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SHORT COMMUNICATION

INHIBITION OF POTASSIUM-GRADIENT-DRIVEN PHENYLALANINE UPTAKE IN LARVAL *LYMANTRIA DISPAR* MIDGUT BY TWO *BACILLUS THURINGIENSIS* DELTA-ENDOTOXINS CORRELATES WITH THE ACTIVITY OF THE TOXINS AS GYPSY MOTH LARVICIDES

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During sporulation, *Bacillus thuringiensis* produces parasporal inclusions with insecticidal activity. The parasporal inclusions produced by most subspecies of *B. thuringiensis* are active only against the larvae of a few lepidopteran insects. Lepidopteran-active parasporal inclusions are usually bipyramidal crystals composed of one or more 130×10^3–140×10^3 M_r polypeptides. These polypeptides are designated as protoxins. The complete insecticidal activity of each protoxin resides in a 55×10^3–70×10^3 M_r protease-resistant toxin which results from solubilization and partial digestion of the crystals in the larval midgut (Aronson *et al.* 1987).

The target of lepidopteran-active *B. thuringiensis* toxins is the brush-border membrane of larval lepidopteran midgut (Lüthy *et al.* 1986). This insect cell membrane contains specific high-affinity receptors, of unknown normal physiological function, for *B. thuringiensis* toxins (Hofmann *et al.* 1988*a*; VanRie *et al.* 1990*b*). After binding to the membrane receptors, the toxin or the toxin–receptor complex forms a pore that is the primary lesion in the mode of action of these cytolytic toxins (Wolfersberger, 1990*a*). In many cases, the larvicidal activity of a *B. thuringiensis* toxin has been found to correlate directly with the concentration and/or affinity of receptors for the toxin in the larval insect midgut (Hofmann *et al.* 1988*b*; VanRie *et al.* 1989, 1990*b*). These observations led to the suggestion that receptor binding was the primary determinant of a toxin's larvicidal activity (VanRie *et al.* 1990*a*). However, toxin alone at very high concentrations is able to form pores in artificial lipid membranes that contain no receptors (Slatin *et al.* 1990) and certain toxins are specifically bound with high affinity by brush-border membrane vesicles (BBMV) prepared from midguts of insects against which the toxins show little or no larvicidal activity (VanRie *et al.* 1990*a*; Wolfersberger, 1990*b*). The latter observation, in particular, favors the suggestion (Wolfersberger, 1990*b*) that the ability of a toxin to form a membrane pore is a more important determinant of its larvicidal activity than its binding characteristics.

Key words: amino acid, brush-border membrane, cotransport, insecticidal protein, *Lymantria dispar*.

The uptake of luminal amino acids by larval lepidopteran midgut cells is mainly by symport with potassium ions (Hennigan and Wolfersberger, 1989). Sacchi *et al.* (1986) were the first to use inhibition of ion-gradient-driven amino acid uptake as a measure of the ability of *B. thuringiensis* toxins to increase the potassium permeability of larval *Pieris brassicae* midgut brush-border membranes. Subsequently, these methods were used to show that certain *B. thuringiensis* toxins increased both the sodium and potassium permeability of larval *Manduca sexta* midgut brush-border membranes (Wolfersberger, 1989; Hendrickx *et al.* 1990).

The studies reported in this communication demonstrate the presence of potassium/phenylalanine symport in larval *Lymantria dispar* midgut brush-border membrane. They also show that this process is inhibited to different extents by two closely related *B. thuringiensis* toxins. The activity of the two toxins as inhibitors of potassium/amino acid symport, unlike the binding of the toxins, correlates directly with their potency as gypsy moth larvicides.

Fifth- and sixth-instar *Lymantria dispar* larvae were used in all experiments. *L. dispar* eggs were obtained from the USDA Forest Service Experiment Station (Hamden, CT). Larval diet was purchased from ICN Biochemicals (Cleveland, OH). The larvae were reared to experimental size at 25°C with constant light.

Larvae were chilled in crushed ice for 15–25 min. Chilled larvae were transected immediately behind the fourth pair of abdominal appendages and again immediately behind the first pair of thoracic appendages. The integument was cut open and spread apart, exposing the midgut. The midgut was rinsed with ice-cold 0.3 mol l^{-1} mannitol in 10 mmol l^{-1} Tris–HCl, pH 8 (mannitol solution), and the tracheoles attaching it to the integument were severed. The midgut was cut longitudinally and opened to form a flat sheet. Midgut contents were rinsed away with ice-cold mannitol solution. The peritrophic membrane and the Malpighian tubules were removed using forceps. The isolated midgut was rinsed with ice-cold mannitol solution, gently blotted and weighed. Isolated midguts were either used immediately for vesicle preparation or placed in a vial with a small amount of mannitol solution and frozen by immersing the vial in liquid nitrogen. Frozen midguts were stored at −80°C until used.

Brush-border membrane vesicles (BBMV) were prepared from either fresh or frozen midguts by the differential magnesium precipitation method of Biber *et al.* (1981), as modified and described by Wolfersberger *et al.* (1987). The protein concentrations of BBMV preparations and of toxin solutions were determined by the method of Bradford (1976) using a Bio-Rad (Richmond, CA) kit with bovine serum albumin as standard.

Bacillus thuringiensis subspecies *kurstaki* strain HD-73 was kindly provided by Dr Howard Dulmage (USDA Cotton Insects Research Unit, Brownsville, TX). *Bacillus thuringiensis* subspecies *kurstaki* strain HD1-9 was kindly provided by Dr Bruce Carlton (Ecogen, Inc., Langhorne, PA). Each of these strains contains a single gene for a lepidopteran-active insecticidal crystal protein (Adang *et al.* 1985; Yamamoto *et al.* 1988). The insecticidal crystal protein gene of strain HD1-9 is classified as type *CryIA(b)* and encodes a 130.6×10^3 M_r polypeptide. The

insecticidal crystal protein gene of strain HD-73 is classified as type *CryIA(c)* and encodes a 133.3×10^3 M_r polypeptide (Höfte and Whiteley, 1989).

The growth of cultures, isolation of parasporal crystals and preparation of crystal protein solutions were as described by Jaquet *et al.* (1987). Trypsin digestion of crystal proteins and purification of the resulting toxins were performed according to the methods of Hofmann and Luethy (1986), as modified and described by Wolfersberger (1989). Both purified toxins gave a single band of approximately 67×10^3 M_r on SDS–PAGE (Laemmli, 1970).

Amino acid uptake measurements were performed at 25°C using a rapid filtration technique (Wolfersberger *et al.* 1987). BBMV were resuspended in 0.1 mol l^{-1} mannitol, 10 mmol l^{-1} Tris–Hepes (pH 8) at a protein concentration of approximately 6 mg ml^{-1} using a syringe equipped with a 3.8 cm long 22 gauge needle. Toxins (8.7 pmol toxin per mg BBMV) were added to BBMV suspensions as small (2% of the volume of the BBMV suspension) samples from stock solutions in 50 mmol l^{-1} sodium carbonate buffer, pH 9.5. An equal amount of 50 mmol l^{-1} sodium carbonate buffer, pH 9.5, was added to control BBMV suspensions. BBMV were preincubated with toxins for 30 min before use in uptake measurements. Uptake determinations were started by mixing a sample of BBMV suspension with an equal-volume sample of radioactive phenylalanine solution (1 mmol l^{-1} phenylalanine, 150 mmol l^{-1} KSCN, 100 mmol l^{-1} mannitol, 10 mmol l^{-1} Tris–Hepes, pH 8). At selected times, uptake was stopped by diluting the mixtures with 100 volumes of ice-cold 0.15 mol l^{-1} NaCl in 1 mmol l^{-1} Tris–Hepes (pH 7) and immediately filtering the dilute suspension through a prewetted cellulose nitrate filter (0.65 μm pore size, Sartorius no. 11305, Hayward, CA). All filters were washed twice with 4 ml of ice-cold 0.15 mol l^{-1} NaCl in 1 mmol l^{-1} Tris–Hepes (pH 7), put into a vial with 10 ml of scintillation cocktail (ScintiVerse BD, Fisher Scientific, Pittsburgh, PA) and counted in a liquid scintillation counter (model 2000CA, Packard Instrument, Downers Grove, IL). Samples of radioactive phenylalanine solutions used in each experiment were spotted on filters and counted. These standard counts were used to convert sample counts per minute into moles of phenylalanine (Hennigan and Wolfersberger, 1989).

L[ring-2,6-^{3}H]phenylalanine, 60 Ci mmol l^{-1}, was purchased from Dupont-NEN (Boston, MA). Dithiothreitol, L-phenylalanine and trypsin (type III) were from Sigma (St Louis, MO). Dextran sulfate (sodium salt) and Sephacryl S-300 (superfine) were from Pharmacia (Uppsala, Sweden). Polyethylene glycol 6000 was from Serva (Heidelberg, FRG). All other chemicals were analytical grade products from either Fisher or Mallinckrodt (St Louis, MO).

The time courses of L-phenylalanine uptake by larval *L. dispar* midgut BBMV in the presence of an initial KSCN gradient as well as in the absence of KSCN are shown in Fig. 1. In the presence of the initial salt gradient there is a large transient accumulation of phenylalanine, whereas in the absence of KSCN phenylalanine uptake was only equilibrative. KSCN-gradient-driven phenylalanine accumulation reached a maximum between 30 s and 2 min. Between 2 min and 60 min the vesicle

phenylalanine concentration gradually declined to its equilibrium value. In the absence of KSCN, vesicle phenylalanine concentration gradually increased to its equilibrium value over a period of approximately 6 min. Phenylalanine uptake, actually binding to the vesicles, at zero time was determined by extrapolation to an infinite osmotic gradient of a plot of equilibrium uptake *versus* osmolarity of the vesicle contents divided by osmolarity of the external solution (Hennigan and Wolfersberger, 1989).

Initial experiments with KSCN gradients showed that maximum L-phenylalanine uptake always occurred between 30 s and 2 min and that equilibrium distribution of phenylalanine between the intravesicular and extravesicular volumes was always reached within 60 min. Therefore, sampling times of 90 s and 60 min were chosen for studies of the effects of *B. thuringiensis* toxins on KSCN-gradient-driven phenylalanine accumulation by *L. dispar* BBMV. The results of these studies are presented in Table 1.

Maximum KSCN-gradient-dependent accumulation of L-phenylalanine by BBMV preincubated with only carbonate buffer was essentially the same as that in untreated BBMV (Fig. 1). Maximum phenylalanine accumulation by BBMV preincubated with HD-73 toxin was approximately 57 % of that by BBMV that had not been preincubated with toxin. There was no significant phenylalanine accumulation by BBMV preincubated with HD1-9 toxin (Table 1).

Amino acid/potassium cotransport systems have been identified in the midgut brush-border membrane of several lepidopteran larvae (Hanozet *et al.* 1980; Giordana *et al.* 1982; Wolfersberger *et al.* 1987; Hennigan and Wolfersberger, 1989). The large, approximately four times the equilibrium value, transient

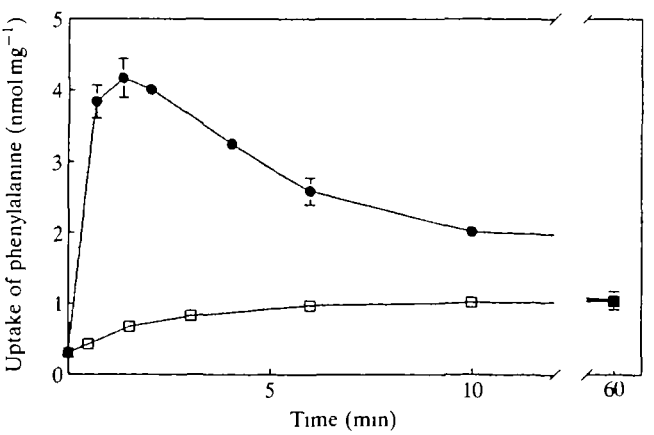

Fig. 1. Time course of L-phenylalanine uptake by brush-border membrane vesicles from larval *Lymantria dispar* midgut. L-Phenylalanine uptake by untreated vesicles in the absence of KSCN (□) and in the presence (●) of an initial KSCN gradient (75 mmol l^{-1} outside and 0 mmol l^{-1} inside). Each point represents the mean of triplicate determinations. When S.D. error bars are not shown they were smaller than the symbol used.

Table 1. *Effects of* Bacillus thuringiensis *toxins on ion-gradient-dependent phenylalanine accumulation by brush-border membrane vesicles from larval* Lymantria dispar *midgut*

Toxin	Accumulation (nmol mg^{-1})
None	2.80±0.13
HD-73	1.61±0.13
HD1-9	0.02±0.06

All uptake mixtures contained 3.1 mg ml^{-1} of BBMV, 0.5 mmol l^{-1} phenylalanine, 75 mmol l^{-1} KSCN, 100 mmol l^{-1} mannitol and 10 mmol l^{-1} Tris–Hepes, pH 8.

BBMV were preincubated with toxin for 30 min before use in uptake experiments.

The reported accumulation is the difference between vesicular phenylalanine uptake at 90 s and that at 1 h (mean±S.E.; N=3).

accumulation of L-phenylalanine in the presence of an initial KSCN gradient (Fig. 1) is clear evidence for amino acid/potassium cotransport in larval gypsy moth midgut.

Sacchi *et al.* (1986) showed that *B. thuringiensis* δ-endotoxin inhibited, in a dose-dependent manner, amino acid/potassium cotransport by BBMV prepared from larval *Pieris brassicae* midgut. They concluded that this inhibition was not due to a direct interaction of the toxin with the cotransporter but rather by the toxin providing an alternative path, a toxin-induced pore, by which the potassium could enter the BBMV and thereby dissipate the initial gradient without causing a large transient accumulation of amino acid. Wolfersberger (1989) and Hendrickx *et al.* (1990) obtained similar results for the effects of *B. thuringiensis* toxins on ion-gradient-driven amino acid uptake by BBMV prepared from larval *Manduca sexta* midgut. The results presented in Table 1 show that *B. thuringiensis* toxins also inhibit ion-gradient-driven amino acid uptake by BBMV prepared from larval *L. dispar* midgut.

The toxin concentrations used in the experiments reported in Table 1 are more than twice the concentration of specific binding sites for HD1-9 or HD-73 toxins on *L. dispar* BBMV (Wolfersberger, 1990*b*), yet the vesicles that were preincubated with HD-73 toxin accumulated more than half as much phenylalanine as the untreated vesicles, while the vesicles that were preincubated with HD1-9 toxin showed no significant phenylalanine accumulation. Assuming that *B. thuringiensis* toxins inhibit ion-gradient-dependent amino acid/potassium symport in larval *L. dispar* midgut by the same mechanism as they do in *P. brassicae* and *M. sexta* midgut, the extent of inhibition, in the presence of excess toxin, should be a measure of the efficiency of pore formation by the toxin or the toxin–receptor complex. Both HD1-9 and HD-73 toxins appear to be bound by the same receptor in gypsy moth midgut (Wolfersberger, 1990*b*). HD-73 toxin is bound with almost 10-fold greater affinity than HD1-9 toxin, but HD1-9 toxin is an approximately 400 times more potent gypsy moth larvicide than is HD-73 toxin (Wolfersberger,

1990*b*). These observations could be explained if bound HD1-9 toxin was much more effective than bound HD-73 toxin in forming the membrane pore that is critical to the cytolytic mode of action of *B. thuringiensis* δ-endotoxins. The results reported in Table 1 are consistent with this explanation.

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References

ADANG, M. J., STAVER, M. J., ROCHELEAU, T. A., LEIGHTON, J., BARKER, R. F. AND THOMPSON, D. V. (1985). Characterized full-length and truncated plasmid clones of the crystal protein of *Bacillus thuringiensis* subsp. *kurstaki* HD-73 and their toxicity to *Manduca sexta*. *Gene* **36**, 289–300.

ARONSON, A. I., BECKMAN, W. AND DUNN, P. (1987). *Bacillus thuringiensis* and related insect pathogens. *Microbiol. Rev.* **50**, 1–24.

BIBER, J., STEIGER, B., HAASE, W. AND MURER, H. (1981). A high yield preparation for rat kidney brush border membranes. Different behavior of lysosomal markers. *Biochim. biophys. Acta* **647**, 169–176.

BRADFORD, M. M. (1976). A rapid sensitive method for the quantitation of protein utilizing the principle of dye–protein binding. *Analyt. Biochem.* **72**, 248–254.

GIORDANA, B., SACCHI, V. F. AND HANOZET, G. M. (1982). Intestinal amino acid absorption in lepidopteran larvae. *Biochim. biophys. Acta* **692**, 81–88.

HANOZET, G. M., GIORDANA, B. AND SACCHI, V. F. (1980). K-dependent phenylalanine uptake in membrane vesicles isolated from the midgut of *Philosamia cynthia* larvae. *Biochim. biophys. Acta* **596**, 481–486.

HENDRICKX, K., DELOOF, A. AND VAN MELLAERT, H. (1990). Effects of *Bacillus thuringiensis* δ-endotoxin on the permeability of brush border membrane vesicles from tobacco hornworm (*Manduca sexta*) midgut. *Comp. Biochem. Physiol.* **95**C, 241–245.

HENNIGAN, B. B. AND WOLFERSBERGER, M. G. (1989). Intestinal amino acid absorption in tobacco hornworm larvae is stimulated by potassium and sodium but not rubidium or lithium. *Archs Insect Biochem. Physiol.* **11**, 21–28.

HÖFTE, H. AND WHITELEY, H. R. (1989). Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiol. Rev.* **53**, 242–255.

HOFMANN, C. AND LUETHY, P. (1986). Binding and activity of *Bacillus thuringiensis* delta-endotoxin to invertebrate cells. *Arch Mikrobiol.* **146**, 7–11.

HOFMANN, C., LUETHY, P., HUETTER, R. AND PLISKA, V. (1988*a*). Binding of the delta-endotoxin from *Bacillus thuringiensis* to brush border membrane vesicles of the cabbage butterfly (*Pieris brassicae*). *Eur. J. Biochem.* **173**, 85–91.

HOFMANN, C., VANDERBRUGGEN, H., HOEFTE, H., VANRIE, J., JANSENS, S. AND VAN MELLAERT, H. (1988*b*). Specificity of *Bacillus thuringiensis* δ-endotoxins is correlated with the presence of high-affinity binding sites in the brush border membrane of target insect midguts. *Proc. natn. Acad. Sci. U.S.A.* **85**, 7844–7848.

JAQUET, F., HUETTER, R. AND LUETHY, P. (1987). Specificity of *Bacillus thuringiensis* delta-endotoxin. *Appl. environ. Microbiol.* **53**, 500–504.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

LÜTHY, P., JAQUET, F., HOFMANN, C., HUBER-LUKAC, M. AND WOLFERSBERGER, M. G. (1986). Pathogenic actions of *Bacillus thuringiensis* toxin. *Zentralbl. Bakt. Mikrobiol. Hyg. I. Suppl.* **15**, 161–166.

SACCHI, V. F., PARENTI. P., HANOZET, G. M., GIORDANA, B., LUETHY, P. AND WOLFERSBERGER, M. G. (1986). *Bacillus thuringiensis* toxin inhibits K-gradient-dependent amino acid transport across the brush border membrane of *Pieris brassicae* midgut cells. *FEBS Lett.* **204**, 213–218.

SLATIN, S. L., ABRAMS, C. K. AND ENGLISH, L. (1990). Delta-endotoxins form cation-selective channels in planar lipid bilayers. *Biochem. biophys. Res. Commun.* **169**, 765–772.

VANRIE, J., JANSENS, S., HOEFTE, H., DEGHEELE, D. AND VAN MELLAERT, H. (1989). Specificity of *Bacillus thuringiensis* delta-endotoxins: importance of specific receptors on the brush border membrane of the midgut of target insects. *Eur. J. Biochem.* **186**, 239–247.

VANRIE, J., JANSENS, S., HOEFTE, H., DEGHEELE, D. AND VAN MELLAERT, H. (1990*a*). Receptors on the brush border membrane of the insect midgut as determinants of the specificity of *Bacillus thuringiensis* δ-endotoxins. *Appl. environ. Microbiol.* **56**, 1378–1385.

VANRIE, J., MCGAUGHEY, W. H., JOHNSON, D. E., BARNETT, B. D. AND VAN MELLAERT, H. (1990*b*). Mechanism of insect resistance to the microbial insecticide *Bacillus thuringiensis*. *Science* **247**, 72–74.

WOLFERSBERGER, M. G. (1989). Neither barium nor calcium prevents the inhibition by *Bacillus thuringiensis* δ-endotoxin of sodium or potassium gradient-dependent amino acid accumulation by tobacco hornworm midgut brush border membrane vesicles. *Archs Insect Biochem. Physiol.* **12**, 267–277.

WOLFERSBERGER, M. G. (1990*a*). Specificity and mode of action of *Bacillus thuringiensis* insecticidal crystal proteins toxic to lepidopteran larvae: recent insights from studies utilizing midgut brush border membrane vesicles. In *Invertebrate Pathology and Microbial Control: 1990* (ed. D. E. Pinnock), pp. 278–282. Adelaide: Society for Invertebrate Pathology.

WOLFERSBERGER, M. G. (1990*b*). The toxicity of two *Bacillus thuringiensis* δ-endotoxins to gypsy moth larvae is inversely related to the affinity of binding sites on midgut brush border membranes for the toxins. *Experientia* **46**, 475–477.

WOLFERSBERGER, M. G., LUETHY, P., MAURER, A., PARENTI, P., SACCHI, V. F., GIORDANA, B. AND HANOZET, G. M. (1987). Preparation and partial characterization of amino acid transporting brush border membrane vesicles from the larval midgut of the cabbage butterfly (*Pieris brassicae*). *Comp. Biochem. Physiol.* **86**A, 301–308.

YAMAMOTO, T., EHMANN, A., GONZALEZ, J. M. AND CARLTON, B. C. (1988). Expression of three genes coding for 135-Kilodalton entomocidal proteins in *Bacillus thuringiensis kurstaki*. *Curr. Microbiol.* **17**, 5–12.