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Activities of *Bacillus thuringiensis* Insecticidal Crystal Proteins Cyt1Aa and Cyt2Aa against Three Species of Sheep Blowfly

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The toxicity of *Bacillus thuringiensis* Cyt1Aa protein to sheep blowfly larvae depends on its solubilization and proteolytic activation. Cyt1Aa crystals were not toxic. Full-length and trypsin-digested Cyt1Aa proteins were toxic to larvae of three species of sheep blowfly. Neither full-length nor trypsin-digested Cyt2A soluble crystal proteins were toxic.

During sporulation, *Bacillus thuringiensis* produces crystalline protein inclusions with insecticidal activity against selected lepidopteran, dipteran, and coleopteran larvae. The insecticidal crystal proteins Cyt1Aa and Cyt2Aa, produced by *B. thuringiensis* subsp. *israelensis* and subsp. *kyushuensis*, respectively, are toxic to mosquito larvae (3, 11). Both proteins are present in the crystals as 27- to 29-kDa proteins but when solubilized can be processed by trypsin to form a protease-resistant core of 22 to 23 kDa with enhanced in vitro activity (1, 11).

Sheep blowflies are a major pest, causing ovine myiasis (flystrike) in New Zealand and Australia, resulting in major economic losses (7, 14). Control of flystrike through regular dipping with insecticides has led to the development of resistant fly populations (5). This has prompted the search for *B. thuringiensis* strains for use as a biopesticide or its insecticidal crystal proteins for incorporation into fleece-colonizing bacteria for the control of sheep blowflies. It has been reported previously that several *B. thuringiensis* subspecies (4) and Cry1Ba (8) have activity against *Lucilia cuprina* larvae. In this paper we describe the toxicities of two *B. thuringiensis* insecticidal crystal proteins, Cyt1Aa and Cyt2Aa, to the larvae of three sheep blowfly species (*L. cuprina*, *Lucilia sericata*, and *Calliphora stygia*).

B. thuringiensis subsp. *israelensis* IPS78/11 strains expressing the cloned Cyt1Aa protein gene and the Cyt2Aa protein gene (11) were grown on nutrient agar plates containing chloramphenicol (5 μg/ml). All cultures were grown at 30°C for 4 days. The sporulated cultures were harvested by washing the plates with distilled water and then were washed with 1 M NaCl followed by three washes with distilled water and stored at −20°C. Crystals were purified by the following method. Two milliliters of washed culture was mixed with 8 ml of 90% sucrose (wt/vol) and centrifuged at 13,000 × *g* at 4°C for 2 h. Eight milliliters of supernatant was removed and mixed with 4 ml of distilled water, and this mixture was centrifuged at 1,000 × *g* for 30 min. The pellet, which contained the crystals, was washed three times with distilled water.

The Cyt proteins were purified by anion-exchange high-performance liquid chromatography. Soluble full-length Cyt proteins were prepared by incubating 5 mg of Cyt1Aa or Cyt2Aa crystals in 5 ml of 50 mM Na_2CO_3, pH 10, containing 2 mM Pefabloc (Boehringer Mannheim) at 37°C for 60 min followed by centrifugation at 10,000 × *g* for 15 min. The pellet was resuspended in 5 ml of 50 mM Na_2CO_3, pH 10.5, containing 2 mM Pefabloc and 10 mM dithiothreitol and incubated at 37°C for 60 min. Trypsin-digested Cyt proteins were prepared by incubating 5 mg of Cyt1Aa or Cyt2Aa crystals in 5 ml of 50 mM Na_2CO_3, pH 10.5, containing 10 mM dithiothreitol at 37°C for 60 min. The preparation was centrifuged at 10,000 × *g* for 15 min. The supernatant was incubated with trypsin (200 μg/ml) at 37°C for 60 min. All preparations were applied to a POROS QE (PerSpective Biosystems) column, equilibrated with 50 mM Tris, pH 8.5, and the proteins were eluted at a flow rate of 5 ml/min with a gradient of 0 to 1 M NaCl (pH 8.5). The protein peaks were dialyzed against 20 mM Na_2HPO_4, pH 8.5, and stored at −20°C. Protein concentrations were determined with the bicinchoninic acid protein assay reagent (Pierce), with bovine gamma globulin as a standard. N-terminal-amino-acid analysis was used to confirm that the full-length Cyt proteins had not undergone proteolysis.

L. sericata larvae were obtained from Biosuppliers (Auckland, New Zealand); *L. cuprina* and *C. stygia* were obtained from N. Haack (AgResearch, Wallaceville, New Zealand). One hundred microliters of diluted crystals or purified proteins was mixed with 1 ml of molten diet (brewer's yeast, 9 g; agar, 9 g; ultra-high-temperature-treated full-cream milk, 250 ml; distilled water, 150 ml, microwave heated to boiling point), and

TABLE 1. LC_{50}s of Cyt1Aa and Cyt2Aa crystals, full-length and trypsin-digested protein, for first-instar *L. sericata*, *L. cuprina*, and *C. stygia* larvae

Prepn	LC_{50} (μg/ml)[a] for:		
	L. sericata	*L. cuprina*	*C. stygia*
Cyt1Aa			
Purified crystals	>2,200	>2,200	>2,200
Full-length soluble protein	236 (203–282)	296 (241–392)	305 (243–391)
Trypsin-digested soluble protein	32 (22–40)	33 (22–39)	27 (19–35)
Cyt2Aa			
Purified crystals	>2,200	>2,200	>2,200
Full-length soluble protein	>2,000	>2,000	>2,000
Trypsin-digested soluble protein	>2,000	>2,000	>2,000

[a] Values in parentheses are 95% fiducial limits.

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1 ml was dispensed into each well of a 24-well microtiter plate (Nunc). Eight newly hatched sheep blowfly larvae were added to each well, and each well was covered with perforated Mylar. Twenty-four larvae were tested at each dose, and all assays were repeated four times. The larvae were incubated at 34°C, and mortality was recorded at 48 h. The 50% lethal concentrations (LC_{50}s) were calculated by log-probit regression analysis with the computer package POLO-PC.

The LC_{50}s of Cyt1Aa and Cyt2Aa crystals, full-length and trypsin-digested proteins, are given in Table 1. Neither Cyt1Aa nor Cyt2Aa crystals were toxic at 2 mg/ml. Full-length Cyt1Aa protein was toxic to all three blowfly species (LC_{50}s ranged from 236 to 305 μg/ml). Trypsin digestion of the Cyt1Aa protein increased toxicity four to six times, with LC_{50}s ranging from 39 to 47 μg/ml. Neither full-length nor trypsin-digested Cyt2Aa proteins were toxic at 2 mg/ml.

The susceptibility of insect larvae to *B. thuringiensis* crystal proteins depends on their midgut environment, including pH (10), specific proteases (6, 12), and the presence of specific receptors (16). Cyt1Aa crystals from strain IPS78/11 are almost insoluble at pH 10 in the absence of dithiothreitol but are soluble at pH 10.5 in the presence of 10 mM dithiothreitol. Cyt2Aa crystals are soluble at pH 10 in the absence of dithiothreitol (11). The optimal pH for trypsin-like activity in *L. cuprina* and *Calliphora vicina* larvae is 7 to 8 (2, 13), suggesting that the larval midgut appears to have a neutral or slightly alkaline pH, which would not be sufficient to dissolve the Cyt1Aa and Cyt2Aa crystals. The increased toxicity of trypsin-digested Cyt1Aa cannot be explained by the lack of proteolytic activity in sheep blowfly protease activity because *L. cuprina* gut extracts could digest the 27-kDa protein (unpublished data).

The specificity of the *B. thuringiensis* insecticidal crystal proteins is determined, in part, by the presence of specific toxin receptors on the insect midgut epithelium. The cytolytic activities of both Cyt1Aa and Cyt2Aa for cultured insect cells and erythrocytes are inhibited by preincubation with phosphatidylcholine or phosphatidylethanolamine liposomes, provided that these contain an unsaturated fatty acyl chain at the syn-2 position (9, 15), suggesting that they have similar receptors. However, the differences that we observe between the activity of Cyt1Aa protein and that of Cyt2Aa protein against sheep blowfly larvae suggest that the larvae lack the receptor for the Cyt2Aa protein.

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REFERENCES

1. **Al-yahyaee, S. A. S., and D. J. Ellar.** 1995. Maximal toxicity of cloned CytA δ-endotoxin from *Bacillus thuringiensis* subsp. *israelensis* requires proteolytic processing from both the N- and C-termini. Microbiology **141:**3141–3148.
2. **Bowles, V. M., P. R. Carnegie, and R. M. Sandeman.** 1988. Characterization of proteolytic and collagenolytic enzymes from the larvae of *Lucilia cuprina*, the sheep blowfly. Aust. J. Biol. Res. **41:**269–278.
3. **Chilcott, C. N., and D. J. Ellar.** 1988. Comparative toxicity of *Bacillus thuringiensis* var. *israelensis* crystal proteins *in vivo* and *in vitro*. J. Gen. Microbiol. **134:**2551–2558.
4. **Cooper, D. J., D. E. Pinnock, and S. T. Were.** 1985. Bacterial pathogens of the Australian sheep blowfly *Lucilia cuprina*, p. 236–243. *In* R. B. Chapman (ed.), Proceedings of the 4th Australasian Conference on Grassland Invertebrate Ecology, Lincoln College, Canterbury, 13 to 17 May 1985. Caxton Press, Christchurch, New Zealand.
5. **Gleeson, D. M., S. C. Barry, and A. C. G. Heath.** 1994. Insecticide resistance status of *Lucilia cuprina* in New Zealand using biochemical and toxicological techniques. Vet. Parasitol. **53:**301–308.
6. **Haider, M. Z., B. H. Knowles, and D. J. Ellar.** 1986. Specificity of *Bacillus thuringiensis* var. *colmeri* insecticidal δ-endotoxin is determined by differential proteolytic processing of the protoxin by larval gut proteases. Eur. J. Biochem. **156:**531–540.
7. **Heath, A. C. G., and D. M. Bishop.** 1995. Flystrike in New Zealand. Surveillance **22:**11–13.
8. **Johnson, C., A. H. Bishop, and C. L. Turner.** 1998. Isolation and activity of strains of Bacillus thuringiensis toxic to larvae of the housefly (Diptera: Muscidae) and tropical blowflies (Diptera: Calliphoridae). J. Invertebr. Pathol. **71:**138–144.
9. **Knowles, B. H., P. J. White, C. N. Nicholls, and D. J. Ellar.** 1992. A broad-spectrum cytolytic toxin from *Bacillus thuringiensis* var. *kyushuensis*. Proc. R. Soc. London B **248:**1–7.
10. **Koller, C. N., L. S. Bauer, and R. M. Hollingworth.** 1992. Characterization of the pH-mediated solubility of *Bacillus thuringiensis* var. *san diego* native δ-endotoxin crystals. Biochem. Biophys. Res. Commun. **184:**692–699.
11. **Koni, P. A., and D. J. Ellar.** 1994. Biochemical characterization of *Bacillus thuringiensis* cytolytic δ-endotoxins. Microbiology **140:**1869–1880.
12. **Ogiwara, K., L. S. Indrasith, S. Asano, and H. Hori.** 1992. Processing of δ-endotoxin from *Bacillus thuringiensis* subsp. *kurstaki* HD-1 and HD-73 by gut juices of various insect larvae. J. Invertebr. Pathol. **60:**121–126.
13. **Pendola, S., and B. Greenberg.** 1975. Substrate-specific analysis of proteolytic enzymes in the larval midgut of *Calliphora vicina*. Ann. Entomol. Soc. Am. **68:**341–345.
14. **Tellam, R. L., and V. M. Bowles.** 1997. Control of blowfly strike in sheep: current strategies and future prospects. Int. J. Parasitol. **27:**261–273.
15. **Thomas, W. E., and D. J. Ellar.** 1983. Mechanism of action of *Bacillus thuringiensis* var. *israelensis* insecticidal δ-endotoxin. FEBS Lett. **154:**362–367.
16. **Van Rie, J., S. Jansens, H. Höfte, D. Degheele, and H. Van Mellaert.** 1990. Receptors on the brush border membrane of the insect midgut as determinants of the specificity of *Bacillus thuringiensis* delta-endotoxins. Appl. Environ. Microbiol. **56:**1378–1385.