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Evidence for Two Different Types of Insecticidal P2 Toxins with Dual Specificity in *Bacillus thuringiensis* Subspecies

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Analysis of polypeptides in the crystalline δ-endotoxins from different *Bacillus thuringiensis* strains revealed two antigenically similar forms of the P2 protein which differed in molecular mass, peptide profile, and amino acid sequence. Purified preparations of the two forms displayed the characteristic dual toxicity of the P2 protein towards members of the orders Lepidoptera and Diptera in vivo but differed markedly in potency for the insects tested. Both species of the P2 protoxin, solubilized and activated by sequential proteolysis with insect gut extract and α-chymotrypsin, retained activity in vivo and in vitro, despite the removal of 144 residues from the N terminus. For the low-molecular-mass form, the dual insecticidal activity was reproducible in the in vitro assays.

During sporulation, strains of the gram-positive bacterium *Bacillus thuringiensis* synthesize proteinaceous, crystalline inclusions (2, 25) that are insecticidal toward larvae within the orders Lepidoptera (3), Diptera (3), and Coleoptera (7, 18). These water-insoluble inclusions (δ-endotoxins) are composed of one or more polypeptides in the form of inactive protoxins. When ingested by susceptible hosts, the protoxins are solubilized and activated by the combination of high pH and proteases in the larval midgut (20). In contrast to broad-spectrum chemical pesticides, these δ-endotoxins are often highly specific for their target organism(s). Although the precise biochemical basis underlying this specificity remains largely undetermined, it has been variously attributed to (i) quantitative differences in the δ-endotoxins produced (28), (ii) proteolytic activation of the protoxin in the host gut (11), and (iii) the presence of unique proteins in the native crystal (33). Support for the latter possibility comes from the demonstration that the *B. thuringiensis* subsp. *kurstaki* HD-1 δ-endotoxin contains two serologically distinct polypeptide species: the 130-kilodalton (kDa) P1 lepidopteran-specific toxin and the broader-spectrum 63-kDa P2 protein (mosquito factor) toxic to both lepidopteran and dipteran larvae (28).

Recently, a combination of the in vivo solubilization and activation of these δ-endotoxins and the availability of susceptible insect cell lines has played a prominent role in characterizing insect specificity and toxin potency and in identifying toxin receptors (8, 10, 13, 14). This approach has not previously been possible with the P2 protein because of its relative insolubility at pHs compatible with cell culture assays. We report here the development of a protoxin activation regimen which yields soluble preparations of these P2 proteins that can be assayed both in vitro and in vivo. The results of a comparative examination of a range of δ-endotoxin crystals characterized by the presence of P2-like polypeptides are also presented.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. thuringiensis* subsp. *kurstaki* HD-1 used in these studies was obtained from H. D. Burges, Institute for Horticultural Research, Littlehampton, England. *B. thuringiensis* subsp. *thuringiensis* HD-117, *B. thuringiensis* subsp. *thuringiensis* HD-770, and *B. thuringiensis* subsp. *galleriae* HD-29 were obtained from H. T. Dulmage (U.S. Department of Argiculture, Brownsville, Tex.). *B. thuringiensis* subsp. *galleriae* 916 was obtained from T. Yamamoto, (E. I. Du Pont de Nemours & Co., Inc., Wilmington, Del.). Conditions for growth and sporulation were as described by Stewart et al. for *B. megaterium* KM (27).

Isolation of crystal δ-endotoxins. The δ-endotoxin inclusions from *B. thuringiensis* subsp. *kurstaki* HD-1 were separated from spores and vegetative cell debris by ultracentrifugation on discontinuous sucrose density gradients, as described by Thomas and Ellar (28). Modified gradients consisting of 67, 72, 79, and 87% (wt/vol) sucrose were used to purify crystals from the remaining strains, with the crystals forming a major band at the interface between the 79 and 87% (wt/vol) sucrose. Protein concentrations of the purified δ-endotoxins were determined by the method of Lowry et al. (21), using bovine serum albumin (Sigma Chemical Co., Poole, England) as a standard.

Electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out by the method of Laemmli and Favre (19), using an acrylamide:*N,N'*-methylenebisacrylamide ratio of 100:1. Electrophoresis conditions and the procedures for staining and destaining were as described by Haider et al. (11).

Peptide mapping by limited proteolysis. The peptide profiles of different P2 proteins were compared after limited proteolysis with α-chymotrypsin (Sigma), using a method based on that of Cleveland et al. (5). Protein samples (20 to 80 μg) were electrophoretically separated on a 13% SDS-polyacrylamide gel. Excised P2 bands were then electrophoresed in the second dimension on an 18% acrylamide gel, as described by Stewart and Ellar (26).

Antiserum preparation and immunoblotting. Antibodies to the *B. thuringiensis* subsp. *kurstaki* P1 and P2 proteins were raised by subcutaneous injection into New Zealand White rabbits, as described by Haider et al. (11). Proteins separated by SDS-polyacrylamide gel electrophoresis were electrophoretically transfered to nitrocellulose filters (Schleicher & Schuell, Inc., Keene, N.H.) in a transblot apparatus (Bio-Rad Laboratories, Watford, England) (29). Immunoblotting

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was carried out by the method of Hawkes et al. (12), using a 1:500 dilution of primary antibody. Bound antibody was detected with peroxidase-conjugated goat anti-rabbit immunoglobulins (I.C.N. Biomedicals Ltd., High Wycombe, England). Nonspecific binding was blocked with 3% (wt/vol) bovine serum albumin (Sigma) in 10 mM Tris hydrochloride–150 mM NaCl, pH 7.4.

Purification of the P2 protein. The two toxic moieties of the HD-1 δ-endotoxin can be separated by virtue of their differential solubilities. Under alkaline (pH 9.5) reducing conditions, the P1 protein is preferentially solubilized, yielding an insoluble P2 fraction. Conversely, treatment with alkaline solutions (pH 11.0 to 12.0) in the absence of a thiol reagent selectively liberates the P2 component (32). Purified crystals were solubilized in 50 mM K_2CO_3–HCl (pH 9.5) containing 10 mM dithiothreitol at 37°C for 60 min. Soluble material (P1) for use in bioassays (see Table 1) was removed by centrifugation at 10,000 × *g* for 10 min in an Eppendorf minifuge at 4°C. The pellet fraction consisting of the P2 crystal protein was then washed three times in ice-cold deionized water, suspended in deionized water, and stored at −20°C.

Solubilization and activation of the P2 protein. The purified P2 crystal protein pellet was activated by a sequential two-step protease digestion in alkaline buffer. A given volume of purified P2 crystal protein, at 1 mg ml^{-1}, was centrifuged at 10,000 × *g* for 5 min, and the pelleted material was suspended in an equal volume of 50 mM K_2CO_3–HCl (pH 10.5). This P2 protein suspension was mixed 10:1 (by volume) with gut extract from *Pieris brassicae*, prepared by the method of Knowles et al. (17), and incubated at 37°C for 90 min. Insoluble material was removed by centrifugation at 10,000 × *g* for 5 min, and the supernatant, containing the soluble P2 crystal protein, was incubated with 10% (vol/vol) α-chymotrypsin from a 5-mg ml^{-1} stock solution at 37°C for a further 90 min. The supernatant fraction resulting from a final centrifugation at 10,000 × *g* for 5 min was used immediately for in vitro cell assays or stored frozen at −20°C.

Cell lines and cytotoxicity assays. The cell lines used in this investigation and their growth conditions have been described previously by Granum et al. (9). Cells at 1×10^6 to 2×10^6 ml^{-1} in tissue culture medium were incubated with soluble activated toxins at a final concentration of 100 μg ml^{-1}. In order to neutralize the effects of insect gut enzymes on the cell lines, the toxins were made 10% in fetal calf serum (GIBCO Diagnostics, Madison, Wis.) prior to addition. Cell viability was assessed by vital staining with trypan blue (28). Controls contained comparable volumes of buffer, gut extract, enzyme, and fetal calf serum.

Bioassay. Bioassay of third-instar *P. brassicae* larvae (obtained from B. Gardiner, Cambridge Biotech Services, Cambridge, England) was carried out as described by Burges et al. (4). Mosquito bioassays were conducted by the method of Tyrell et al. (30), using 4- to 6-day-old *Aedes aegypti* or *Anopheles gambiae* larvae, grown from eggs obtained from D. Funnel (Shell Research, Sittingbourne, England) and C. Curtis (London School of Hygiene and Tropical Medicine, London, England), respectively.

N-terminal amino acid analysis. Solubilized toxin preparations, resolved by SDS-polyacrylamide gel electrophoresis, were transferred to polyvinylidene difluoride membranes (Millipore Corp., Watford, England), using a transblot apparatus (Hoeffer) operating at 50 mA constant current for 1 h (22). Transfer buffer consisted of 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid, pH 6.0. The polyvinylidene difluoride membrane was rinsed in deionized water for 5 min, stained for 5 min in 0.1% Coomassie brilliant blue and then in 50% methanol–10% acetic acid, and destained in 50% methanol–10% acetic acid for 10 min. The membrane was then briefly rinsed in deionized water, air dried, and stored at −20°C until required. Coomassie brilliant blue-stained protein bands were excised from the polyvinylidene difluoride membrane with a clean razor blade, divided into fine strips, and applied to the sample cartridge of a 470A gas phase sequencer (Applied Biosystems). Sequence analysis was performed by using standard RUN 470-1 software under the control of a 900A data-controller module. The phenylthiohydantoin amino acids were analyzed on-line, using a 120A PTH-amino acid analyzer.

RESULTS

Crystal polypeptide composition and antigenic relationship. The crystal δ-endotoxins used in the present study are characterized by two prominent protein components, P1 and P2 (Fig. 1a). The P1 component frequently contains more than one polypeptide and migrates in the region of 130 to 140 kDa, and the P2 component has an apparent molecular mass of 60 to 63 kDa. Electrophoretic analysis of these δ-endotoxins demonstrates the considerable variation in the contributions made by individual polypeptides to the total crystal protein. Visual estimation from the gels indicate that the P2 component of the *B. thuringiensis* subsp. *kurstaki* HD-1 δ-endotoxin (Fig. 1a, lane 3), for example, constitutes only a small fraction (10%) of crystal protein, whereas in strains such as *B. thuringiensis* subsp. *thuringiensis* HD-117 (Fig. 1a, lane 4) the P2 moiety is the major (80%) component. For *B. thuringiensis* subsp. *galleriae* HD-29 and 916 (Fig. 1a, lanes 6 and 7), the P2 band of 61 kDa migrated slightly faster than the equivalent band (63 kDa) in HD-1, HD-117, and HD-770, suggesting the existence of a different P2-type protein in these strains.

To establish the antigenic relationship between the HD-1 P2 toxin and the P2-like proteins of the other strains, a duplicate gel was immunoblotted with antiserum raised against purified HD-1 P2 (Fig. 1b). All strains, except for *B. thuringiensis* subsp. *israelensis* (included as a negative control), contained at least one band showing strong cross-reactivity with the P2 antiserum. The similarities of the various P2 toxins were further investigated by peptide mapping the purified P2 proteins. Figure 2 compares the peptide maps of all five strains. The P2 peptide profiles from HD-1 (Fig. 2, lane 1), HD-117 (Fig. 2, lane 2), and HD-770 (Fig. 2, lane 3) show that they are very similar. The peptide maps of HD-29 (Fig. 2, lane 4) and 916 (Fig. 2, lane 5) revealed a number of peptides common to all strains but were sufficiently dissimilar to suggest the presence of a second type of P2 protein in these strains. Further evidence supporting the separate identities of the two P2 protein species came from studies (see below) of the in vivo and in vitro insecticidal activities of both insoluble and soluble preparations of these toxins.

In vivo toxicity. The two toxic moieties of the *B. thuringiensis* subsp. *kurstaki* HD-1 δ-endotoxin can be separated under alkaline reducing conditions, yielding a soluble P1 fraction and an insoluble P2 pellet. For each of the native δ-endotoxins, incubation in K_2CO_3 buffer at pH 9.5 in the presence of 10 mM dithiothreitol for 60 min at 37°C solubilized all the P1 protein, leaving P2 crystal protein as an insoluble pellet. The purity of these P2 crystal protein pellets was confirmed by immunoblotting of the insoluble pellets with antisera raised against purified HD-1 P1 protein. In each

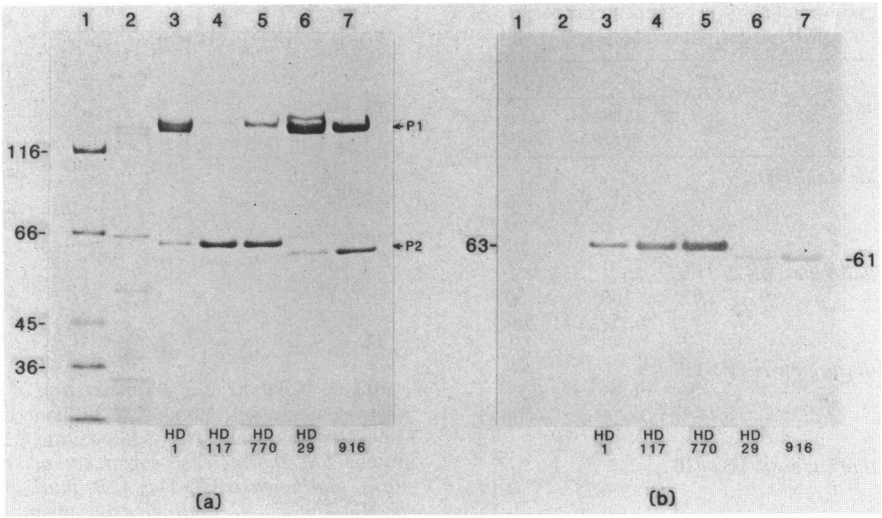

FIG. 1. Polypeptide composition and antigenic relationships of the crystal proteins of six *B. thuringiensis* strains. (a) SDS-13% polyacrylamide gel, Coomassie brilliant blue stained, of crystal δ-endotoxins. Lanes: 1, Molecular mass standards (in kilodaltons); 2, *B. thuringiensis* subsp. *israelensis* native crystal; 3, *B. thuringiensis* subsp. *kurstaki* HD-1 native crystal; 4, *B. thuringiensis* subsp. *thuringiensis* HD-117 native crystal; 5, *B. thuringiensis* subsp. *thuringiensis* HD-770 native crystal; 6, *B. thuringiensis* subsp. *galleriae* HD-29 native crystal; 7, *B. thuringiensis* subsp. *galleriae* 916 native crystal (50 μg in each lane). (b) Immunoblot of the gel in panel a, developed using antiserum raised against the *B. thuringiensis* subsp. *kurstaki* 63-kDa polypeptide, showing the high-molecular-mass (63-kDa) and low-molecular-mass (61-kDa) forms of the P2 crystal protein.

case, no significant cross-reactivity could be detected (data not shown).

Aqueous suspensions of the P2 crystal protein pellets were then assayed for in vivo insecticidal activity, and the data were compared with results from similar bioassays using the corresponding native δ-endotoxins. The native crystals and P2 crystal protein pellets proved to be toxic to both lepidopteran and dipteran larvae. Certain differences were observed, however, in the degree of larval susceptibility, and they were seen to be directly correlated to the nature of the P2 species used. Table 1 summarizes the results of quantitative bioassays using fourth-instar *P. brassicae* larvae. The toxin concentrations giving 50% inhibition of feeding after 36 h for both the native crystals and P2 crystal proteins of HD-1 and HD-770 (characterized by a high-molecular-mass P2) were observed, on average, to be some 10-fold less than those for similar preparations of the low-molecular-mass P2

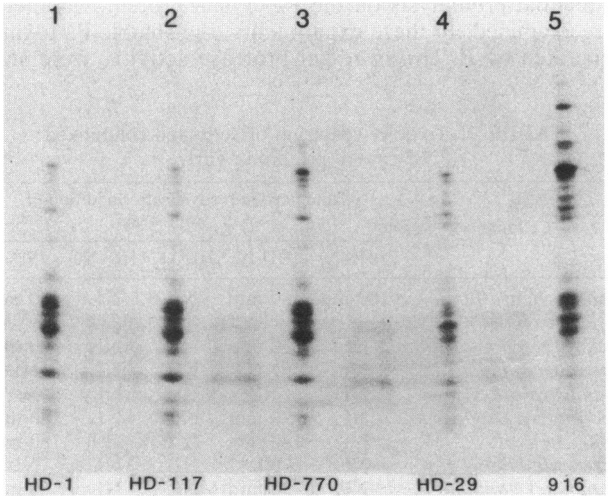

FIG. 2. Peptide mapping by limited proteolysis with α-chymotrypsin of electrophoretically separated P2 polypeptides (Coomassie brilliant blue-stained gel). Lanes: 1, *B. thuringiensis* subsp. *kurstaki* HD-1; 2, *B. thuringiensis* subsp. *thuringiensis* HD-117; 3, *B. thuringiensis* subsp. *thuringiensis* HD-770; 4, *B. thuringiensis* subsp. *galleriae* HD-29; 5, *B. thuringiensis* subsp. *galleriae* 916.

TABLE 1. In vivo toxicities of crystal proteins for *P. brassicae*[a]

Toxin	IC_{50} (μg ml^{-1})[b]
B. thuringiensis subsp. *kurstaki* HD-1	
Native crystal	0.01
Insoluble P2[c]	0.1
Activated soluble P2[d]	1.0
Soluble P1[c]	0.1
B. thurginiensis subsp. *galleriae* HD-29	
Native crystal	1.0
Insoluble P2	1.0
Activated soluble P2	1.0
Soluble P1	1.0
B. thuringiensis subsp. *thuringiensis* HD-770	
Native crystal	0.01
Insoluble P2	0.1
Activated soluble P2	1.0
Soluble P1	0.01

[a] Twelve fourth-instar larvae.
[b] IC_{50}, Toxin concentration giving 50% inhibition of feeding after 36 h.
[c] Native crystal incubated for 60 min at 37°C in 50 mM K_2CO_3–HCl, pH 9.5, containing 10 mM-dithiothreitol yielded a soluble fraction (P1), which was used directly, and the P2 crystal protein was suspended in deionized water by sonication.
[d] Insoluble P2 protein incubated for 90 min at 37°C in 50 mM K_2CO_3, pH 10.5, and *P. brassicae* gut extract (10:1 by volume) yielded a soluble P2 crystal protein fraction, which was incubated for 90 min at 37°C with 10% (vol/vol) α-chymotrypsin from a 5-mg ml^{-1} stock solution.

TABLE 2. In vivo toxicities of crystal proteins for members of the order Diptera[a]

Toxin	LC_{50} ($\mu g\ ml^{-1}$)[b] for:	
	Aedes aegypti	*Anopheles gambiae*
B. thuringiensis subsp. *kurstaki* HD-1		
Native crystal	12.5	0.05
Insoluble P2[c]	6.25	0.13
B. thuringiensis subsp. *galleriae* HD-29		
Native crystal	100	50
Insoluble P2	50	100
B. thuringiensis subsp. *thuringiensis* HD-117		
Native crystal	25	25
Insoluble P2	25	0.25
B. thuringiensis subsp. *thuringiensis* HD-770		
Native crystal	6.25	0.13
Insoluble P2	2.50	0.13
B. thuringiensis subsp. *galleriae* 916		
Native crystal	250	100
Insoluble P2	>500	150

[a] Twenty-five 4- to 6-day-old larvae.
[b] LC_{50}, Toxin concentration giving 50% mortality after 24 h.
[c] Native crystal incubated for 60 min at 37°C in 50 mM K_2CO_3–HCl, pH 10.5, containing 10 mM dithiothreitol yielded a soluble fraction, which was discarded, and an insoluble P2 crystal protein fraction, which was suspended in deionized water by sonication.

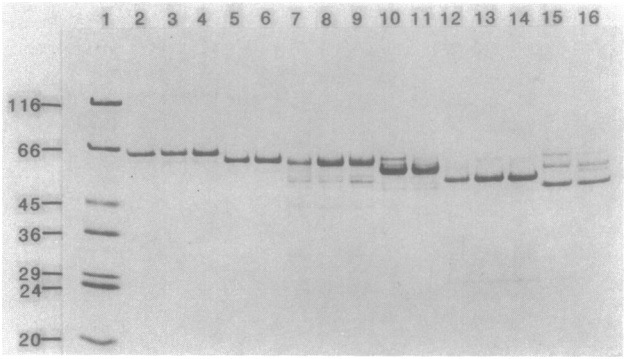

FIG. 3. Solubilization and activation of the P2 toxin (13% SDS-polyacrylamide gel, Coomassie brilliant blue stained). Lanes: 1, Molecular mass standards (in kilodaltons); 2 to 6, 15 μg of P2 crystal protein (2, *B. thuringiensis* subsp. *kurstaki* HD-1; 3, *B. thuringiensis* subsp. *thuringiensis* HD-117; 4, *B. thuringiensis* subsp. *thuringiensis* HD-770; 5, *B. thuringiensis* subsp. *galleriae* HD-29; 6, *B. thuringiensis* subsp. *galleriae* 916); 7 to 11, 25 μg of P2 crystal protein solubilized in 50 mM K_2CO_3–HCl (pH 10.5) and activated with 10% (vol/vol) *P. brassicae* gut extract (7, *B. thuringiensis* subsp. *kurstaki* HD-1; 8, *B. thuringiensis* subsp. *thuringiensis* HD-117; 9, *B. thuringiensis* subsp. *thuringiensis* HD-770; 10, *B. thuringiensis* subsp. *galleriae* HD-29; 11, *B. thuringiensis* subsp. *galleriae* 916); 12 to 16, 25 μg of P2 crystal protein treated first with *P. brassicae* gut extract and then with 10% (vol/vol) α-chymotrypsin from a 5-mg ml^{-1} stock solution (12, *B. thuringiensis* subsp. *kurstaki* HD-1; 13, *B. thuringiensis* subsp. *thuringiensis* HD-117; 14, *B. thuringiensis* subsp. *thuringiensis* HD-770; 15, *B. thuringiensis* subsp. *galleriae* HD-29; 16, *B. thuringiensis* subsp. *galleriae* 916).

of HD-29. Moreover, further bioassays using dipteran larvae confirmed the differential toxic properties of the two P2 species. The larvae of both *Aedes aegypti* and *Anopheles gambiae* were more susceptible to the high-molecular-mass P2 crystal proteins of HD-1, HD-117, and HD-770 (Table 2). P2 protein from HD-1 and HD-770 was 10 to 50 times more active against *Anopheles gambiae* than against *Aedes aegypti*.

Solubilization and activation of the P2 toxin. The 130-kDa P1-type toxins of *B. thuringiensis* subsp. *kurstaki* HD-1 and other strains are synthesized as inactive protoxins and initially require proteolytic processing to generate an active toxic moiety, which can be assayed in vitro. We therefore investigated the effects of various activation regimens on the structure and activity of purified P2 preparations. Incubation of P2 crystal protein pellets in 50 mM K_2CO_3–NaOH, pH 12.0, for 60 min at 37°C resulted in complete solubilization of the toxin. However, this soluble preparation proved to have no cytolytic activity in vitro. Yamamoto and Iizuka (32) have previously observed that treatment of HD-1 P2 with lepidopteran proteases at pH 10.2 removes a 5-kDa fragment from the P2 protein. Confirmation of this observation came when the various P2 crystal protein pellets were exposed to *P. brassicae* gut proteases in the presence of an alkaline buffer at pH 10.5 and the results were monitored by SDS gel electrophoresis. For both high- and low-molecular-mass P2 species, treatment with *P. brassicae* gut extract removed a fragment of approximately 5 kDa from both the 63- and the 61-kDa polypeptides (Fig. 3, lanes 7 to 11). The most important outcome of these experiments was, however, the finding that in each case the P2 crystal protein pellet was converted into a form that was now soluble in carbonate buffer at pH 10.5. When these soluble preparations were subsequently assayed against a variety of cell lines, no significant cytotoxicity could be demonstrated. However, such preparations still retained in vivo toxicity against *P. brassicae* larvae (Table 1).

In their ability to kill members of both Lepidoptera and Diptera, P2 crystal proteins resemble the 130-kDa *B. thuringiensis* subsp. *aizawai* (formerly designated subsp. *colmeri* [10]) IC-1 protoxin (11). The differential specificity of this toxin was shown to be correlated with sequential proteolysis by gut proteases from two different insect orders or by a combination of gut proteases and trypsin (11). The effect of sequential proteolysis on the P2 crystal protein was therefore examined. In these experiments, the soluble P2 toxins, generated by *P. brassicae* gut protease activity, were sub-

TABLE 3. Toxicity spectrum of activated solubilized P2 crystal protein in vitro

Cell line[a]	Time needed for lysis after addition of toxin[b] from strain:				
	HD-1	HD-29	HD-117	HD-770	916
Choristoneura fumiferana	180 min	180 min	NL	NL	180 min
Heliothis zea	NL	210 min	NL	NL	60 min
Lymantria dispar	NL	NL	NL	NL	NL
Mamestra brassicae	NL	NL	NL	NL	NL
Spodoptera frugiperda	NL	90 min	NL	NL	120 min
Aedes aegypti	NL	180 min	22 h	22 h	180 min
Aedes albopictus	NL	NL	NL	NL	NL
Anopheles gambiae	NL	30 min	NL	NL	60 min
Culex quinquefasciatus	NL	NL	NL	NL	NL

[a] Concentration, 1×10^6 to 2×10^6 cells ml^{-1}.
[b] Time taken for 50% lysis after addition of soluble toxin (100 μg ml^{-1}) activated with *P. brassicae* gut extract and α-chymotrypsin. Lysis was assessed by the inability to exclude trypan blue. NL, No lysis; indicates that no cytopathic effect was observed after 24 h.

	Residue 50					
HD-1 P2	V	A	P	V	V	G
HD-117 P2 (58 kDa)	V	A	P	V	V	G

	Residue 146									
HD-1 P2	S	I	T	S	S	V	N	T	M	Q
HD-117 P2 (51 kDa)	S	I	T	S	S	V	N	T	M	Q
HD-29 P2 (49 kDa)	S	I	I	D	S	V	N	T	L	Q

FIG. 4. Comparison of the N-terminal amino acid sequences of the activated forms of HD-117 and HD-29 P2 crystal proteins with the corresponding regions in the *B. thuringiensis* subsp. *kurstaki* HD-1 P2 protein.

sequently exposed to α-chymotrypsin. Treatment with this enzyme promoted a further limited proteolysis, during which an additional 7 kDa was cleaved from both high- and low-molecular-mass derivatives of the P2 toxin, generating major proteolytic products of 51 and 49 kDa, respectively (Fig. 3, lanes 12 to 16). Prolonged incubation under these conditions resulted in no further significant degradation of these products, suggesting that the 51- and 49-kDa derivatives might represent the protease-resistant cores of the two P2 species. In contrast to the gut protease-treated toxins, which exhibited only in vivo activity, the second proteolytic step yielded toxins that proved to be both larvicidal and cytolytic.

In vitro toxicity. Table 3 shows the results of the in vitro assay of activated soluble P2 toxins on a range of lepidopteran and dipteran cell lines. For a direct comparison of cytolytic activity against different cell lines, the time taken for 50% cell mortality (as assessed by trypan blue staining) upon addition of 100 μg of toxin ml^{-1} was used. Among the high-molecular-mass P2 toxins, HD-1 was active only against *Choristoneura fumiferana* cells, and the equivalent P2 proteins from HD-117 and HD-770 showed weak activities against the *Aedes aegypti* cell line. In contrast, the activated P2 proteins of HD-29 and 916 proved to be cytolytic for a much broader spectrum of lepidopteran and dipteran cell lines. The finding that mortality in control cells incubated with equal volumes of buffer, gut extract, enzyme, and fetal calf serum rarely exceeded 10% confirmed that the observed cytolysis is directly attributable to toxin activity. These observations were confirmed by experiments in which toxicity was neutralized by preincubation of toxin with specific antisera. Equal volumes of activated solubilized P2 protein (100 μg ml^{-1}) and *B. thuringiensis* subsp. *kurstaki* HD-1 P2 antiserum were incubated together for 30 min at 20°C prior to addition to cells. In each case, this pretreatment resulted in a complete neutralization of toxicity.

N-terminal amino acid analysis. N-terminal amino acid analysis of the activated derivatives of the P2 toxins was carried out in an attempt to align these proteolytic products with the recently published sequence of an HD-1 P2 crystal protein (6). The sequences of the 58- and 51-kDa proteolytic derivatives of the HD-117 P2 toxin exactly matched sequences beginning at residues 50 and 146, respectively, in the *B. thuringiensis* subsp. *kurstaki* P2 protein (Fig. 4), supporting the results from peptide mapping. These data show that both proteolytic events involved extensive processing at the N terminus of the protein; treatment with *P. brassicae* gut extract and α-chymotrypsin sequentially removed 49 and 96 amino acids. The differences observed between the N-terminal sequence of the 49-kDa proteolytic derivative from the HD-29 toxin and the corresponding regions in the HD-117 and *B. thuringiensis* subsp. *kurstaki* P2 sequences (Fig. 4) provided further evidence for the existence of two forms of the P2 toxin. Additional data supporting this view are contained in the reports by Donovan et al. (6) and Widner and Whiteley (31).

DISCUSSION

Recent analysis of the genes encoding several 130-kDa lepidopteran-specific δ-endotoxins has revealed extensive protein sequence similarity to the P1 protoxin of *B. thuringiensis* subsp. *kurstaki* HD-1 (1, 23, 24). Despite this similarity, however, bioassays of these proteins both in vivo and in vitro (13) demonstrated that they differed markedly in both specificities and potencies. Such observations suggest that even minor modifications in the amino acid sequences of these toxins may be sufficient to generate significant differences in their capacity to recognize and bind to specific insect receptors.

A further illustration of the emerging complexity of the family of P1-type polypeptides comes from a recent study of *B. thuringiensis* subsp. *aizawai* HD-249 (15). This strain synthesizes two immunologically related P1-type polypeptides of approximately 130 kDa. Upon activation with insect gut proteases, one of these protoxins is cleaved to a 60-kDa product which is cytolytic to CF1 cells but not to a *Spodoptera frugiperda* cell line. Conversely, proteolytic processing of the second protoxin yields a 65-kDa fragment active against *Spodoptera* cells but nontoxic to CF1 cells. The three different 50% inhibitory concentrations for *P. brassicae* of the P1 preparations in Table 1 suggest that they contain one or more different P1 toxins. This suggestion is supported by our finding (data not shown) that the three P1 preparations differ in their in vitro cytolytic spectra.

To investigate the applicability of these observations to other antigenically cross-reacting *B. thuringiensis* δ-endotoxins, we have compared the specificities and potencies of five P2 crystal proteins from strains of *B. thuringiensis* subsp. *kurstaki, galleriae*, and *thuringiensis*. The results suggest that these toxins may constitute a family of related proteins analogous to the P1 toxins. Although immunoblotting with P2-specific antiserum indicated that all five P2 δ-endotoxins shared a common antigenic determinant, differences in their apparent molecular masses and peptide profiles suggest that there are at least two distinct P2 species. In the past, study of the P2 crystal protein has been limited by the lack of a suitable in vitro system for the qualitative and quantitative assay of these toxins. The development of such a system based on the sequential proteolytic processing of the P2 protein has enabled us to characterize the different in vitro activities of these toxins and to compare these data with insect bioassays.

Bioassay of insoluble and activated soluble preparations of the P2 crystal proteins against lepidopteran and dipteran larvae revealed a clear correlation between the molecular masses of the protoxins and their ultimate larvicidal activities. Against *P. brassicae* larvae, insoluble preparations of the higher-molecular-mass P2 forms (HD-1 and HD-770) consistently exhibited higher quantitative toxicities than similar preparations of a low-molecular-mass P2 (HD-29). However, it is of interest that when activated and solubilized, the high-molecular-mass P2s become, on average, 10-fold less toxic, whereas the potency of the HD-29 P2 is apparently undiminished. Comparison of the 50% inhibitory concentrations of the insoluble high-molecular-mass P2 crystal proteins with those obtained for the corresponding native

crystals (P1 and P2) reveals the latter to be 10 times more toxic toward *P. brassicae*. Interestingly, no such difference was observed for the low-molecular-mass (HD-29) insoluble P2 protein. Since comparisons of the in vivo toxicities of purified preparations of P2 and P1 toxins from HD-1 show them to be equally toxic to larvae of *P. brassicae* (Table 1) and *Trichoplusia ni* (11), these results might imply a synergistic effect between the P1 and the high-molecular-mass P2 toxins in the HD-1 strain. No such effect was observed, however, with the HD-770 and HD-29 strains. It will be interesting to see whether this difference persists in bioassays against a wider variety of insects.

Further support for the division of the P2 protein into subgroups was provided by the dipteran larvae assays. Table 2 shows that, in general, the high-molecular-mass insoluble P2 crystal proteins (HD-1, HD-117, and HD-770) were more active against *Anopheles* larvae than against *Aedes* larvae and were 1 to 2 orders of magnitude more toxic to *Anopheles* larvae than the corresponding low-molecular-mass toxins from the HD-29 and 916 strains. Against *Aedes aegypti* larvae, the same difference was observed but on a smaller scale. Although these data suggest that the low-molecular-mass forms of P2 are not particularly effective dipteran toxins, firm conclusions cannot be drawn until bioassays against a more extensive range of dipteran larvae have been completed. Further data for and against synergism between P1 and P2 proteins are contained in Table 2. Thus, although visual estimates from gels indicate that the P2 toxin represents only 10% of total protein in the HD-1 and HD-29 crystal proteins and approximately 40% in strain 916 crystals, the activities of these crystals against *Anopheles gambiae* were significantly greater in each case than that of the insoluble P2 protein alone. This effect was not seen in HD-117, in which P2 represents some 80 to 90% of total crystal protein, or in HD-770, in which P1 and P2 are present in approximately equal amounts in the crystal.

In marked contrast to the results of the in vivo assays, the low-molecular-mass P2 toxins exhibited the most potent cytolytic activity and were active against a broader range of cell lines. At the concentration tested, the HD-1 P2 toxin lysed only CF1 cells. Interestingly, this is the only cell line which we have found to be susceptible to the activated P1 toxin from this strain (16). The possibility that this result with P2 from HD-1 was attributable to contamination of the preparation with small amounts of the P1 toxin was ruled out by the demonstration that the preparation did not cross-react with P1 antiserum and that the cytolysis could be completely blocked by antiserum specific to P2. Further experiments are needed to determine whether activated P1 and P2 preparations bind to different receptors on the CF1 cells and whether synergism can be demonstrated.

In vitro, the low-molecular-mass P2 toxins displayed the dual toxicity toward members of the orders Lepidoptera and Diptera that is a feature of their in vivo activity. These results suggest either that the receptors for these toxins may be more widely distributed in the cell lines studied or that they may bind to more than one receptor type. By analogy with the multiple P1-type toxins found in many strains, several P2 toxins may also reside in some strains. This conclusion is reinforced by recent reports from Donovan et al. (6) and Widner and Whiteley (31). The fact that only a single N-terminal sequence was detected in the purified P2 proteins that we examined suggests that this is not likely to explain the broad spectrum of in vitro activity that we observed for the low-molecular-mass forms of P2. The need for caution in interpreting results with cell lines that are not necessarily representative of the gut epithelial cell targets of these toxins is emphasized by the poor correlation observed between the in vivo and in vitro assays of the purified P2 toxins. Nevertheless, a combination of results from the bioassays, antibody cross-reactivity, peptide mapping, and N-terminal sequencing strongly suggests that the P2 toxins are likely to constitute a group of proteins with extensive sequence similarity but different entomocidal activities.

One interesting difference between the P1 and the P2 toxins is the apparent retention of activity in the latter despite extensive N-terminal proteolysis. Removal of approximately 600 amino acids from the C terminus is typically seen in the activation of P1 toxins. Comparison of the apparent molecular masses of the activated P2 toxins with the N-terminal sequences of the purified products suggests that little or no C-terminal processing is taking place during the activation. In this respect, it is interesting that Widner and Whiteley (31) have recently found that deletion of the last 11 residues at the C terminus of the *cryB1* gene of *B. thuringiensis* subsp. *kurstaki* HD-1 results in a loss of toxicity. Alignment of the sequences of P1 and P2 from HD-1 (6) revealed a region of similarity in P2 extending from residues 162 to 260. Sequences in this region are also conserved in a number of other *B. thuringiensis* δ-endotoxins of widely differing specificities, suggesting that they may constitute a functional domain which plays an important role in toxicity. It is noteworthy, therefore, that after sequential activation with *P. brassicae* gut enzymes and chymotrypsin, which removes some 145 residues from the N terminus, this region is retained at the N terminus in the protease-resistant P2 core.

The P2 *B. thuringiensis* δ-endotoxins are particularly interesting because of their dual toxicity toward members of the orders Lepidoptera and Diptera (31). Our development of a regimen for the activation of these proteins and the duplication of this dual toxicity in vitro for the toxins from HD-29 and 916 will allow us to investigate the molecular basis for this broad specificity in more detail.

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