Vol. 198, No. 3, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 15, 1994 Pages 940-947

ALTERED PROTOXIN ACTIVATION BY MIDGUT ENZYMES FROM A *BACILLUS THURINGIENSIS* RESISTANT STRAIN OF *PLODIA INTERPUNCTELLA*

Brenda Oppert[1,2*], Karl J. Kramer[1], Donovan E. Johnson[1],
Susan C. MacIntosh[3], and William H. McGaughey[1]

[1]U. S. Grain Marketing Research Laboratory, ARS-USDA, Manhattan, KS 66502-2736
[2]Department of Biochemistry, Kansas State University, Manhattan, KS 66506-3702
[3]Novo Nordisk Entotech, Davis, CA 95616-4880

Received December 16, 1993

Processing of *Bacillus thuringiensis* protoxins to toxins by midgut proteinases from a strain of the Indianmeal moth, *Plodia interpunctella* (Hubner), resistant to *B. thuringiensis* subspecies *entomocidus* (HD-198) was slower than that by midgut proteinases from the susceptible parent strain or a strain resistant to *B. thuringiensis* subspecies *kurstaki* (HD-1, Dipel). Midgut extracts from *entomocidus*-resistant insects exhibited five-fold lower activity toward the synthetic substrate α-N-benzoyl-DL-arginine ρ-nitroanilide than extracts from susceptible or *kurstaki*-resistant insects. Midgut enzymes from susceptible or *kurstaki*-resistant insects converted the 133 kDa CryIA(c) protoxin to 61-63 kDa proteins, while incubations with *entomocidus*-resistant enzymes resulted in predominantly products of intermediate size, even with increased amounts of midgut extract. The 61-63 kDa proteins were only produced by *entomocidus*-resistant midgut extracts after long term incubations with the protoxin. The data suggest that altered protoxin activation by midgut proteinases is involved in some types of insect resistance to *B. thuringiensis*. © 1994 Academic Press, Inc.

Crystalline inclusions of the bacterium *Bacillus thuringiensis* (Bt) contain proteins that, after ingestion, solubilization, and proteolytic processing, are toxic to a wide range of insects in the orders Lepidoptera, Coleoptera, and Diptera. Bt insecticidal proteins and their genes are classified according to their structures, antigenic properties, and activity spectra, and the group designated CryI are toxic to Lepidoptera (1). Inclusions generally consist of multiple protoxins, but Bt subspecies *kurstaki* HD-73 is unique in that it contains only one protoxin form, designated CryIA(c), with an apparent molecular mass of approximately 133 kDa.

Insect resistance to Bt was first demonstrated in *Plodia interpunctella*, where it was found to be stable and recessively inherited (4, 5). Subsequently, other resistant strains have been described (reviewed in 6). Because the mode of action of Bt is complex, a number of physiological or behavioral alterations could result in insect adaptation to Bt toxins. Physiological mechanisms proposed for the development of resistance involve primarily midgut receptor- or proteinase-mediated processes (7).

*Corresponding author. FAX: (913)537-5584.

Abbreviations: BApNA: α-N-benzoyl-DL-arginine ρ-nitroanilide; Bt: *Bacillus thuringiensis*; SDS-PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis.

0006-291X/94 $5.00
Copyright © 1994 by Academic Press, Inc.
All rights of reproduction in any form reserved.

Data supporting the development of resistance due to changes in midgut receptors have been reported for several lepidopteran species. Midgut toxin binding affinity was correlated to toxin susceptibility in *P. interpunctella* (8) and some other Lepidoptera (9-14), but no correlation was found in *Lymantria dispar* (15). In studies of one strain of *Heliothis virescens*, data suggested that a broad-spectrum resistance to multiple Bt toxins was not related to alterations in midgut receptors (16).

Because proteinases are involved in protoxin activation, enzymatic alterations that affect Bt activation could result in resistance. To date, proteinase-mediated mechanisms have not been identified in insect strains resistant to Bt, but proteinases have been proposed to influence target specificity of Bt toxins (17, 18). In addition, crystalline protoxin solubility, which is in part determined by midgut proteinases, can affect the potency of Bt endotoxins (19, 20).

We present data that demonstrate differences in the proteolytic processing of Bt protoxins by midgut extracts from Bt susceptible and resistant strains of *P. interpunctella*. Digestion of CryIA(c) protoxin by a strain resistant to Bt subsp. *entomocidus* was slower than that by the susceptible parent strain or a strain resistant to Bt subsp. *kurstaki*. Incomplete or slowed processing of Bt protoxins in the gut has the potential to reduce toxicity to the insect and lead to adaptation to this important microbial insecticide.

MATERIALS AND METHODS

Strains used in this study have been previously described (5, 22). They include: 1) Bt susceptible strain RC688^s, collected from a farm grain storage bin in Riley County, Kansas in 1988; 2) Bt resistant strain 198^r, derived from RC688^s that was selected for resistance using isolate HD-198 (Bt subsp. *entomocidus*); and 3) Bt resistant strain Dpl^r, also derived from RC688^s and selected for resistance using HD-1 (Dipel; Bt subsp. *kurstaki*).

Midguts from susceptible or resistant strains of *P. interpunctella* were obtained from late 4th instar larvae after chilling on ice for 30 min. The posterior and anterior ends of the larvae were removed, and the midguts were extracted with forceps and immediately submersed in ice-cold dissection buffer (200 mM Tris, pH 8.0, 20 mM $CaCl_2$). Midguts were homogenized in the same buffer (20 per 0.5 ml) in a 1.5 ml microcentrifuge tube on ice using a tissue grinder, followed by centrifugation at 15,000 x g for 5 min. The supernatant was divided into 0.025 ml aliquots and stored at -20°C.

Proteolytic activity was determined using the substrate α-N-benzoyl-DL-arginine ρ-nitroanilide (BApNA) (1 mg per ml) in dissection buffer. One unit of activity was defined as the amount of enzyme required to produce an increase in absorbance of one in one minute when monitored at 412 nm.

CryIA(c) protoxin crystals were obtained from Bt subsp. *kurstaki* HD-73. The culture was grown on glucose-yeast extract-salts medium (23) at 30°C for 2-3 days with agitation until sporulation was complete. Crystals were purified by density gradient centrifugation (24), washed with water, and solubilized at approximately 10 mg/ml in 2x protoxin buffer (100 mM sodium carbonate, pH 10.0, 800 mM sodium chloride, 10 mM ethylenediamine tetraacetic acid, 20 mM β-mercaptoethanol), vortexing occasionally while incubating on ice for several hours. Glycerol was added 1:1 (v:v), and the solubilized protoxin was concentrated in a centrifugal filter (Amicon) with a molecular weight cutoff of 30 kDa.

Purified protoxin (10μg) was incubated with a normalized amount of midgut extract or tryptic BApNA activity at either room temperature or 37°C in a total volume of 20 μl. Dissection buffer was used for incubations at pH 8.0, while 100 mM sodium carbonate was used for those at pH 10.0. To stop the reaction, samples were heated at 95°C for 5 min, cooled, mixed with 20 μl of 2x sample buffer containing β-mercaptoethanol, and again heated at 95°C for 5 min. Without heating prior to the addition of sodium dodecyl sulfate (SDS)-containing buffer, non-specific proteolysis resulted, as has been previously reported for HD-1 protoxins in SDS-containing solutions (25).

Samples were subjected to SDS polyacrylamide gel electrophoresis (SDS PAGE) according to Laemmli (26). Protoxin activation to toxin was monitored using either 7% single percentage or 4-12% gradient gels that were stained with 0.1% Coomassie brilliant blue.

Protein concentration was determined by the dye-binding method of Bradford (27). Mark-12 molecular mass markers, gradient gels, and electrophoresis buffers were from NOVEX. Porcine trypsin (Type IX) was from Sigma, and all other chemicals were obtained from BioRad, Fisher, or Sigma.

RESULTS

The toxicities (LD_{50} values) of three Bt protoxins, CryIA(b), CryIA(c), and CryIC, to a Bt susceptible *P. interpunctella* strain, designated RC688^s, and two resistant strains, one selected for resistance to Bt subsp. *kurstaki* (HD-1) and designated Dpl^r, and the other selected for resistance to Bt subsp. *entomocidus* (HD-198) and designated 198^r, are presented in Table 1. The two resistant strains were most resistant to CryIA(c) followed by CryIA(b). The Dipel-resistant strain was not significantly resistant to CryIC, whereas the *entomocidus*-resistant strain was about 6-fold resistant.

Since trypsin-like enzymes are probably involved in protoxin activation, proteolytic enzyme activity was compared between larval midgut extracts of the three *P. interpunctella* strains, using the trypsin synthetic substrate BApNA (Table 2). The lowest level of enzyme activity was observed in resistant strain 198^r. Strains RC688^s and Dpl^r exhibited approximately the same level of BApNA-hydrolyzing specific activity, but that from strain 198^r was about five-fold lower.

In order to determine if the differences in BApNA proteolytic activity correlated with differences in Bt protoxin activation, the proteins resulting from incubations containing solubilized CryIA(c) protoxin and midgut extracts or porcine trypsin were analyzed using SDS-PAGE. After normalizing proteolytic activity using BApNA as the substrate, proteolysis of CryIA(c) protoxin at pH 8.0 by midgut extracts or porcine trypsin in two hour incubations was compared (Fig. 1). One mU of midgut enzymes from the Bt susceptible strain (RC688^s) digested the protoxin to an approximately 61 kDa protein, whereas digestion by trypsin resulted in a slightly larger protein of about 63 kDa. Extracts from Dpl^r processed the protoxin similar to those from RC688^s. Extracts from 198^r, however, produced few low molecular mass proteins, even with five mU of enzyme activity. Proteins larger than 63 kDa, including the 133 kDa

Table 1. LD_{50} values of three *Bacillus thuringiensis* protoxins for three strains of *Plodia interpunctella*[1]

Strain	CryIA(b)	CryIA(c)	CryIC
RC688^s	0.1	0.3	0.1
Dpl^r	12.3	719.9	0.2
198^r	1.3	38.4	0.6

[1]Adapted from McGaughey and Johnson (32). Microgram protoxin per larva using bioassay according to Johnson et al. (35).

Table 2. Comparison of the proteolytic activities of midgut extracts from a Bt susceptible strain, RC688^s, and two Bt resistant strains, 198^r and Dpl^r, of *Plodia interpunctella*, using the synthetic substrate BApNA

Strain	Specific Activity (ΔA_{412}/mg protein/sec)[1]
RC688^s	0.94 ± 0.07 (a)
Dpl^r	1.05 ± 0.12 (a)
198^r	0.21 ± 0.06 (b)

[1]Values given are the means ± s. e. (n = 4). Data with the same letter were not statistically different ($\alpha = 0.05$) as determined by Tukey analysis.

protoxin, were observed in the 198^r digests. Since increased levels of protein were necessary in order to normalize 198^r midgut activity, it was difficult to distinguish protoxin products from midgut proteins in digests containing higher levels of gut activity. We estimate that, on a gut equivalent basis, the 198^r extract digested CryIA(c) more than an order of magnitude slower than extracts from the other two strains. The same experiment, performed at a pH comparable to the midgut physiological pH (10.0), yielded similar results (data not shown). However, since trypsin hydrolysis of CryIA(c) was much slower at pH 10, the number of trypsin activity units required to process the protoxin was ten-times greater than that of RC688^s or Dpl^r.

With 2 mU of midgut activity and an increased time of incubation from 10 to 1000 min in pH 10 buffer, 198^r gut extracts processed the protoxin to an approximately 61 kDa protein (Fig. 2). A range of proteins of intermediate size was observed after 10 min incubations with RC688^s and Dpl^r extracts. Proteins of approximately 62-63 kDa were prominent after 100 min incubations with RC688^s and Dpl^r extracts. After 1000 min, proteolysis of CryIA(c) by RC688^s or Dpl^r extracts was completed, and no proteins of molecular masses greater than 61 kDa were present. In 10 and 100 minute digestions containing 198^r midgut enzymes, it is apparent that the

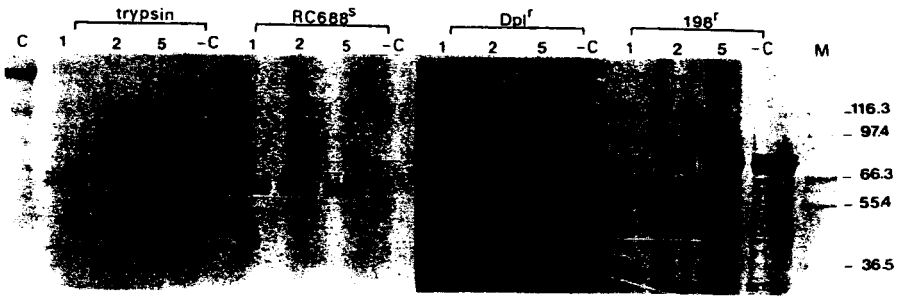

Fig 1. SDS-PAGE (4-12%) of incubations at pH 8.0 containing 10 μg of CryIA(c) and increasing activities (as indicated in mUnits) of trypsin or *Plodia interpunctella* larval midgut extracts from strains RC688^s, Dpl^r, and 198^r. Samples were incubated for 2 h at 37°C. M: molecular mass markers, as indicated on the right; C: CryIA(c) protoxin only; -C: enzyme only, at the highest level tested.

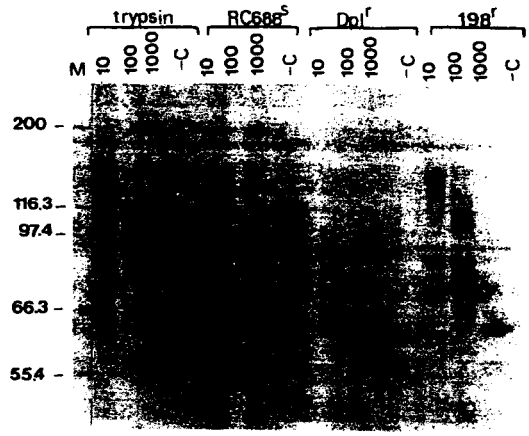

Fig. 2. SDS-PAGE (7%) of incubations at pH 10.0 containing 10 μg of CryIA(c) and 20 mUnits of trypsin or 2.0 mUnits of *Plodia interpunctella* larval midgut extracts. Samples were incubated at room temperature for the time (in minutes) indicated. M: molecular mass markers, as indicated on the left; -C: enzyme only, incubated overnight.

protoxin is processed at a slower rate when compared to the other enzyme sources. Although 198^r midgut extracts produced a 61-63 kDa protein after the longest incubation time, minor proteins of approximately 65-80 kDa were still present.

DISCUSSION

Larval gut proteinases play a critical role in the proteolytic activation of Bt protoxins. Results from our study demonstrate that incomplete and slowed processing of protoxin CryIA(c) by midgut extracts from Bt resistant strain 198^r occurred at high enzyme:protoxin ratios, at two different pH values, and after long incubation times when compared to extracts from the corresponding susceptible parent strain, $RC688^s$, another resistant stain, Dpl^r, or a mammalian serine proteinase, porcine trypsin.

Lepidopteran insects have highly alkaline conditions in their midguts, and serine proteinases constitute the major digestive enzymes in *P. interpunctella* (21, 28, 29). Hydrolysis of BApNA was used as a normalizing measure of overall midgut proteolytic activity in the strains, because some proteinases involved in protoxin activation have properties of trypsin-like enzymes (30, 31). BApNA specific activity of 198^r was approximately five-fold lower than that of $RC688^s$ or Dpl^r. Trypsin or Bt resistant midguts were adjusted to a level of BApNA hydrolyzing activity equivalent to that in the susceptible strain. Using pH 8.0 buffer, this normalization resulted in the use of less total protein in the case of trypsin, more in the case of 198^r midgut extracts, and approximately the same amount of protein in the case of Dpl^r when compared to the level of protein used in $RC688^s$ extracts.

Digestion of CryIA(c) by midgut extracts from Bt resistant strain Dpl^r was similar to the processing by extracts from the susceptible strain $RC688^s$. The final product was an

approximately 61 kDa protein, which is within the reported range of proteolytic products reported for HD-73 protoxin (2, 3). In a 2 hour, 37°C incubation containing relatively high levels of enzyme activity, 198^r generated none of the 61 kDa product from the protoxin. Porcine trypsin digestion of CryIA(c) produced a slightly larger (~63 kDa) protein. A difference in processing of CryIA(c) by bovine trypsin and gut juice from *Choristoneura fumiferana* was previously reported by Milne and Kaplan (31). Since the final products of CryIA(c) processing by gut enzymes versus purified trypsin differ in size, then the insect gut enzymes have properties unique from those of mammalian trypsin.

Previously, we reported no difference in the processing of Bt protoxins HD-1, HD-133, and HD-144 by midgut extracts from two other strains of *P. interpunctella*, one susceptible (UE-343) and the other resistant (UE-343R) to Bt subsp. *kurstaki* (HD-1) and suggested that proteinases did not play a role in the resistance mechanism of Bt subsp. *kurstaki* (21). Our present results, obtained using another *kurstaki*-resistant strain, Dpl^r, are consistent with the earlier findings. A receptor-mediated resistance mechanism has been demonstrated for UE-343R (8). However, it should not be assumed that all *P. interpunctella* resistance to Bt will be receptor-mediated. In this study, we observed a difference in protoxin processing between two strains, one resistant to Bt subsp. *entomocidus* and the other to Bt subsp. *kurstaki*. Our results suggest that some strains of Bt may elicit a proteinase-mediated mechanism of resistance in *P. interpunctella*. Strain Dpl^r has a higher LD_{50} for CryIA(b) and CryIA(c) than 198^r, but the opposite is true for CryIC. The inclusion body from Bt subsp. *entomocidus* isolate HD-198 contains protoxins CryIA(a), CryIA(b), CryIC, and CryID, while that from *kurstaki* isolate HD-1 contains CryIA(a), CryIA(b), CryIA(c), CryIIA, and CryIIB (32). At present, no correlation can be made between the specific protoxin composition used in resistance selection and the resulting mechanism of resistance.

The reduced ability of 198^r gut enzymes to produce protein products similar to those obtained with gut enzymes from a susceptible strain could result in a novel mechanism of *P. interpunctella* resistance to Bt. If the protoxin must be activated rapidly in order to interact with gut receptors before elimination from the gut, this slowed rate of activation would reduce the concentration of active toxin and effectively decrease the toxicity of Bt. In the 198^r strain, specific proteinases may be involved in Cry protoxin activation that are less abundant, less active due to an alteration in structure or catalytic activity, or inhibited. The identification, purification and characterization of Bt protoxin processing enzymes and their inhibitors, together with midgut factor(s) that alter Bt processing, will be important in understanding why strains such as 198^r are not as efficient in activating the protoxin, and whether a slower rate of protoxin activation is involved in the mechanism of insect resistance to Bt.

Altered processing by 198^r gut extracts was observed with not only CryIA(c), but also with two other protoxins, CryIA(b) and CryIC (Oppert et al., unpublished data). This result indicates that enzyme-mediated resistance could be broadly-based and a potentially serious problem in field applications of Bt. If slowed protoxin activation is linked to resistance, recombinant genes coding for activated toxins instead of protoxins would be a possible method to circumvent this type of resistance mechanism.

We are not aware of other reports that describe a possible proteinase involvement in insect resistance to Bt. However, Bai et al. (33) suggested that differences in proteinase levels in the

midguts of three lepidopteran species influence their susceptibility to Bt protoxins. Dai and Gill (34) indicated that the dipteran-active CryIVD protoxin was processed more slowly by a midgut extract from a resistant strain of *Culex quinquefasciatus* than by an extract from a susceptible strain, but they did not provide supporting data.

Conflicting data regarding *Heliothis* resistance to Bt has led investigators to search for resistance mechanisms other than those that are midgut receptor-mediated (14, 16). Our results suggest that altered proteolytic processing is another resistance mechanism, but more data are needed to establish a causal relationship between protoxin processing and insect resistance to Bt. If altered processing proves to be a second mechanism of resistance, a more detailed characterization of its biochemical basis will not only facilitate an evaluation of its occurrence in field and laboratory-selected resistant strains, but will also lead to a better understanding of the influence of protoxin processing on the susceptibility of insects to different Bt endotoxins.

ACKNOWLEDGMENTS

We would like to express our appreciation to Rich Hammel, Mary Alice Schrick, and Cris Oppert for excellent technical assistance. Dipel is a registered trademark of Abbott Laboratories, Chicago, IL. This research was a cooperative investigation between the Agricultural Research Service of the USDA, the Kansas Agricultural Experiment Station (contribution #94-236-J) and Novo Nordisk Entotech and was supported in part by an ARS Postdoctoral Fellowship (BO) and by USDA grant #93-37302-9570 (BO). Mention of a proprietary product does not constitute a recommendation or endorsement by the USDA.

REFERENCES

1. Höfte, H. and Whiteley, H. R. (1989) *Microbiol. Rev.* 53: 242-255.
2. Choma, C. T., Surewicz, W. K., Carey, P. R., Pozsgay, M., Raynor, T., and Kaplan, H. (1990) *Eur. J. Biochem.* 189: 523-527.
3. Ogiwara, K., Indrasith, L. S., Asano, S., and Hori, H. (1992) *J. Invertebr. Pathol.* 60: 121-126.
4. McGaughey, W. H. (1985) *Science* 229: 193-195.
5. McGaughey, W. H. and Beeman, R. W. (1988) *J. Econ. Entomol.* 81: 28-33.
6. McGaughey, W. H. and Whalon, M. E. (1992) *Science* 258: 1451-1455.
7. Gill, S. S., Cowles, E. A., and Pietrantonio, P. V. (1992) *Annu. Rev. Entomol.* 37: 615-36.
8. Van Rie, J., McGaughey, W. H., Johnson, D. E., Barnett, B. D., and Van Mellaert, H. (1990b) *Science* 247: 72-74.
9. Haider, M. Z. and Ellar, D. J. (1987) *Biochem. J.* 248: 197-201.
10. Hofmann, C. H., Vanderbruggen, H., Höfte, J., Van Rie, S., Jansens, S., and Van Mellaert, H. (1988) *Proc. Natl. Acad. Sci. USA* 85: 7844-7848.
11. Van Rie, J., Jansens, S., Höfte, H., Degheele, D., and Van Mellaert, H. (1989) *Eur. J. Biochem.* 186: 239-247.
12. Van Rie, J., Jansens, S., Höfte, H., Degheele, D., and Van Mellaert, H. (1990a) *Appl. Environ. Microbiol.* 56: 1378-1385.
13. Ferre, J. M., Real, M. D., Van Rie, J., Jansens, S., and Peferoen, M. (1991) *Proc. Natl. Acad. Sci. USA* 88: 5119-5123.
14. MacIntosh, S. C., Stone, T. B., Jokerst, R. S., and Fuchs, R. L. (1991) *Proc. Natl. Acad. Sci. USA* 88: 8930-8933.
15. Wolfersberger, M. G. (1990) *Experientia* 46: 475-477.
16. Gould, F., Martinez, Ramirez, A., Anderson, A., Ferre, J., Silva, F. J., and Moar, W. J. (1992) *Proc. Natl., Acad. Sci. USA* 89: 7986-7990.
17. Haider, M. Z., Knowles, B. H., and Ellar, D. J. (1986) *Eur. J. Biochem.* 156: 531-540.
18. Lecadet, M. M., and Martouret, D. (1987) *J. Invertebr. Pathol.* 49: 37-48.
19. Jaquet, F., Hutter, R., and Luthy, P. (1987) *Appl. Environ. Microbiol.* 53: 500-504.

20. Aronson, A. I., Han, E. -S., McGaughey, W., and Johnson, D. (1991) *Appl. Environ. Microbiol.* 57: 9481-986.
21. Johnson, D. E., Brookhart, G. L., Kramer, K. J., Barnett, B. D., and McGaughey, W. H. (1990) *J. Invertebr. Pathol.* 55: 235-244.
22. McGaughey, W. H. and Johnson, D. E. (1992) *J. Econ. Entomol.* 85: 1594-1600.
23. Nickerson, K. W., Julian, G. St., and Bulla, Jr., L. A. (1974) *Appl. Microbiol.* 28: 129-132.
24. Ang, B. J. and Nickerson, K. W. (1978) *Appl. Environ. Microbiol.* 36: 625-626.
25. Inagaki, S., Miyasono, M., Ishiguro, T., Takeda, R., and Harashi, Y. (1992) *J. Invertebr. Pathol.* 60: 64-68.
26. Laemmli, U. K. (1970) *Nature (London)* 227: 680-685.
27. Bradford, M. (1976) *Analyt. Biochem.*, 72: 248-254.
28. Chen, M. S., Johnson, B., Wen, L., Muthukrishnan, S., Kramer, K. J., Morgan, T. D., and Reeck, G. R. (1992) *Protein Express. Purification* 3: 41-49.
29. Christeller, J. T., Laing, W. A., Markwick, N. P., and Burgess, E. P. J. (1992) *Insect Biochem. Molec. Biol.* 22: 735-746.
30. Lecadet, M. M., and Dedonder, R. (1967) *J. Invertebr. Pathol.* 9: 310-321.
31. Milne, R., and Kaplan, H. (1993) *Insect Biochem. Molec. Biol.* 23: 663-673.
32. McGaughey, W. H. and Johnson, D. E. *J. Econ. Entomol.*, (in press).
33. Bai, C., Yi, S. X., and Degheele, D. (1990) *Meded. Fac. Landbouwwet., Rijksuniv. Gent* 55: 519-525.
34. Dai, S. and Gill, S. (1993) *Insect Biochem. Molec. Biol.* 23: 273-283.
35. Johnson, D. E., McGaughey, W. H., and Barnett, B. D. (1991) *J. Invertebr. Pathol.* 57: 159-165.