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PURIFICATION AND CHARACTERIZATION OF THE ACTIVE FRAGMENT FROM Bacillus Thuringiensis DELTA-TOXIN

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Limited tryptic hydrolysis of a partially purified delta-toxin (M_r=100,000) from Bacillus thuringiensis, has produced a polypeptide fragment of M_r=60,000 containing the full biological activity. The fragment was the only polypeptide observed in the polyacrylamide-gel electrophoresis of the delta-toxin after treatment with trypsin and could be purified by DEAE-cellulose chromatography. Amino acid and partial sequence analyses indicate that the 60,000 M_r fragment has been derived from the mid-section of the holotoxin peptide; over 80% of Lys, 65% of Pro and 50% of His residues in the holotoxin have been lost in the active fragment. This section must contain the active site since its specific insecticidal activity is approximately twice that of the holotoxin. The active fragment shows complete cross-reactivity with the antiserum raised against the native toxin, and appeared to possess higher thermal stability than the mother protein. It provides a powerful tool for studies of the structure involved in the insecticidal activity. © 1986 Academic Press, Inc.

Strains of Gram-positive spore-forming bacteria, Bacillus thuringiensis have been known to produce an intracellular protein called delta-toxin which is toxin to lepidoptera insect larvae. The protein is formed as an insoluble refractile body at the time of sporulation and released as such together with spores upon autolysis (For reviews see Refs. 1 and 2). Upon ingestion of the delta-toxin, the insect larvae stop feeding and die in a few days following an apparent paralysis.

We have recently separated a protein of M_r=100,000 with full delta-toxin activity from the spore-delta toxin mixture of B. thuringiensis strain HD-1, by extraction with a bicarbonate buffer, pH 9 containing mercaptoethanol (3). At a later stage of culture, the solubilized delta-toxin also contained a protein of 130,000 M_r, the molecular size generally reported for the polypeptide of delta-toxin from most strains of B. thuringiensis including HD-1 (4-6). In order to facilitate structure-function studies, we have attempted to obtain

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an active polypeptide fragment from the solubilized delta-toxin using limited proteolysis. Trypsin has been found effective in producing a 60,000 M_r polypeptide from the 100,000 M_r or 130,000 M_r native toxin without a loss in the insecticidal activity. The purification and characterization of this polypeptide are reported here.

MATERIALS AND METHODS

Bacillus thuringiensis strain HD-1, kindly provided by Dr. Dulmage of Cotton Insects Research, ARS, USDA, Brownsville, TX, was grown in the medium described by Yamamoto et al. (7), at 30°C in a gyratory shaker at 220 rpm. After 18 hrs of growth, 40 ml of culture was transferred to a 2 L flask containing 340 ml of the prewarmed medium and incubation continued for 48- The spore and crystals (delta-toxin) were centrifuged and immediately washed five times with 0.1 mM phenylmethane sulfonyl fluoride (PMSF). The precipitate may be stored at -70°C until use. Delta-toxin was separated from the mixture by extraction with 0.1M Na-carbonate buffer, pH 9 containing 1% mercaptoethanol and 0.1 mM PMSF as described (3).

Proteolysis of delta-toxin was carried out in 0.05M Tris-HCl buffer, pH 8 containing 0.1% mercaptoethanol with either trypsin or chymotrypsin at a substrate to enzyme ratio of 50 (w/w). Delta-toxin was found to be soluble in this buffer after it was precipitated from the extract with one-third saturation of $(NH_4)_2SO_4$. Crude insect larvae protease was prepared by homogenization of 4g of seven-day-old tobacco budworm larvae in 100 ml of phosphate buffer-saline (Dulbecco's Phosphate Buffered Saline), followed by centrifugation at 10,000 rpm for 10 min. Digestion of delta-toxin with crude insect protease was carried out in the above buffer, using substrate to enzyme ratio of 10.

Polyacrylamide electrophoresis (PAGE) was carried out in 7.5% gels in 0.1% Na-dodecylsulfate and 7M urea (8). The gels were then stained with Coomassie Brilliant Blue.

Protein determination was carried out by the dye-binding method of Schaffner and Weismann (9). Amino acid analysis and partial sequence determination were performed essentially as described previously by Lai and Dietzschold (1).

Insecticidal activity was assayed by a modified procedure of Dulmage et al. (11) in which 5 ml feed containing serially diluted toxin was used in each cup and the surviving tobacco budworms were counted after three days at 30°C (3).

RESULTS

Limited proteolysis of delta-toxin. No loss of insecticidal activity was observed when delta-toxin was incubated either with trypsin or crude insect juice for up to 22 hrs at room temperature. With chymotrypsin partial inactivation (about 10%) occurred in 22 hrs.

Polyacrylamide gel-electrophoresis (PAGE) of the digests taken at different intervals revealed that a major polypeptide with M_r=60,000 was produced by trypsin digestion of the native protein (Fig. 1). Since no other protein bands are visible at the end of 22 h, the 60,000 M_r fragment must represent the core region containing the active site. Digestion with chymotrypsin and insect juice protease showed similar patterns, but the major band produced by chymotrypsin was not well defined. With the insect proteases, two protein bands around M_r=60,000 and several minor bands were visible at the end of 24 hrs (data not shown).

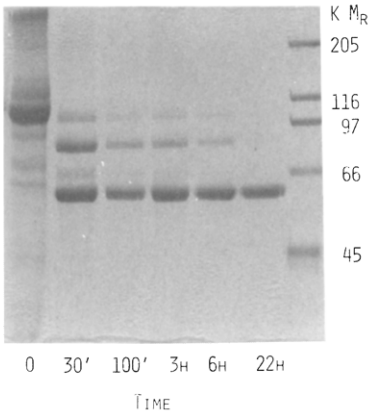

Fig. 1. Formation of 60,000 M_r peptide from delta-toxin (M_r=100,000) on tryptic digestion: Delta toxin (1.5 mg) in 5 ml 0.05 M Tris-HCl buffer, pH 8, 0.1% mercaptoethanol was treated with 30 µl of TPCK trypsin (1 mg/ml 1 mM HCl) at room temperature. Immediately after mixing (0 hr) and at indicated times, 20 µl aliquots were removed and added to 0.2 ml cold 10% trichloroacetic acid. The precipitate was washed once with 0.2 ml of acid-acetone, dissolved in 100 µl sample buffer and 50 µl was electrophoresed as described in "Methods."

It is noteworthy that both 100,000 M_r and 130,000 M_r species of delta-toxin have yielded 60,000 M_r fragment by proteolysis. The 130,000 M_r delta-toxin was produced at the later stage of culture and some preparations contained both species of delta-toxin (3) (also see Materials and Methods). In the experiments shown in Fig. 1, the preparation used for trypsin digestion contained mainly the 100,000 M_r delta-toxin.

Purification of the active fragment produced by trypsin digestion. When a 7h-digest of delta-toxin (M_r=100,000 or M_r=130,000) with trypsin was chromatographed on a DEAE-cellulose column, a single symmetrical peak of A_{280} was obtained which coincided with the insecticidal activity (Fig. 2). This peak contained **98.5%** of the total activity recovered from the column. **The specific activities** of the parent protein (M_r=100,000) and the active fragment were **1640 units/mg** and **3470 units/mg**, respectively. PAGE of the active peak showed its molecular homogeneity, with M_r=60,000 (Fig. 3).

Double diffusion analysis of the 60,000 M_r active fragment towards rabbit anti-serum raised against the native delta-toxin, and vice versa, showed immuno-precipitation lines completely fused to each other (data not shown). It indicated that the active fragment contained the major epitope of the holotoxin.

Amino acid and the NH_2-terminal sequence analyses. Comparison of amino acid composition of delta-toxin and that of active fragment indicated that over 80% of Lys, 65%

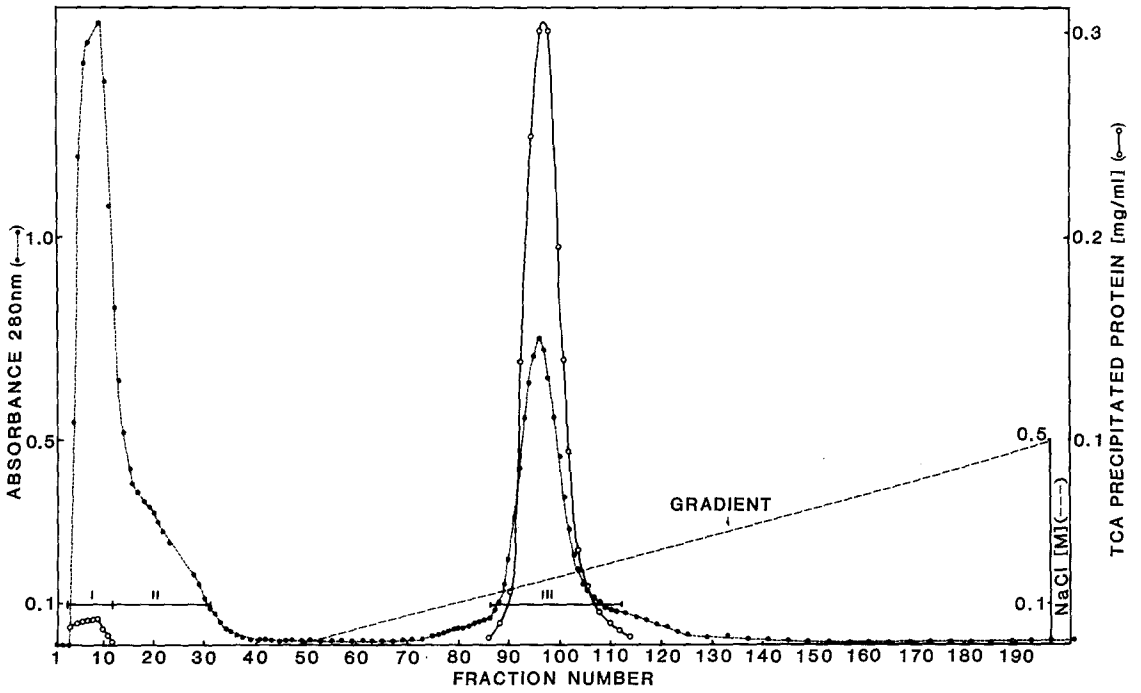

Fig. 2. DEAE-cellulose chromatography of the tryptic digest of delta-toxin. Fifteen mg of delta-toxin was digested with 0.3 mg of trypsin for 7 hr at room temperature as described in Fig. 1. The digest was applied directly onto a DE-52 column (1.5 x 25 cm) equilibrated with 50 mM Tris-HCl, pH 8 and eluted with the NaCl gradient as indicated.

of Pro and 50% of His residues in the holotoxin were cleaved away during the tryptic hydrolysis (Table 1). It further indicated that most of Lys, but not Arg residues were located in the 40% segment of the molecule "exposed" where trypsin came in contact.

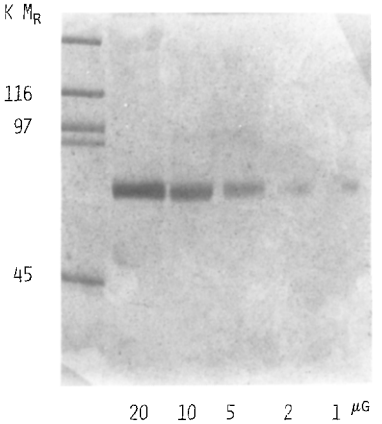

Fig. 3. Homogeneity of purified active fragment. Indicated amounts of the active fragment from DE-52 chromatography were subjected to PAGE as described in "Methods."

Table 1
Amino Acid Composition of Delta-Toxin and Its Active Fragment

Amino Acid	Delta-toxin M_r=100,000	Active Fragment M_r=60,000
Aspartic Acid	95	61
Threonine	48	38
Serine	51	34
Glutamic Acid	119	59
Proline	66	23
Glycine	92	42
Alanine	68	36
Cysteine	3	3
Valine	72	42
Methionine	8	7
Isoleucine	55	38
Leucine	70	46
Tyrosine	27	28
Phenylalanine	35	29
Lysine	47	8
Histidine	18	9
Arginine	45	32

The values are in residues per mole. Sample was hydrolyzed in 5.7N HCl for 24 hr, and analyzed with an amino acid analyzer equipped with fluorescamine detector (8). The numbers are the average of three determinations.

Edman degradation of the holotoxin and the 60K M_r fragment indicated that the NH_2-terminal sequences of these protein were **Met-Asx-** and **Gly-Ala-**, respectively.

DISCUSSION

Yamamoto and McLaughlin (9) reported isolation of two proteins with M_r=62,000 (P1) and M_r=135,000 (P2) from parasporal crystal of B. thuringiencis var. Kurstaki (HD-1), and that P1 was also toxic to mosquito larva. When P2 (M_r=135,000) was incubated with the cabbage looper larva guts, formation of a fragment with M_r=62,000 was detected. Since P1 and P2 were serologically different, they concluded that P1 was not the 62,000 M_r fragment from P2 (9). We have now purified a 60,000 M_r fragment from the tryptic digest of 100,000 M_r insecticidal protein from the same strain of bacteria that is fully active and serologically identical to the parent protein. This active fragment is thus different from P1 of Yamamoto and McLaughlin (9).

The insecticidal protein (delta-toxin) of B. thuringiensis has been notorious for its poor solubility and molecular heterogeneity; M_r ranging from 100,000 to 135,000 have been

reported (4-6). The best preparation we obtained (3) contained 5% of 130,000 M_r material. Nevertheless, digestion with trypsin yielded a single polypeptide fragment of M_r=60,000 and with full insecticidal activity. This strongly suggests that all forms of delta-toxin contain the same active core of 60,000 M_r.

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