THE BIOCHEMISTRY OF THE PROTEIN CRYSTAL TOXIN OF *BACILLUS THURINGIENSIS*

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The crystal consists of dimeric protein subunits. The monomer peptide chains are held together in the subunit and the subunit in the crystal by disulfide and non-covalent bonds. The monomer peptide has a molecular weight of about 130 kdaltons which, in the presence of proteases, is hydrolyzed to a protease-resistant-protein of 65 kda that is toxic both to larvae by injection and to tissue culture cells and thus is the active toxin. The gene for this protein is on a plasmid which greatly simplifies the work of the genetic engineers. The gene has been partially sequenced and the secondary structure of this part has been predicted but not demonstrated.

Bacillus thuringiensis (Bt) is divisible into 20 varieties or serovars based on serological and biochemical tests on the vegetative cell. The protein that is responsible for most of the toxicity, and especially for the insecticidal activity of commercial preparations, is produced as a bipyramidal crystal during sporulation of the Bacillus (Fig. 1). The surface pattern observed is due to the regular positioning of rod-shaped molecules with dimensions of about 9 X 13 nm and a calculated molecular weight of 230,000 daltons (1 dalton is the mass of a hydrogen atom) (Holmes and Monroe, 1965). The shapes of the crystals vary from almost cuboidal to slender needle shapes depending on the serovar. Isolates can often be recognized by crystal shape alone.

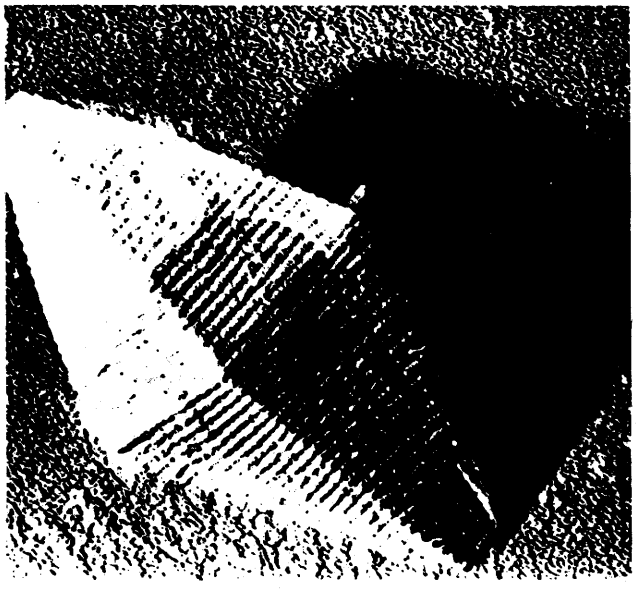

Figure 1. Electron micrograph of a parasporal crystal of *Bacillus thuringiensis* (Courtesy of C. Hannay)

Serology of the crystal broadly reflects the groupings referred to above but with considerable overlap (Krywienczyk et al., 1978, 1981). In this review I shall refer to serovars by their full names but crystal types by their first 3 letters, or in the case of *kurstaki* crystal types to K-1 and K-73, as suggested by Krywienczyk et al. (1978).

Many of the problems of dissolving and characterizing the crystal that have historically plagued the study of Bt crystal toxin have been overcome in the past 5-8 years. Crystals are now readily separated from spores (Milne et al., 1977, Sharpe et al., 1975) and can be freed of the proteases that are adsorbed to the crystal which have, in the past, caused confusion about the molecular weight of the constituent peptide chains (Chestukhina et al., 1978; Nickerson and Swanson, 1981).

The techniques for dissolution of the crystal are also now reasonably defined. The crystal is held together principally by intermolecular disulfide bonds of which there are as many per peptide chain as in wool keratin (Huber, 1982) and by noncovalent interactions such as salt bridges and hydrogen bonds. Highly alkaline conditions (pH 11-13) dissolve the crystal by alkaline hydrolysis of disulfide bonds but a considerable loss of toxicity proportional to the length of time in alkali occurs (Trumpi, 1976). Under less alkaline conditions (pH 9.5-11) dissolution will occur in the presence of disulfide reducing agents such as dithiothreitol (Dtt) or mercaptoethanol and toxicity is preserved. Under near neutral pH conditions denaturants such as ionic detergents, guanidine hydrochloride, urea, or chaotropic salts are required along with disulfide reducing agents and in most cases toxicity is totally destroyed. Huber (1982), however, describes conditions for the dissolution of crystals in guanidine hydrochloride in which toxicity is retained. The toxicity is also destroyed by heat as well as by denaturants, leading to the conclusion that secondary structure (helical or beta organization) or tertiary structure (packing of the helical or beta structural elements) of the crystal protein is required.

A protein with a molecular weight of about 230 kilodaltons (Kda) is obtained when the crystal is dissolved under carefully controlled conditions without reducing agents (Huber 1982). This is in good agreement with the molecular

weight calculated from the x-ray data (see above). In the presence of reducing agents a single molecule with a molecular weight of 130 kda is observed indicating that the unreduced molecule is probably a dimer. Although this has generally been the accepted interpretation, Huber (1982) was the first to present data clearly supporting this interpretation. The discrepancy in the molecular weights observed in the monomer and the dimer remains unresolved where molecular weights are determined by electrophoresis in SDS. Ultracentrifugation (Huber, 1982; Glatron et al., 1972) gave lower values for the monomer but the dimer molecular weight is exactly 2X that of the monomer. For the sake of clarity let us accept the 130 kda molecular weight in the balance of this discussion.

The amino acid compositions vary from serovar to serovar (Table I). The greatest variability is seen in the charged amino acids and glycine. The dicarboxylic amino acids aspartate and glutamate are present in unusually high amounts in all serovars, about half as their amides glutamine and asparagine (Glatron et al., 1972; Fast, 1981).

Table I. Amino acid compositions and minimal molecular weights

AA/type	*thu* (8)[a]	*fin* (2)[b]	*sot* (3)[c]	*ale* (3)[d]	*tol* (2)[e]	*gal* (2)[f]	*ken* (2)[g]	*and* (1)[h]	*kur-1* (1)[i]
Asp	14	14	13	14	15	16	15	12	14
Thr	8	8	7	8	9	9	8	7	7
Ser	8	9	8	9	12	11	9	8	9
Glu	13	12	15	14	12	13	14	15	13
Pro	5	6	8	6	6	7	5	7	4
Gly	10	15	9	12	16	11	9	10	8
Ala	8	13	7	8	11	10	8	8	6
Val	8	9	6	9	8	8	8	7	8
Cys	1	2	1	2	1	2	2	2	2
Met	1	1	1	1	1	1	1	1	1
Ile	7	8	8	8	8	8	8	7	6
Leu	11	11	13	11	10	13	13	11	9
Tyr	5	4	5	5	3	5	5	4	5
Phe	5	4	6	5	4	6	6	5	4
Lys	4	6	4	4	3	4	5	4	3
His	2	3	2	2	2	3	2	3	2
Arg	6	3	8	7	5	7	7	6	9
Try	1	1	1	1	ND	1	ND	1	1
min. mol. wt.	12820	13231	13231	11895	12796	14291	13042	13282	12400

Considerable controversy exists as to whether carbohydrate is bound to the crystal protein and is essential to toxicity. Several workers have reported concentrations of carbohydrate in the crystal ranging from 0.5% to 12% in crystals (Holmes and Monroe, 1965; Bateson and Stainsby, 1970; Bulla et al., 1977). Other investigators working with related isolates failed to detect carbohydrate in rigorously purified crystals (Lecadet, 1965; Huber et al., 1981). Treatment with carbohydrases to remove sugars or with immobilized concanavalin A to remove glycoproteins containing glucose and mannose (the sugars reported to be present) from solution failed to materially reduce toxicity (Fast, 1981). Amino sugars or sialic acids, often associated with glycoproteins, were not detected in the crystal (Fast, 1981; Bulla et al., 1977; Bateson and Stainsby, 1970). Whether the carbohydrates are covalently bound to the peptide chain or only adsorbed to it, they do not appear to participate in toxicity to insects. There is no lipid or nucleic acid associated with the crystal.

The monomeric 130 kda peptide chain is a protoxin that must be activated by proteolytic hydrolysis (Lecadet and Martouret, 1965; and many others). The activated product is generally a protease resistant protein (PRP) with a molecular weight in the range of 50-70 kda although smaller toxic fragments have frequently been reported but generally not well documented (Fast and Angus, 1970; Pendleton, 1973; Faust et al., 1974). Lecadet and Dedonder, (1967) isolated and partially characterized 2 small toxic peptides from 350mg of starting proteolytic digest. Work on this scale has not been repeated.

The amino acid composition of PRP is significantly different from that of the crystal. Amino acid compositions of PRP from 3 serovars are shown in Table II (Chestukhina et al., 1982). A major reduction in the amount of lysine and an increase in hydrophobicity in PRP over that of the protoxin is observed. In the light of Beegle's finding (Abstract, Ithaca meeting of the SIP, 1983) that the toxicity spectra of activated PRPs is the same as the spectra of the crystal, the differences in amino acid composition seen in the table are probably significant.

There are significant sequence differences in PRP of these serovars as well. SDS gels of cyanogen bromide cleavage products of PRP from 3 different serovars have been published (Chestukhina et al., 1982) (Fig.2). Cyanogen bromide cleaves peptides only at methionine. Because the products of cleavage have different molecular weights in the three serovars shown, sequence differences must exist and again are likely to affect toxicity spectra.

Table II. Comparison of the amino acid composition of the PRP of 3 serovars of *Bacillus thuringiensis* (from Chestukhina 1982).

Amino Acid	*galleriae* 65 kda (mol%)	*tolworthi* 65 kda (mol%)	*alesti* 70 kda (mol%)
1/2 cys	1.0	1.0	1.0
asp	11.7	11.7	11.1
thr	6.6	8.0	6.3
ser	11.8	9.8	9.4
glu	11.2	10.5	11.0
pro	5.1	4.8	5.7
gly	7.6	7.5	8.0
ala	6.2	5.7	5.7
val	4.9	4.4	7.3
ile	5.5	6.8	6.3
leu	8.5	9.3	9.8
tyr	3.6	3.9	3.1
phe	4.2	5.5	6.0
his	1.7	1.4	2.1
lys	1.3	1.0	1.0
arg	6.2	6.9	5.0
met	0.7	1.0	0.7
trp	0.7	0.6	0.6

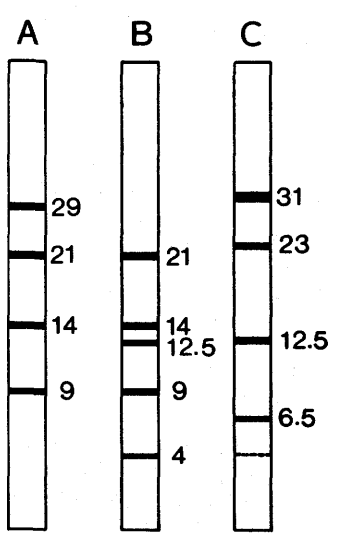

Figure 2. SDS-Page patterns of PRP of serovars (A) *tolworthi* (B) *alesti* and (C) *galleriae* after treatment with cyanogen bromide. Numbers are molecular weight in kda.

Recently the gene for the 130 kdalton peptide has been cloned and expressed in both *E. coli* and *Bacillus subtilus* (Schnepf and Whiteley, 1981; Klier et al., 1982). The expressed protein in both these hosts has been shown to be toxic to lepidoptera. The sequence of the first 333 residues of this protein from HD-1 Dipel have been inferred from the nucleotide sequence of the gene (Wong et al., 1983). In fig. 3 I have reproduced that sequence and have marked the hydrophobic and charged amino acids.

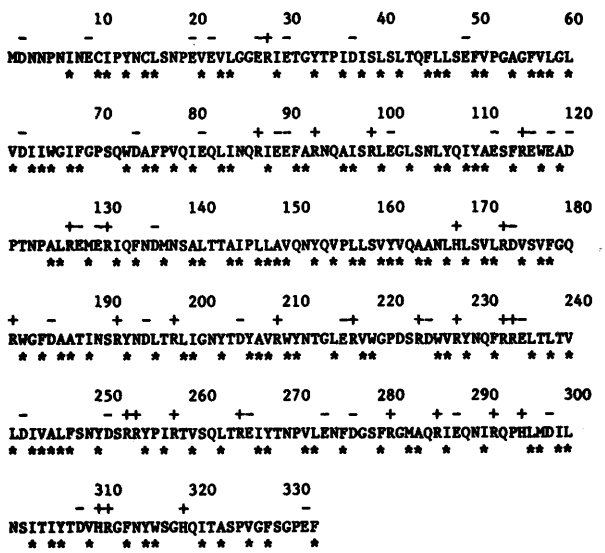

Figure 3. Amino acid sequence of the N-terminal 333 amino acids of the HD-1 Dipel crystal protein. Amino acids are designated by the single letter code. * designates hydrophobic amino acids. Charged amino acids are designated by + or -.

Techniques are available for predicting the secondary structure of a protein from sequence information (Chou and Fasman, 1978). I have applied these techniques to the crystal protein sequence and show the probable position of turns and of alpha helical and beta structures (Fig.4).

Because many of the turns are so tight, only 4-8 residues, it is likely that at least some of the beta structure is antiparallel. While such predictions of structure are of limited accuracy there are good indications that turns and secondary structure are highly conserved, even more highly than sequence, in homologous proteins. The availability of additional sequences, which are presently being derived, should greatly assist in identifying the structure(s) responsible for toxicity.

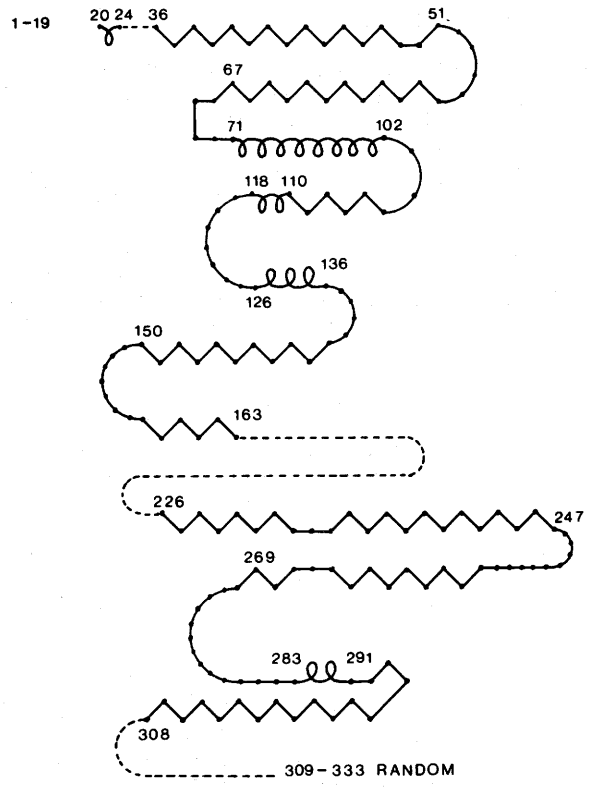

Figure 4. Secondary structure of the N-terminal 333 residues of HD-1 dipel crystal protein as predicted by the method of Chou and Fasman (1978). Zigzag indicates beta structure, coils indicate helices, and dashed lines indicate random structures. Chain reversal indicates predicted turns.

Utilizing a program developed to detect repetition of sequences, I have found no repeating sequences in these first 333 residues. This and the observation that the gene product has a molecular weight of 130 kda indicates that the subunit cannot be composed of aggregates of small peptides as previously suggested (Fast and Martin, 1980).

So what? The subtitle of this session is "Where do we go from here?" What I have reviewed in this paper is the barest of beginnings, but it is a beginning. We do not know what the structure of PRP is, nor even where it comes from in the sequence of the monomer. We do not know what the active structures are in PRP. We do not know how different isolates within a serotype with different toxicity spectra differ from each other, much less how toxins from different serotypes differ. We do not know why the toxicity of a single isolate varies so much between target insects; it may be because of differences in their proteases or their toxin receptors if there are such (I think there are!). In some cases we have hints, but nothing solid. When we know the answers to these questions then I predict that molecular biologists will be able to tailormake a crystal protein for each pest or crop, and Bt or related products could dominate the insecticide market for defoliating insects. The potential is enormous.

If Bt is to reach this potential it will be necessary to recruit and support much more fundamental work on crystal toxin. Because of its economic interest, its potential for much more benign pest control, and its potential for genetic manipulation interest is growing but support is meager. We need to convince our colleagues doing fundamental work on protein structure and molecular genetics that Bt is a good model for study. More particularly we need to convince the funding agencies of the value of such work so that it is supported. Significant growth in support of such fundamental studies is required to realize the potential of this organism for environmentally benign insect control.

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