation, and it was discarded. The 12 to 24% precipitate accounted for 15% of the protein and was easily suspended in buffer A and dialyzed against 20 mM $NaHCO_3$ (pH 9.0). A very large amount of protein precipitated during this dialysis, and much of the protein could not be redissolved. The final yield could be increased with a slight sacrifice in purity by including the 24 to 30% precipitate in the subsequent steps.

The elution profile of the DEAE BioGel A column after application of the solution of the 12 to 24% $(NH_4)_2SO_4$ precipitate (A_{300}, 0.382) is shown in Fig. 2. After the unbound protein which contained the toxin (peak I) had been eluted, the column buffer was changed to one containing 0.1 M KSCN. A second peak of protein (peak II) was then eluted, containing less than 2% of the protein applied to the column. It was necessary to measure A_{300}s of the fractions because the A_{300} of the 2-ME in the buffer was much less than the A_{280}, whereas the A_{300} of the eluted proteins was still large.

SDS-PAGE analysis of the two peaks from the anion-exchange column showed that peak I contained a major band at 63 kDa and faint bands at 41 and 55 kDa (Fig. 1), whereas peak II contained three major bands at 55, 58, and 63 kDa.

Peak I from the DEAE BioGel A column was dialyzed against water, lyophilized, and dissolved in buffer C containing 4 M NaCl. The dilute sample (0.23 mg/ml; A_{300}, 0.075) was applied to the phenyl-Sepharose CL-4B column previously equilibrated to room temperature. The elution profile of the chromatography is shown in Fig. 3. Peak I contained the protein not bound to the resin (approximately 12% of that applied), peak II contained the protein eluted by washing with buffer C lacking 4 M NaCl (2%), and peak III contained the protein eluted by buffer C plus 2 M KSCN (40%). A very small amount of protein was eluted by the final two buffers, i.e., buffer C plus 4 M KSCN and 6 M urea. Peak III showed a single band on SDS-PAGE when 1 to 20 μg of protein was applied (Fig. 1). The molecular size was estimated to be 63

TABLE 1. Purification of toxic peptide

Fraction	Protein (mg)	LC_{50} (μg/cup)[a]	Toxic units[b]	% Yield	Purification
B. thuringiensis fermentation	1,320[c]	0.03	44,000		
TD supernatant	987	0.4	2,500	100	1.0
$(NH_4)_2SO_4$ precipitate, 12 to 30% saturation[d]	270	0.3	900	36	1.3
DEAE BioGel A, peak I	75	<0.06[e]	>1,200	>48	>6.3
Phenyl-Sepharose CL-4B, peak III	29	0.04	700	28	9.5

[a] Surface application; 50% mortality produced in second-instar larvae within 5 days.
[b] Total protein/LC_{50}, 10^3.
[c] Dry weight of spore-parasporal crystal mixture.
[d] The 12 to 24% precipitate gave a slightly more homogeneous fraction with a reduction in yield.
[e] Data did not allow a narrower estimate to be made.

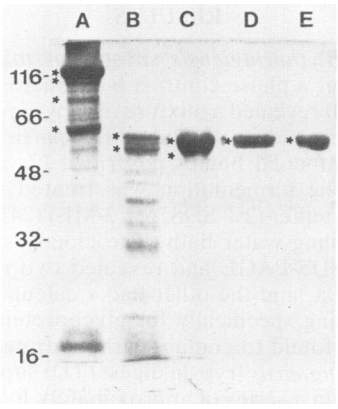

FIG. 1. SDS-PAGE of the most toxic fraction at each step in the purification of the caterpillar toxin. Lanes: A, unfractionated *B. thuringiensis* subsp. *kurstaki* fermentation; B, *B. thuringiensis* TD supernatant; C, 12 to 24% $(NH_4)_2SO_4$ precipitate; D, first peak from DEAE BioGel A chromatography; E, third peak from phenyl-Sepharose CL-4B chromatography. The gel was stained with Coomassie blue to detect proteins. *, Carbohydrate-sensitive-staining bands. Molecular sizes (kilodaltons) of standard proteins are indicated on the left at the appropriate migration distances.

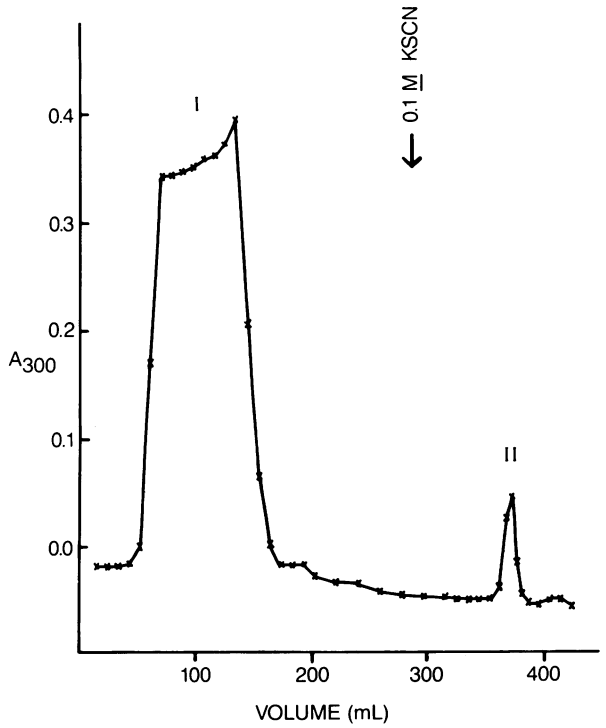

FIG. 2. DEAE BioGel A chromatography elution profile (see text for details). A sample (82 ml) of the 12 to 24% $(NH_4)_2SO_4$ precipitate was applied to a column (bed volume, 50 ml; flow rate, 28 ml/h; temperature, 4°C) and eluted with 20 mM $NaHCO_3$ (pH 9.5)–1 M 2-ME.

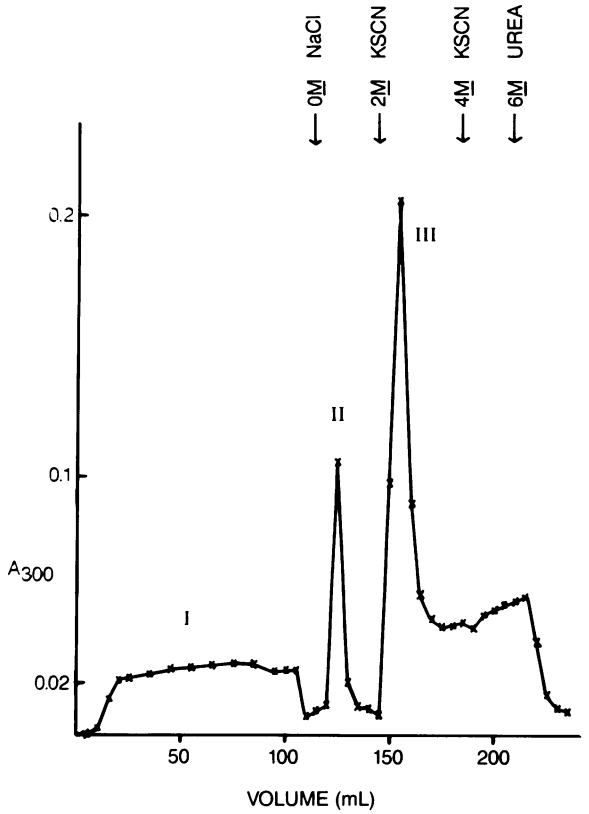

FIG. 3. Phenyl-Sepharose CL-4B chromatography elution profile (see text for details). The sample for peak I was DEAE BioGel A fraction I applied to a column (bed volume, 55 ml; flow rate, 28 ml/h; temperature, 4°C) and eluted with 20 mM $NaHCO_3$ (pH 9.5)–0.1 M 2-ME.

kDa by using SDS-PAGE with bovine serum albumin (66 kDa) and catalase (60 kDa) as molecular size standards.

The purified toxin allowed to reach equilibrium in isoelectric focusing gels containing 6 M urea showed a single band with an apparent pI of 6.5 ± 0.1 in three runs. The toxic fraction III from the phenyl-Sepharose column had a single N-terminal amino acid, i.e., isoleucine, and was analyzed to be 5.3 ± 1.5% carbohydrate by the phenol-sulfuric acid method (4).

The amino acid composition of the purified toxin was measured after 24, 48, and 72 h of hydrolysis in 3 N mercaptoethanesulfonic acid at 110°C. The calculated measurements are listed in Table 2. The striking aspects of the composition are the small numbers of residues of Lys (2 residues), His (7 residues), Met (7 residues), and Trp (10 residues), the large numbers of residues of Pro (28 residues) and Ser + Thr (88 residues), and the total number of the eight nonpolar amino acids, Phe, Trp, Pro, Ala, Val, Met, Ile, and Leu (230 residues; 43% of the total residues). The 537 residues gave an estimated molecular size of 59.6 kDa, which would be 62.8 kDa when the approximately 5% (wt/wt) carbohydrate content of the toxin is added.

Two difficulties encountered in the purification were the low solubility of the toxic fragment in aqueous solutions and the aggregation of the soluble proteins in the original TD supernatant. The first condition was observed in the precipitation of much of the toxic material from the TD supernatant when it was stored at 4°C for 1 week. Also, as the purity of the toxic fraction increased, its maximum protein concentration in 20 mM $NaHCO_3$ (pH 9.0) decreased from approximately 5 mg/ml in the *B. thuringiensis* TD supernatant to 0.2 mg/ml for the final purified toxin.

The presence of high-molecular-weight aggregates was detected when the *B. thuringiensis* TD supernatant was applied to chromatography columns containing BioGel A-0.5m or BioGel P-150 resin equilibrated with 20 mM $NaHCO_3$ (pH 9.0). Although SDS-PAGE indicated that little, if any, of the component polypeptides were larger than 70 kDa (Fig. 1), more than 75% of the protein was eluted at the void volumes of the size exclusion columns. Second, minor peaks at elution volumes similar to that of bovine serum albumin were also present. Both the excluded fraction and the second peaks were toxic to *T. ni* larvae.

Both the solubility and aggregation difficulties were solved by the addition of the chaotropic agent KSCN and the thiol reagent 2-ME. For example, the 12 to 30% $(NH_4)_2SO_4$ precipitate was soluble to 0.1 mg of protein per ml in 20 mM $NaHCO_3$ (pH 9.0), to 0.4 mg/ml when 2-ME was added to 0.1 M, and to 6.8 mg/ml when KSCN was also added to 2 M. Also, a partially purified sample in 50 mM $NaHCO_3$ (pH 9.5)–0.1 M 2-ME–2 M KSCN gave no eluted protein at the void volume in size exclusion chromatography. Since KSCN weakens hydrophobic interactions (11), the low solubility and the aggregation condition are thought to arise from intermolecular hydrophobic interactions. That the toxin itself could participate in these processes was demonstrated by its strong binding to the phenyl-Sepharose CL-4B resin.

It was found that the presence of 2 M KSCN did not decrease the potency of the toxin. A partially purified sample was stored for 35 days in 20 mM $NaHCO_3$ (pH 9.5)–0.1 M 2-ME–2M KSCN, and it retained its high toxicity.

TABLE 2. Amino acid composition of purified toxic peptide

Amino acid	No. of residues by hydrolysis method[a]	No. of residues estimated from gene sequence[b]
Alanine	32	32
Arginine	37	42
Aspartic acid + asparagine	58	55
Cysteine	ND[c]	0
Glutamic acid + glutamine	47	50
Glycine	45	39
Histidine	7	8
Isoleucine	38[d]	39
Leucine	46	52
Lysine	2	2
Methionine	7	6
Phenylalanine	31	30
Proline	28	31
Serine	63[e]	51
Threonine	25[e]	40
Tryptophan	10	9
Tyrosine	23	20
Valine	38[d]	32

[a] 3 N mercaptoethanesulfonic acid, 110°C. The values were calculated by averaging measurements from three hydrolysis time periods (24, 48, and 72 h).

[b] Residues 29 to 566 were predicted from the crystal protein gene of *B. thuringiensis* subsp. *kurstaki* HD-1-Dipel (26).

[c] ND, Not detected. Cysteine would have been converted to cysteic acid by the hydrolysis method.

[d] Values of Ile and Val measured in 72-h hydrolysate were taken as the total amount present in the protein.

[e] Total amount of Ser determined by plotting the time of hydrolysis versus the amount measured and extrapolating back to time zero. Since the analyzer did not adequately resolve Ser from Thr, it is more accurate to express the sum of the two.

The protein concentration of the gut juice digest supernatant was 21 mg/ml. About 4 mg/ml was solubilized during the digestion, similar to the amount solubilized by trypsin digestion. The gut juice supernatant and the gut juice digest supernatant were analyzed by SDS-PAGE. Both showed complex patterns and both had several protein bands in the range of 60 to 65 kDa. Antigenic similarity between the proteins in the gut juice digest supernatant and those in the purified toxin was evidenced by the formation of a precipitin line between the gut juice digest supernatant and rabbit antitoxin serum (Fig. 4). One continuous precipitin line was formed, and no spurs were visible.

DISCUSSION

The lepidopteran-toxic property of *B. thuringiensis* varieties has long been known to result from the midgut digestion of the parasporal crystals (8). Cooksey (3) even showed that one could digest the unpurified fermentation preparation with laboratory grade enzymes in vitro and obtain toxic material apparently identical to that produced in the caterpillar midgut. We followed the general procedure of Cooksey and with a simple three-step purification scheme obtained milligram quantitites of a homogeneous, toxic protein fragment. This toxic material was immunologically cross-reactive with the toxin produced by in vitro gut juice digestion of *B. thuringiensis*. The size of this toxin (63 kDa including carbohydrate) is within the range of 58 to 70 kDa reported by others (2, 21, 30). Differences in *B. thuringiensis* isolates, fermentation conditions, and methods of proteolytic activation may in part account for these differences. Chemical sequencing must be directly correlated with gene sequencing to clarify these differences. The presence of carbohydrate in this toxic fragment is in contrast to some reports for similar fractions (14, 26). Carbohydrate probably represents a nonessential property of parasporal protein which varies with different *B. thuringiensis* isolates and possibly even with fermentation conditions. Although we detected carbohydrate in the purified fraction by gel staining and by chemical analysis, we cannot rule out a very tight noncovalent association, such as that implied by Knowles et al. (16).

The amino acid composition (Table 2) and the N-terminal isoleucine correspond well both with the 538 amino acid residues (29 through 566) deduced from the gene sequence for the HD-1-Dipel crystal protein (26) and with a very similar sequence obtained from the crystal protein gene for *B. thuringiensis* subsp. *sotto* (28). The toxin also appears to be similar to a 516-amino-acid-residue toxic fragment of M_r 58,000 isolated from *B. thuringiensis* subsp. *dendrolimus* T84A1 (21) and to a fragment from *B. thuringiensis* subsp. *kurstaki* HD-1 which appears on SDS-PAGE gels with M_r 59,000 (30).

The LC_{50} of the purified toxin toward second-instar *T. ni* larvae was 0.3 μg per assay cup, i.e., 10-fold more toxic than was the original *B. thuringiensis* TD. Studies on the site of toxicity and binding characteristics of the toxin associated with the toxicity have been done using an unpurified toxic digest (16). Our purified toxin was shown to be toxic to the cells in culture of three lepidopteran species (W. McCarthy, Pennsylvania State University). In brief, three lepidopteran cell lines show 50% or more cell lysis (10^5 cells per ml) in 4 h at 27°C when treated with 2.5 to 8.5 μg of homogeneous toxin per ml (W. McCarthy, personal communication).

Andrews et al. (1) recently discussed the in vitro preparation of a toxic cleavage product, starting with purified crystalline protoxin. Their procedure is somewhat similar to that of Cooksey (3) and the one which we describe, except that we found it satisfactory to begin directly with the unfractionated fermentation residue. In addition, we observed that ion-exchange chromatographed material sometimes appeared homogeneous on SDS-PAGE but actually had a nontoxic component that did not separate from the toxic peptide.

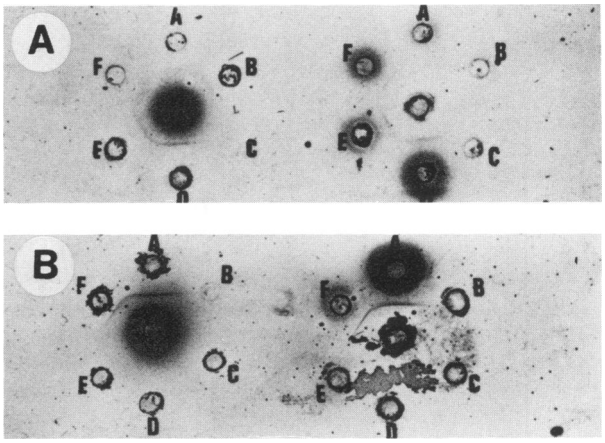

FIG. 4. Ouchterlony double-diffusion assay comparing the reactivity of the antitoxin serum to the *B. thuringiensis* TD supernatant (A) and to the gut juice supernatant (B). Microscrope slides were covered with a thin layer of an agar solution, and wells were cut out of the hardened agar. In panel A on the left, undiluted (100%) antiserum (4 μl) was placed in the center well, and dilutions of the *B. thuringiensis* TD supernatant (4 μl) were placed in the surrounding wells. Wells: A, ×8 dilution; B, ×16 dilution; C, buffer control; D, undiluted; E, ×2 dilution; and F, ×4 dilution. In panel A on the right, undiluted *B. thuringiensis* TD supernatant was placed in the center well, and the same dilutions of the antiserum as on the left panel were placed in the surrounding wells. In panel B on the left, undiluted antitoxin serum was placed in the center well, and dilutions of the gut juice supernatant were placed in the surrounding wells. Wells: A, undiluted; B, buffer control; C, ×256 dilution; D, ×64 dilution; E, ×16 dilution; and F, ×4 dilution. In panel B on the right, undiluted gut juice supernatant was placed in the center well, and the same dilutions of the antiserum as on the left were placed in the surrounding wells. The precipitin lines were detected by using Coomassie blue.

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