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 Mr polypeptides. These polypeptides are designated as protoxins. The complete insecticidal activity of each protoxin resides in a 55×10^3–70×10^3 Mr 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. 1988a; VanRie et al. 1990b). 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, 1990a). 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. 1988b; VanRie et al. 1989, 1990b). These observations led to the suggestion that receptor binding was the primary determinant of a toxin's larvicidal activity (VanRie et al. 1990a). 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. 1990a; Wolfersberger, 1990b). The latter observation, in particular, favors the suggestion (Wolfersberger, 1990b) 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 *CrylA(b)* and encodes a 130.6×10\(^3\) M\(_{r}\) polypeptide. The
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insecticidal crystal protein gene of strain HD-73 is classified as type CryIA(c) and encodes a $133.3 \times 10^3 \, M_r$ 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 $67 \times 10^3 \, M_r$ 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-2,6-3H]phenylalanine, 60 Ci mmol$^{-1}$, 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 Mallinkrodt (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

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Accumulation (nmol mg⁻¹)</th>
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<tr>
<td>None</td>
<td>2.80±0.13</td>
</tr>
<tr>
<td>HD-73</td>
<td>1.61±0.13</td>
</tr>
<tr>
<td>HD1-9</td>
<td>0.02±0.06</td>
</tr>
</tbody>
</table>

All uptake mixtures contained 3.1 mg ml⁻¹ of BBMV, 0.5 mmol⁻¹ phenylalanine, 75 mmol⁻¹ KSCN, 100 mmol⁻¹ mannitol and 10 mmol⁻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, 1990b), 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, 1990b). 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,
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 \textit{B. thuringiensis} \(\delta\)-endotoxins. The results reported in Table 1 are consistent with this explanation.

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References


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