TRYPSIN ACTIVATION OF THE
BACILLUS THURINGIENSIS DELTA-ENDOTOXIN IN
OSTRINIA NUBILALIS LARVAE

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INTRODUCTION

The aerobic spore-forming soil bacterium, *Bacillus thuringiensis* (Bt), is well known for its ability to produce a proteinaceous parasporal crystal during sporulation (Eller, *et al.*, 1985; Jaquet, *et al.*, 1987). This crystal protein is toxic, following ingestion, to many insect larvae from the orders Lepidoptera, Diptera, and Coleoptera. After exposure to the alkaline conditions within the insect gut, the crystalline protein (230 kda) is reduced to a 130 kilodalton (kda) protoxin, which is then converted to the activated toxin (55-68 kda) by proteolytic action of the insect gut proteases.

Bacillus thuringiensis strains are grouped into subspecies based on antigenic properties of the flagella and biochemical characteristics (Jacquet, *et al.*, 1987). More than 30 subspecies have been characterized to date. Most of these subspecies are toxic to lepidopteran larvae, while one subspecies is specific for mosquitoes and blackflies. Partly because of their potential as biological insecticides, the study of these delta-endotoxins is of extreme commercial and scientific interest.

The delta-endotoxins of *Bacillus thuringiensis* differ in their activity and potency toward different insect species. The reasons for these differences are not known. However, several factors may be involved, since the crystal protein has to pass through a two-step process, solubilization and activation, before reaching the gut epithelium, where the toxin appears to act (Jacquet, *et al.*, 1987). An alkaline gut pH and proteases of the insect gut have been reported as being potential factors that govern this two-step process and thus the specificity of these delta-endotoxins (Jacquet, *et al.*, 1987; Luthy, *et al.*, 1987). Recent studies by Ellar, *et al.* (1985; Knowles, *et al.*, 1984) indicate that glycoprotein receptors on the insect gut epithelium play a role in determining potency and specificity of *B. thuringiensis* delta-endotoxins. However, their findings also revealed that binding of the delta-endotoxin to the gut epithelium is at least partially controlled by the insect gut environment, i.e. pH and gut proteases.

This paper represents an attempt to assess the role of insect gut juices in determining specificity and potency of two *Bacillus thuringiensis* delta-endotoxins. For this study, larvae of an insect considered not susceptible to Bt delta-endotoxins, *Ostrina nubilalis* Hbn. (European corn borer), were selected to assess the ability of gut proteases and pH to regulate the potency of *B. thuringiensis* Berliner and *B. t.* var. *kurstaki* HD-73 delta-endotoxins. Larvae of the corn borer have demonstrated almost no susceptibility to the delta-endotoxins of either of these strains. Rivers and Zimmack (unpublished data) have observed that the pH (6.8-7.2) of corn borer larval gut juices is not favorable for solubilization of Berliner and HD-

73 delta-endotoxins, which may account for the low susceptibility of corn borer larvae toward these endotoxins.

In order to determine whether the pH found in the gut contents of corn borer larvae is a factor governing the potency of Berliner and HD-73 delta-endotoxins, the pH of corn borer larvae gut contents was adjusted to that found in highly susceptible insects (9.5-11.0), and the gut juices were incubated with crystals from both Bt varieties. The concentration of proteolytic enzymes in the gut contents was also altered, and observations of changes in the solubilization or activation of crystals from Berliner and HD-73 were noted. Sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) was used to monitor solubilization (protein band at 130 kda) and activation (band at 55-68 kda) of the crystals from both Bt varieties incubated with gut juices from corn borer larvae. A protein band of 230 kda was observed, if neither solubilization or activation occurred. Bioassays were performed to determine whether alterations to the gut pH and/or concentration of proteases in the gut juices would effect the susceptibility of corn borer larvae toward crystals from Berliner and HD-73. Corn borer larvae displayed a high susceptibility to crystals incubated in gut juices that had been altered with the addition of a proteolytic enzyme and the pH adjusted to 10.2.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. thuringiensis* var. *kurstaki* HD-73 was supplied by D.H. Dean (*Bacillus* Genetic Stock Center, Columbus, Ohio) and maintained in the laboratory on nutrient agar slants. *B. thuringiensis* Berliner was isolated from a refrigerated Dipel formulation (Abbott Laboratories, North Chicago, Illinois) and maintained as above. Both subspecies were grown in half strength BBL nutrient broth medium at 30°C for 4 days on a rotary shaker.

An *Escherichia coli* strain (JM103) containing the plasmid pOS4201, which produces the C protein (Kronstad and Whitley, 1986) from *B. thuringiensis* var. *kurstaki* HD-1, was obtained from the *Bacillus* Genetic Stock Center and maintained on minimal media slants. This plasmid encodes a 130 kda protein, which is believed to be the protoxin derived from intact crystal of *kurstaki* HD-1. In order to obtain protoxin, plasmid pOS4201 was grown in Luria broth medium containing ampicillin (100 µg/ml) at 37° C for 24 hours. Isopropyl-beta-D-thio-galactopyranoside was added (5 x 10^{-5} M) to induce the *lac* promotor to produce the 130 kda protein.

Isolation and purification of crystals. The crystals were separated from the spores by a two-phase system with dextran sulfate and polyethylene glycol (Goodman, *et al.*, 1967). The crystal suspensions, containing less than 1% spores, were stored frozen at -20° C. Protein from pOS4201 was isolated by sonication of the cells five times for 30 seconds at the maximum setting using a Sonic Dismembrator (Quigley-Rochester). The resulting suspension was centrifuged in 12% NaBr at 3500 x g, and the crystals were washed twice by centrifugation at 3500 x g in distilled water at 4° C. The protein suspensions were stored frozen at -20° C.

Preparation of gut enzyme. Gut proteases were prepared by removing the digestive tracts of 12 fifth-instar corn borer larvae and placing them in 50 mM carbonate buffer/HCL (pH 9.5) containing 10 mM dithiothreitol as a reducing agent. The guts were homogenized and then centrifuged at 10,000 x g for 5 minutes. The supernatant was stored frozen at -20°C.

TABLE 1. Bioassays using first instar European corn borer larvae fed crystals from *B. thuringiensis* Berliner and *B.t.* var. *kurstaki* HD-73.

| | | Mortality | |
Strains	24 hours	48 hours	72 hours
Bt Berliner untreated	315	32%	74%
Bt Berliner treated	84%	100%	100%
Bt *kurstaki* HD-73 untreated	46%	54%	75%
Bt *kurstaki* HD-73 treated	92%	100%	100%
pOS4201 untreated	36%	41%	41%
pOS4201	62%	62%	62%

* Intact Bt cells containing spores and crystals were used as well as crystal alone to observe the effects of trypsin activation at pH 10.5. Cell concentrations used were 1×10^8 cells/ml; crystal concentrations were 9.0 μg/ml of liquid food medium. One hundred first instar larvae were used at each concentration.

Solubilization of crystal into protoxin. The crystals were solubilized by the method of Thomas and Ellar (1983). Solubilization was also performed by incubating the crystals in gut enzymes at 37° C for 60 minutes. The insoluble fraction was removed by centrifugation (10,000 x g) for 5 minutes, and the supernatant stored at -20° C.

Preparation of active toxin. Protoxin was incubated with trypsin (2.8 mg/ml) or gut enzymes (10:1 w/v) for 30 minutes at 37° C for sodium dodecyl sulfate polyacylamide gel analysis (SDS-PAGE). For insect bioassays, crystals were incubated for 30 minutes at 30° C with trypsin (2.8 mg/ml) at a pH of 10.2.

SDS polyacrylamide gel electrophoresis. SDS-PAGE was performed by the method of Knowles, Thomas, and Ellar (1984).

Insect larvae and bioassays. Newly hatched first instar European corn borer larvae were used for the bioassays. Corn borer egg masses were supplied by the USDA-ARS, Ankeney, Iowa. Bioassays were performed by the method of Reichelderfer (1987). Crystals, protoxin, and toxin were mixed with the liquid diet at 60° C; the mixture was then poured into sterile shell vials and allowed to cool. A single, newly hatched larva was placed in each vial. Mortality was determined after 3 days incubation at 29° C.

RESULTS

Table 1 contains data collected from bioassays that exposed first instar European corn borer larvae to treated and untreated crystal protein from *B. t.* Berliner,

B. t. var. *kurstaki* HD-73, and the plasmid pOS4201. Bioassays that used native crystals (untreated) taken directly from Berliner, HD-73, and pOS4201 demonstrated relatively low morality (32-54% after 48 hours), when crystals were fed to larvae of the corn borer. Treated crystals (preincubated at a pH of 10.2 with 2.8 mg/ml trypsin) from Berliner, HD-73, and pOS4201, however, displayed insecticidal activity toward the corn borer that is typical of the activity found in highly susceptible insects (Sutter and Raun, 1967). Mortality reached 100% in larvae fed treated crystals from Berliner and HD-73 within 48 hours, while treated crystals from the plasmid pOS4201 induced only 62% mortality after 96 hours.

SDS-PAGE was used to determine whether crystals from Berliner, HD-73, and the plasmid pOS4201 were activated or solubilized. Preliminary SDS-PAGE data (not shown) revealed that crystals from Berliner and HD-73 incubated in gut enzyme from corn borer larvae for 2 hours were solubilized but not activated in the existing gut conditions. A protein band observed at 130 kda from crystals of Berliner and HD-73 indicated that the crystals from both varieties had been solubilized. The appearance of a protein band in the 55-68 kda range would have indicated that the crystals from both varieties had undergone both solubilization and activation. However, a protein band in this range was not observed for crystal protein from Berliner and HD-73. Crystal protein from the plasmid pOS4201 was neither solubilized nor activated, when incubated in corn borer gut enzyme. A band at 130 kda was observed after incubation in corn borer gut enzyme. Since this plasmid encodes only crystal protein in the protoxin form (130 kda), solubilization was not expected. However, activation was expected to occur due to the proteolytic action of corn borer gut proteases.

Crystals from Berliner and HD-73 were further examined by presolubilizing the crystal protein before incubating with gut enzyme. This was accomplished by incubating the crystals in a carbonate buffer containing the reducing agent dithiothreitol at a pH of 10.2. SDS-PAGE data revealed that the crystals from both Berliner and HD-73 formed a major band at 130 kda, indicating that the crystals had been solubilized. These presolubilized crystals were then incubated with corn borer gut enzyme for 1 hour. Using SDS-PAGE, a protein band was observed at 68 kda (not shown) for both Berliner and HD-73 crystals. This observed protein band at 68 kda indicated that the crystal protein from both varieties had been activated by the corn borer gut proteases.

DISCUSSION

Larvae of the European corn borer have been reported as showing almost no susceptibility to the delta-endotoxins of *Bacillus thuringiensis* varieties Berliner and *kurstaki* HD-73 (Sutter and Raun, 1967). This study using native crystals from both varieties fed to first instar corn borer larvae (Table 1) confirmed the conclusions of Sutter and Raun (1967.). Several reports (Haider, *et al.*, 1986; Jaquet, *et al.*, 1987; Tojo and Aizawa, 1983) have indicated that the pH and composition of gut juices from insects highly susceptible to the delta-endotoxins of *Bacillus thuringiensis* play a part in governing potency and specificity of these endotoxins. This research addressed whether the lack of susceptibility that European corn borer larvae display toward Bt delta-endotoxins may be attributed, at least in part, to the pH and composition of gut contents found within these larvae.

Tojo and Aizawa (1983) previously determined that two gut factors are essential for solubilization and activation of *B. thuringiensis* crystals: 1) an insect

gut pH of 10.2-10.5; and 2) gut proteases similar to trypsin-chymotrypsin. The highly alkaline gut pH found to be necessary for solubilization by Tojo and Aizawa (1983) has been observed in most highly susceptible insects tested thus far. European corn borer larvae possess gut contents with a pH near neutral (Raun, et al., 1966; Rivers and Zimmack, unpublished) and appear to possess gut environments not capable of solubilizing Bt delta-endotoxins. SDS-PAGE data presented here agree with this observation.

Crystals from Berliner and HD-73 incubated in corn borer gut enzymes and subjected to SDS-PAGE both formed a major band at 130 kda. This would suggest that solubilization of the crystals to protoxin occurred, but the protoxin was not activated. This observation might be attributed to a lack of proteoltic enzymes within the gut contents. However, SDS-PAGE revealed that presolubilized crystals were transformed into the activated toxin, when incubated in gut juices from corn borer larvae. Therefore, it appears that corn borer larvae possess proteolytic enzymes capable of activating the protoxins derived from Berliner and HD-73 crystals. The inability of corn borer gut juices to convert the intact crystals of Berliner and HD-73 to the activated toxin appears not to be due to a lack of proteases in the corn borer gut. The lack of an alkaline gut pH appears to be responsible for the inability of corn borer gut juices to transform crystals into the activated form.

The gut proteases of certain Lepidopteran species have been observed to be capable of cleaving the intact crystal of Bt and releasing the 130 kda protoxin (Tojo and Aizawa, 1983). This suggests that the 130 kda bands observed by SDS-PAGE for crystals from Berliner and HD-73 may not have been solubilized by the pH conditions of the corn borer gut but rather by proteolytic processing of the crystals. This mechanism is supported by the observed protease activity in corn borer midguts discussed above and by the observations of Tojo and Aizawa (1983).

Observations by Jaquet, et al. (1987) suggest that larvae of Heliothis virescens (a nonsusceptible insect) are not good convertors of the intact crystals of Bt morrisoni into the activated toxin, apparently due to a lack of gut proteases. By incubating morrisoni crystals in a proteolytic enzyme before feeding the crystals to H. virescens larvae, mortality was induced that resembled that of a highly susceptible insect. These larvae possess a gut pH between 9.0-10.2, so the concentration of proteolytic enzyme within their guts appears to be the limiting factor for the activation of Bt crystals.

With Heliothis virescens larvae, both gut factors described by Tojo and Aizawa (1983) had to be met for mortality to be induced that resembled that of a highly susceptible insect. Both gut factors appear to be required for corn borer larvae as well. Since the required proteases appear to be present, it seems that the neutral pH found within the gut juices of European corn borer larvae is not favorable for solubilization and is the limiting factor for the activation of the crystals of Berliner and HD-73.

The protein crystals produced by the plasmid pOS4201 were neither solubilized nor activated when incubated in corn borer gut enzymes. Solubilization was not expected to occur, since this plasmid only produces crystals in the 130 kda protoxin form. However, activation of the 130 kda protoxin was expected to occur due to the apparent proteolytic activity observed in the corn borer's gut contents using crystals from Berliner and HD-73. Since only one band of 130 kda was observed after two hours incubation in the gut contents of corn borer larvae, it

appears that corn borer larvae lack the proteases necessary to activate the crystals produced by the plasmid pOS4201. However, as discussed above, corn borer gut contents appear to possess proteases capable of activating solubilized crystals from Berliner and HD-73. Perhaps, some other factor is involved in the solubilization and activation of the protein products from the plasmid pOS4201, or the amount of proteolytic enzyme required to induce activation of the crystals *in vivo* (bioassays) is much higher than that needed for *in vitro* (SDS-PAGE) studies. Bioassay data in Table 1 would appear to support the latter possibility.

Data obtained from bioassays revealed that crystals from Berliner, HD-73, and the plasmid pOS4201 required incubation with a proteolytic enzyme (trypsin) in an alkaline environment (pH 10.2) to induce 100% mortality in corn borer larvae. This seems to conflict with our SDS-PAGE observations which suggest that adjusting the pH of corn borer gut juices to 10.2-10.5 should induce high mortality with crystals from Berliner, HD-73, and the plasmid pOS4201 incubated in these gut juices, when fed to corn borer larvae. A third factor in the gut of Lepidoteran insects has been suggested to be involved in regulating potency of Bt delta-endotoxins and may play a role in our conflicting data between SDS-PAGE and bioassays.

Recent studies by Ellar, *et al.* (1985; Knowles, *et al.*, 1984) have suggested that glycoprotein receptors on the insect gut epithelium may be a third factor, aside from gut pH and proteases, governing potency of *B. thuringiensis* delta-endotoxins. However, these cell receptors would not be a factor in the solubilization and activation of Bt crystals. Therefore, glycoprotein receptors on European corn borer gut epithelium did not play a role in the inconsistencies observed between our SDS-PAGE observations and bioassay data. Differences in the concentration of gut proteases required to activate solubilized crystal for *in vitro* and *in vivo* studies may account for the need to add trypsin to altered gut contents (pH adjusted to 10.2) of corn borer larvae to induce high levels of mortality. Studies are currently being conducted in our laboratory to further examine this possibility.

This study suggests that the transformation of *Bacillus thuringiensis* crystals into the activated toxin depends upon the insect gut environment. Solubilization of crystals from a given variety of *Bacillus thuringiensis* appears to be dependent upon the pH and possibily the proteases found within the insect gut. Our observations suggest that the European corn borer is not a good activator of the intact crystal into the solubilized form. This is important, since commercial preparations of *B. thuringiensis* are presented to the target insect in the form of the intact crystal. This work suggests that the effect of the delta-endotoxin may be increased toward a target insect by applying crystal that is transformable to the activated toxin within the insect gut or by directly applying the activated toxin.

ACKNOWLEDGMENTS

LITERATURE CITED

Ellar, D., W. Thomas, B. Knowles, S. Ward, J. Todd, F. Drobniewski, J. Lewis, T. Sawyer, D. Last, and C. Nichols. 1985. Biochemistry, genetics, and mode

of action of *Bacillus thuringiensis* delta-endotoxins. Molec. Biol. Microb. Differ., pp. 230-240.

Goodman, N., R. Footfried, and M. Rogoff. 1967. Biphasic system for separation of spores and crystals of *Bacillus thuringiensis*. J. Bacteriol. 94: 485.

Haier, M., B. Knowles, and D. Ellar. 1986. Specificity of *Bacillus thuringiensis* var. *colmeri* insecticidal delta-endotoxin is determined by differential proteolytic processing of the protoxin by larval gut proteases. Eur. J. Biochem. 156: 531-540.

Jaquet, F., R. Hutter, and P. Luthy. 1987. Specificity of *Bacillus thuringiensis* delta-endotoxins. Appl. Environ. Microbiol. 36: 500-504.

Knowles, B., W. Thomas, and D. Ellar. 1984. Lectin-like binding of *Bacillus thuringiensis* var. *kurstaki* lepidopteran-specific toxin is an initial step in insecticidal action. FEBS 168: 197-202.

Kronstad, J. and H. Whiteley. 1986. Three classes of homologous *Bacillus thuringiensis* crystal-protein genes. Gene 43: 29-40.

Luthy, P., F. Jaquet, C. Hofmann, M. Huber-Lukac, and M. Wolfersberger. 1987. Pathogenic actions of *Bacillus thuringiensis* toxin. Proc. Europ. Work. Bact. Protein Toxins, Gustav Fisher Verlag, Stuttgart, pp. 1-6.

Raun, E., G. Sutter, and M. Revelo. 1966. Ecological factors affecting the pathogenicity of *Bacillus thuringiensis* var. *thuringiensis* to the European corn borer and the fall armyworm. J. Invertebr. Pathol. 8: 365-375.

Reichelderfer, C. 1987. Biological assays with insect pathogens. CRC Handbook of Natural Pesticides, pp. 489-516.

Thomas, W. and D. Ellar. 1983. *Bacillus thuringiensis* var. *israelensis* crystal delta-endotoxin: Effects on insect and mammalian cells *in vitro* and *in vivo*. J. Cell Sci. 60: 181-197.

Sutter, G. and E. Raun. 1967. Histopathology of European corn borer larvae treated with *Bacillus thuringiensis*. J. Invertebr. Pathol. 9: 90-103.

Tojo, A. and K. Aizawa. 1983. Dissolution and degradation of *Bacillus thuringiensis* delta-endotoxin by gut juice protease of the silkworn *Bombyx mori*. Appl. Environ. Microbiol. 45: 576-580.