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BACILLUS THURINGIENSIS PARASPORAL CRYSTAL TOXIN: DISSOCIATION INTO TOXIC LOW MOLECULAR WEIGHT PEPTIDES

P.G. FAST and W.G. MARTIN

Canadian Forestry Service
Forest Pest Management Institute
Sault Ste. Marie, Ontario
P6A 5M7

and

National Research Council
Sussex Drive
Ottawa, Ontario

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SUMMARY: Parasporal crystals of *bacillus thuringiensis* can be dissociated into low molecular weight peptides (< 5000 daltons) by dissolving them in 0.1 M N-morpholinopropane sulfonic acid buffer pH 7.8 containing 0.05 M dithiothreitol and 2M-4M KSCN, or by performic acid oxidation. The peptides obtained by dissolving in KSCN were still toxic to silkworm larvae.

The parasporal proteinaceous inclusion body (crystal) of *Bacillus thuringiensis* is toxic to a number of economically important insects and is the major toxic component in commercially available insecticides based on this bacterium. The crystal is made up of subunits having a mol. wt. of 230,000 daltons (1) but there is confusion in the literature about the number and size of the polypeptide components. Recent reports suggest, on the basis of polyacrylamide gel electrophoresis in sodium dodecyl sulphate (SDS), that the polypeptide chains range from 50,000-150,000 daltons (2, 3, 4). The toxic component(s) produced by insect gut proteases acting on the crystal also are reported to be of substantial molecular weight and the best evidence indicates a mol. wt. of 60,000 daltons for this component (4).

However, Sayles et al. (5) reported that crystal protein dissolved in 8M urea-dithiothreitol (Dtt), consisted of small peptides of about 1,500 daltons, and Aronson and Tillinghast (6) recently confirmed that *B.t.* crystals of their isolate dissociated into low mol. wt. peptides. Toxicity was not reported. They detected nine different N-terminal amino acids.

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There have also been unsubstantiated reports of toxic low-mol. wt. peptides in proteolytic digests of *B.t.* crystals (7, 8, 9).

Much of the confusion referred to above arises because the crystal is so difficult to dissolve. Alkali (pH $\geq$ 9.5) in the presence of disulfide reducing agents or even higher pH ($\geq$ 11) in their absence are required to dissolve the crystal. Strong denaturants will also dissolve the crystal but toxicity is lost. Aronson and Tillinghast (6) have shown that 1% SDS with 0.15% Dtt is not sufficient to completely disperse the crystal.

Recently Fast and Milne published a method for dissolving crystals of *B.t.* at moderate pH (7.5-8) in the presence of 1M KSCN, a strongly chaotropic salt, and 0.05M Dtt with full retention of toxicity (10). We report here that higher concentrations of KSCN (2-4M) result in dissociation of the crystal into peptides with mol. wts. in the vicinity of 1000 daltons.

MATERIALS & METHODS: Production of sporulated cultures and purification of parasporal crystals have been described previously (11, 12). Purified [^{3}H]leucine labelled crystals were dissolved in 0.1M N-morpholinopropane sulfonic acid (MOPS) buffer pH 7.8 that was 0.05 M dithiothreitol and appropriate concentrations of KSCN. Gel filtration utilized a 2.5 x 35 cm column of Sephacryl S-300 (Pharmacia). Sample was eluted in dissolving solvent less dithiothreitol (Dtt). Eluant radioactivity was monitored using Insta-gel (Packard) in a Beckman LS9000 scintillation counter. Crystals of *B.t.* var. *kurstaki*-k-1 were oxidized with performic acid as described by Hirs (14).

The molecular weights were determined by the equilibrium method in a Beckman Model E Ultracentrifuge equipped with a photoelectric scanner and multiplex accessory. A relatively short fluid column ($\sim$3 mm) in double sector centrifuge cells with sapphire windows was used. The procedure followed that described by Chervenka (13). The samples in 4M KSCN and 98% HCOOH were centrifuged at 48,000 RPM and 60,000 RPM respectively, at 25°C, until unchanging patterns, scanned at 280 nm, were observed ($\sim$20 hrs.). Flourocarbon oil was not used in runs with HCOOH. Baselines were established by rinsing the cells carefully with solvent and then scanning solvent vs. solvent at equilibrium speeds.

Bioassays utilized 4th instar *Bombyx mori* larvae "Nichi" strain, 10 larvae/replicate. Each larva was force-fed 2 $\mu\ell$ of a solution containing peptides or suspended crystals. Five dilutions were used in each assay. Mortality was recorded after 24 hrs and the LD_{50} determined by probit analysis.

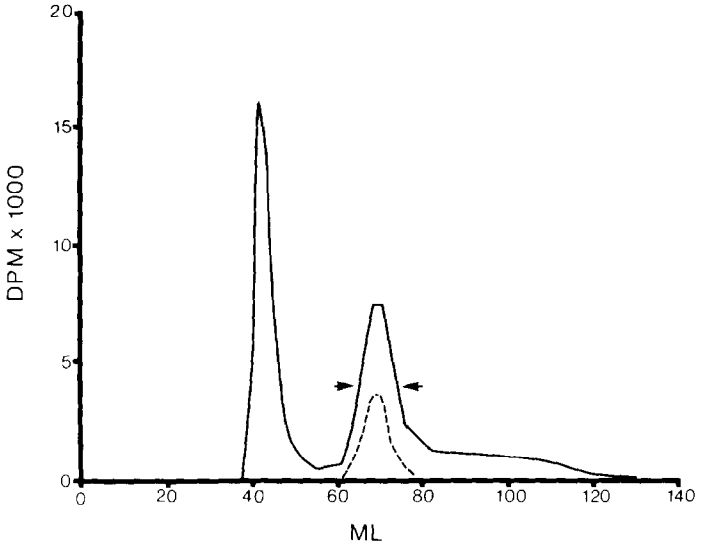

Fig. 1. Elution diagram of *kurstaki*-k-1 crystals dissolved in 0.1M MOPS pH 7.8 + 1M KSCN + 0.05M Dtt then eluted from Sephacryl S-300 with MOPS + 1M KSCN. Eluant between the arrows was pooled, concentrated and rechromatographed (-----).

RESULTS: When crystals of *Bacillus thuringiensis* var. *kurstaki*-k-1 were dissolved in MOPS buffer pH 7.8 + 1M KSCN and 0.05M Dtt the digest was as toxic as the original crystals (10). Gel filtration of the digest on Sephacryl S-300 with MOPS-1M KSCN as eluting buffer yielded the pattern shown in Fig. 1. The included peak between the arrows (mol. wt. $\sim$ 80,000 daltons) was as toxic as the starting crystal preparations. When this peak was concentrated and gel filtered again it eluted in the same position (dashed line Fig. 1). This elution pattern was reproducible.

Because the tail on the 80,000 dalton peak in Fig. 1 is indicative of aggregation, crystals of the same strain were dissolved under the same conditions except that KSCN concentration was increased to 2M. The dissolved crystal was gel filtered in 0.1M MOPS buffer pH 7.8, 2M KSCN (Fig. 2). No 80,000 dalton peak was obtained. The peak at $\sim$ 120 ml is outside the range where mol. wt. is linearly related to elution volume and therefore of $<$ 10,000 daltons. The toxicity of this low mol. wt. material was equivalent to that of the crystal prepration from which it was de-

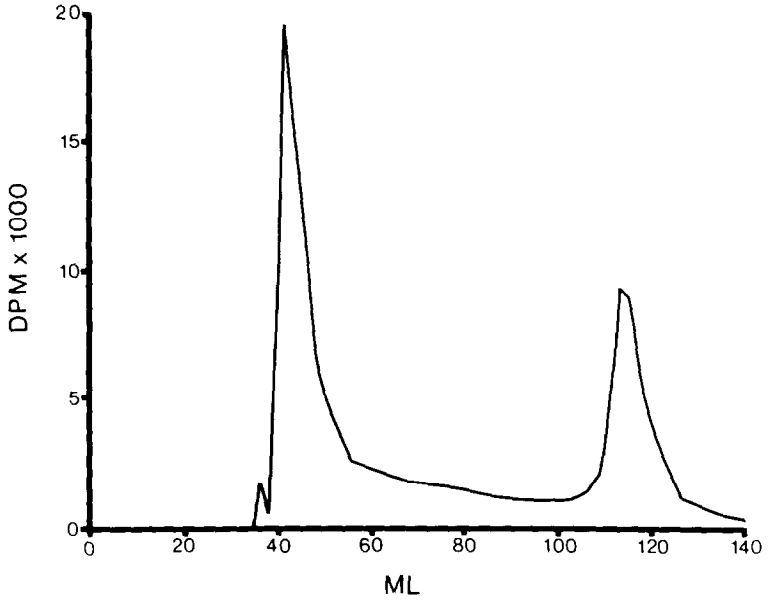

Fig. 2. Elution diagram as in Fig. 1 except that KSCN concentration was 2M throughout.

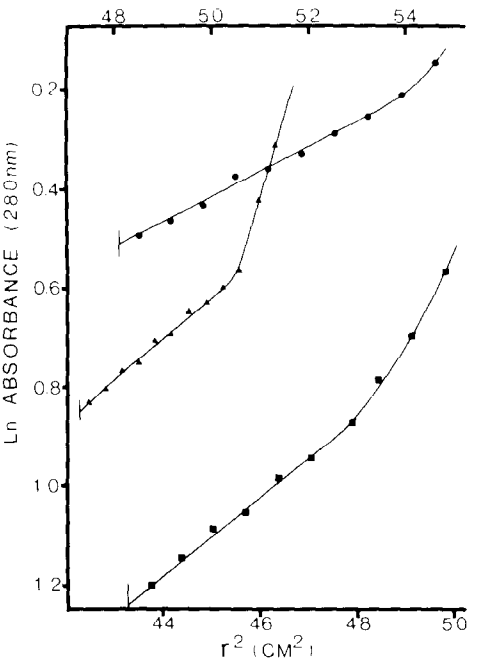

Fig. 3. Sedimentation equilibrium plots of pooled low molecular weight peaks run in 4M KSCN and of whole oxidized crystal in 98% HCOOH. Molecular weight is directly proportional to the slope of the curve of the relation between ln absorbance and the square of the distance from the center of rotation (r^2). Runs were at 25°C, meniscus position is indicated by short vertical bars, lower abscissa refers to runs in 4M KSCN and upper to 98% HCOOH. Runs at 48 x 10^3 RPM in 4M KSCN, —■— *thaumatin*, —●— *actin*; *kurstaki*-K-1 at 60 x 10^3 RPM in 98% HCOOH, —▲—.

Table 1

Molecular Weight of Crystal Peptides

Varieties	Solvent[1]	Molecular Weight[2]	
thuringiensis	4M KSCN	1200	∿ 3000
sotto	4M KSCN	760	∿ 2000
kurstaki-k-1	4M KSCN	aggregates	
kurstaki-k-1	98% HCOOH	740	∿ 3000

[1]Partial specific volume taken as 0.74 in KSCN and 0.72 ml/g in HCOOH (15).

[2]Higher values were estimated from data near the base of the solution columns.

rived (LD_{50} 0.026 and 0.025 µg/gm larvae, respectively). The eluting buffer was not toxic at the levels used for bioassay.

Crystals dissolved in 3M or 4M KSCN gave similar patterns. The relative quantities of the 2 peaks were not reproducible suggesting that a varying amount of reaggregation was taking place before or during elution.

Crystals of two other taxonomic varieties, var. *sotto* and var. *thuringiensis*, gave similar elution diagrams when dissolved and gel filtered in the 2M-4M KSCN system. These low mol. wt. peptides of var. *sotto* and var. *thuringiensis* were also toxic to silkworm larvae.

Equilibrium centrifugation (Fig. 3) of pooled low mol. wt. material in 4M KSCN gave the mol. wt. shown in Table 1. These data show that the peak position was not due to column artifacts and establish that much of the peptidic material has a molecular weight in the range of 1000 daltons. The larger peptides, which piled up at the bottom of the cell were smaller than 5000 daltons as judged by the slope near the base of the cell. The aggregated *Kurstaki*-k-1 low molecular weight material could again be disaggregated by addition of Dtt.

To establish whether all the crystal could be disaggregated to low molecular weight peptides, purified crystals were oxidized with performic

acid to convert thiols and disulphides to sulfonic acid and thereby prevent reformation of disulfide bonds. The resulting peptides were fully soluble only in 98% formic acid and when centrifuged to equilibrium in this solvent gave mol. wts. in good agreement with those observed in KSCN (Table I). There was no evidence of any material larger than about 3000 daltons in this solvent. The entire crystal, therefore, is composed of short peptides held together by disulfide bonds and non-covalent interactions. The performic acid oxidized crystal material was no longer toxic to insects.

DISCUSSION: The crystal preparations used were free of adsorbed proteinases as determined by Azocoll assay. Furthermore, the pattern observed in Fig. 1 was reproducible; low molecular weight peptides were not observed until 2M KSCN was used. Hydrolytic cleavage would have been observable at lower salt concentrations. Therefore the small peptides are not products of peptide chain hydrolysis.

The results presented here confirm the earlier observations by Sayles et al. (5) and of Aronson and Tillinghast (6) that parasportal crystals can be disaggregated yielding peptides of about 1000 daltons. The data establish that toxicity to insects is carried on such small peptides and establish (although the possibility that these peptides reaggregate with reformation of disulfide bonds in the larval gut cannot be ruled out) that the entire crystal is composed of such low mol. wt. material. The detection by Aronson and Tillinghast (6) of at least 9 N-terminal acids is in accord with the amino acid analyses which suggest a minimal molecular weight of 13,000 and thus a multiplicity of peptides all or only some of which may be toxic to insects.

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