Involvement of chitinases of *Bacillus thuringiensis* during pathogenesis in insects

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***Bacillus thuringiensis* subsp. *israelensis* IPS78 and *B. thuringiensis* subsp. *aizawai* HD133 both secreted exochitinase activity when grown in a medium containing chitin. Allosamidin, a specific chitinase inhibitor, inhibited activity from both strains, with IC_{50} values of about 50 μ M with colloidal chitin as substrate and between 1 and 10 μ M with 4-methylumbelliferyl-diacetylchitobioside and 4-methylumbelliferyl-triacetylchitotrioside as substrates. The involvement of these chitinolytic activities during pathogenesis in insects has been investigated with *B. thuringiensis* subsp. *israelensis* IPS78 against larvae of the midge *Culicoides nubeculosus*, and with *B. thuringiensis* subsp. *aizawai* HD133 against caterpillars of the cotton leafworm *Spodoptera littoralis*. Presence of 100 μ M allosamidin increased the LD_{50} by factors of 1.3 and 1.4, respectively, demonstrating a role for bacterial chitinases in the attack on the insects. Presence of chitinase A from *Serratia marcescens* considerably decreased the values for LD_{50} , confirming previous observations with different systems of the potentiation of entomopathogenesis of *B. thuringiensis* by exogenous chitinases. The most likely action of the endogenous chitinases of *B. thuringiensis* is to weaken the insects' peritrophic membranes, allowing more ready access of the bacterial toxins to the gut epithelia. Addition of exogenous chitinases will then increase this effect. Complementary cross-infection experiments, strain HD133 against midge larvae and strain IPS78 against caterpillars, were performed to investigate the pathogen/host specificities of the effects. Results showed that much higher concentrations of bacteria were required to achieve even low mortalities, and addition of chitinase A gave no increase in death rate.**

Keywords: chitinase, peritrophic membrane, *Bacillus thuringiensis* toxin, allosamidin, entomopathogenesis

INTRODUCTION

Chitin, the (1,4)- β -linked homopolysaccharide of N-acetylglucosamine, occurs in insects as a major component of the cuticle and of the peritrophic membrane, a protective sleeve lining the gut of many insects. Pathogens that infect through the gut must penetrate this chitin-rich barrier. Chitinase activities may play key roles in the virulence of some pathogens that infect insects via the peritrophic membrane, including the

malarial parasite *Plasmodium gallinaceum* (Huber *et al.*, 1991), the trypanosome *Leishmania* (Schlein *et al.*, 1991), the nematode *Brugia malayi* (Fuhrman *et al.*, 1995) and *Autographa californica* nucleopolyhedrovirus (Hawtin *et al.*, 1997). In this study we have examined the role of chitinases in the pathogenesis of *Bacillus thuringiensis*, which produces a range of insecticidal protein crystalline inclusion bodies during sporulation. Several strains are used worldwide as biological control agents against major insect pests. Classification of *B. thuringiensis* isolates is on the basis of their flagellar serotypes, with different serotypes displaying distinctive virulence profiles against a range of dipteran or lepidopteran larvae (deBarjac, 1981).

There have been reports that addition of commercial chitinase preparations (Smirnoff, 1974), crude chitinase

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Abbreviations: (GlcNAc)₂, N,N'-diacetylchitobiose; (GlcNAc)₃, N,N',N''-triacetylchitotriose; (GlcNAc)₄, N,N',N'',N'''-tetraacetylchitotetraose; IC_{50} , concentration causing 50% inhibition; LD_{50} , dose causing 50% mortality; 4-MeUmb, 4-methylumbelliferone.

preparations from *Bacillus circulans* (Wiwat *et al.*, 1996), and even of chitinolytic bacteria extracted from the insect's gut (Sneh *et al.*, 1983), enhance the insecticidal activity of *B. thuringiensis*. These preparations, however, will have contained contaminating enzymes, such as proteases, which may also enhance insecticidal activity. Therefore the exogenous bacterial chitinase used in this study was a single enzyme, chitinase A from *Serratia marcescens*, expressed in *Escherichia coli*. Regev *et al.* (1996) reported that this recombinant enzyme synergistically enhanced the activity of recombinant *B. thuringiensis* δ -endotoxin CryIC against larvae of *Spodoptera littoralis*.

Chitin is degraded to its monomer by the chitinolytic enzyme system. Chitinases (EC 3.2.1.14) consist of endochitinases, which randomly cleave within the chitin chain, and exochitinases, which progressively hydrolyse diacetylchitobiose units from the end of the chitin chain. The products of chitinase action are further degraded to *N*-acetylglucosamine by *N*-acetylglucosaminidases (EC 3.2.1.52). Chitinases serve a variety of functions in nature including morphogenesis, defence, nutrition and pathogenesis (Gooday, 1994). As part of the study we have used allosamidin, a specific chitinase inhibitor produced by *Streptomyces* spp. (Sakuda *et al.*, 1987a, b). It is a pseudo-trisaccharide containing *N*-acetylglucosamine and has been used to study inhibition of chitinase activity in a number of biological systems (Gooday, 1990; Gooday *et al.*, 1988; Milewski *et al.*, 1992; Shahabuddin *et al.*, 1993).

Here we report on the chitinolytic activity of two strains of *B. thuringiensis*, inhibition of activity by allosamidin and the effect of addition of exogenous chitinase or allosamidin on their insecticidal activity. *B. thuringiensis* subsp. *israelensis* has strong larvicidal activity against a range of dipteran pests (deBarjac, 1990) and here we have used it against the larvae of the blood-sucking midge *Culicoides nubeculosus*. Many species of *Culicoides* are pests of man and domestic animals and are vectors of virus diseases in commercial livestock (Kettle, 1977). We have also used *B. thuringiensis* subsp. *aizawai* against caterpillars of the cotton leafworm *Spodoptera littoralis*. The cotton leafworm is found throughout Africa, Europe and the Middle East and causes damage to a wide range of crops (Carter, 1984).

METHODS

Chemicals. Glycol chitin was prepared from glycol chitosan (Sigma) using the method of Trudel & Asselin (1989). Regenerated chitin was prepared from practical grade chitosan (Sigma) using the method of Molano *et al.* (1977). 4-Methylumbelliferyl glycosides of *N*-acetylglucosamine oligosaccharides [4-MeUmb-(GlcNAc)₁₋₄] (Sigma) were prepared at 0.8 mM: in water for the monomer, dimer and trimer glycosides and in 50% ethanol for the tetramer glycoside. All stocks were stored at -20°C .

Bacterial strains. *B. thuringiensis* subsp. *israelensis* IPS78 and *B. thuringiensis* subsp. *aizawai* HD133 were provided by Dr D. J. Ellar (University of Cambridge, UK) and were routinely grown and maintained on nutrient agar (Oxoid CM3). *E. coli*

strain A992 harbouring the pSL3ChiAII plasmid expressing the chitinase A gene from *S. marcescens* was the gift of Professor I. Chet (Hebrew University of Jerusalem, Israel) and was grown on L-broth containing ampicillin ($100\text{ }\mu\text{g ml}^{-1}$).

Preparation of culture supernatants. The two *B. thuringiensis* strains were grown in a chitinase-inducing medium consisting of regenerated chitin (0.4%), Oxoid yeast extract (0.05%), $(\text{NH}_4)_2\text{SO}_4$ (0.1%), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.03%), KH_2PO_4 (0.6%), K_2HPO_4 (1%); the pH was adjusted to 7 prior to autoclaving. Cultures were grown with shaking for 72 h at 30°C and the supernatant fraction harvested by centrifugation (13000 g , 30 min). Culture supernatants were concentrated tenfold using an Amicon Centriprep 10 Microconcentrator with a 10 kDa cut-off membrane, and stored at -20°C until used for chitinase assays.

Chitinase assays. Chitinase activity and inhibition by allosamidin were measured by assaying the supernatant extracts in the presence of increasing concentrations of allosamidin (0–100 μM) using two microtitre plate fluorescence assays. The first utilizes glycol chitin as a substrate in the presence of Calcofluor White M2R (4,4'-diamino-2,2'-stilbene disodium sulfonic acid), a fluorescent brightener (Sampson & Gooday, 1996). Breakdown of glycol chitin was monitored by measuring the decrease in fluorescence against time and activities were calculated as katal, by equating chitin degradation with the equivalent number of moles of *N,N'*-diacetyl-glycolchitobiose. Calcofluor assay mixtures contained 65 μl sample, 75 μl glycol chitin suspension (0.18%, w/v), 10 μl Calcofluor White M2R stock solution (0.1% w/v), 10 μl allosamidin solution (0–2 mM) or water and 40 μl 0.1 M citric acid/0.2 M dibasic sodium phosphate buffer (pH 6). All assays were read every 5 min for 30 min at 37°C , and performed in six replicates.

Levels of activity were also determined using the 4-methylumbelliferyl glycoside assay according to the method of McCreath & Gooday (1992). Samples were assayed against the four fluorogenic substrates [4-MeUmb-(GlcNAc)₁₋₄], which allow measurement of different enzyme activities within the chitinolytic system, with hydrolysis of 4-MeUmb-GlcNAc, 4-MeUmb-(GlcNAc)₂, 4-MeUmb-(GlcNAc)₃₋₄ detecting *N*-acetylglucosaminidase, exochitinase and endochitinase activities, respectively (Kuranda & Robbins, 1987; McCreath & Gooday, 1992). For each of the substrates, enzyme activity was calculated using a calibration curve which converted change in fluorescence to concentration of free 4-methylumbelliferone released (McCreath & Gooday, 1992). The 4-methylumbelliferyl assay mixtures contained 25 μl of sample, 5 μl of one of the four fluorogenic substrate solutions (0.8 mM) [4-MeUmb-(GlcNAc)₁₋₄], 10 μl allosamidin solution (1 mM–10 μM) and 60 μl of the citrate/phosphate buffer (pH 6), and were incubated for 30 min at 37°C . The reaction was stopped by the addition of 100 μl 1 M glycine/NaOH stop buffer and fluorescence was measured immediately. All assays were performed in six replicates. Fluorescence readings for all assays were measured at excitation 355 nm, emission 460 nm, using a Fluoroskan II microtitre plate fluorimeter.

Preparation of chitinase A extract. *E. coli* A992 cells carrying plasmid pSL3ChiAII were grown for 20 h at 37°C in L-broth containing ampicillin ($100\text{ }\mu\text{g ml}^{-1}$) and 0.1 mM phenylmethylsulfonyl fluoride. The cells were removed by centrifugation and the supernatant fraction concentrated 20-fold by ultrafiltration using an Amicon Centriprep 10 Microconcentrator with a 10 kDa cut-off membrane, and then lyophilized to a final concentration of 500 $\mu\text{g protein ml}^{-1}$. Preparations were stored at -20°C until used for bioassay purposes.

Insect larvae and bioassays. Larvae of the midge *C. nubeculosus* and the cotton leafworm *S. littoralis* were used for bioassays. The bacteria were grown for 2 d at 30 °C on nutrient agar plates and cultures were then scraped off, resuspended in 1 ml sterile deionized water and lyophilized. Stock suspensions of lyophilized plate cultures were prepared for both strains of *B. thuringiensis* by resuspending cells in sterile deionized water at a concentration of 10 mg ml⁻¹.

The effect of chitinase on the insecticidal activity of strain IPS78 against midge larvae was studied using five treatments. Portions of 500 µl of the bacterial stock suspension (10 mg ml⁻¹) were added to 500 µl of distilled water, or of 1 mM allosamidin solution, or of chitinase A extract at three concentrations (50, 125 or 250 µg protein ml⁻¹). The resultant bacterial suspensions were diluted to give a range of dose levels, and 250 µl portions of each were added to 3 ml deionized water containing 25 fourth-instar midge larvae and incubated at 27 °C. Bioassay readings were performed in duplicate and mortalities were recorded after 3 d. Each experiment was repeated on a separate day. All data from replicates were pooled and analysed by probit analysis (Finney, 1962). The lethal dose required to kill 50% of the larvae (LD₅₀) was determined by linear regression of these values.

The rate of killing of strain IPS78, with and without added chitinase, was assessed by adding 500 µl of the stock bacterial suspension to 500 µl of either chitinase A solution (125 µg protein ml⁻¹) or distilled water. The resultant suspensions were diluted to give suspensions containing 1.5 mg and 0.8 mg bacteria ml⁻¹. Portions of 250 µl of these were added to 3 ml deionized water containing 25 fourth-instar midge larvae, and incubated at 27 °C. All readings were performed in duplicate and mortalities were recorded every 12 h for 4 d. Each experiment was repeated on a separate day.

The effect of chitinase on the insecticidal activity of strain HD133 against newly hatched first-instar larvae of *S. littoralis* was studied in a similar fashion. Portions of 500 µl of the bacterial stock suspension were added to 500 µl of distilled water, or of 1 mM allosamidin solution, or of chitinase A extract at two concentrations (50 and 100 µg protein ml⁻¹). The resultant bacterial suspensions were diluted to give a range of dose levels, and 250 µl portions of each were added to 2 cm cubes of an artificial diet (Bot, 1967). Twenty newly hatched larvae were put onto each cube and incubated at 27 °C. Experiments were done in duplicate and mortalities recorded after 4 d. Each experiment was repeated on a separate day. All data from replicates were pooled and analysed as before.

The rate of killing of strain HD133, with and without added chitinase, was assessed by adding 500 µl of the stock bacterial suspension to 500 µl of either chitinase A solution (100 µg protein ml⁻¹) or distilled water. The resultant suspensions were diluted to give suspensions containing 1.2 mg and 0.6 mg bacteria ml⁻¹. Portions of 250 µl of each were added to cubes of artificial diet and larvae were added and incubated at 27 °C as before. All readings were performed in duplicate and mortalities were recorded every 24 h for 5 d. Each experiment was repeated on a separate day.

RESULTS

Chitinase activity and inhibition by allosamidin

Supernatant extracts both of *B. thuringiensis* subsp. *israelensis* IPS78 and *B. thuringiensis* subsp. *aizawai* HD133 exhibited very similar chitinolytic activities

Fig. 1. Inhibition of chitinolytic activities of *B. thuringiensis* subsp. *aizawai* HD133 by allosamidin. Supernatant extracts were assayed against increasing concentrations of allosamidin (0–100 µM). Activity was measured against a range of substrates: glycol chitin (○), 4-MeUmb-GlcNAc (●), 4-MeUmb-(GlcNAc)₂ (□), 4-MeUmb-(GlcNAc)₃ (■) and 4-MeUmb-(GlcNAc)₄ (△) for 30 min at 37 °C in the citrate/phosphate buffer pH 6.0. Results are expressed as a percentage of the respective control value and are means of six replicates. Bars represent standard error values. Control values [fkcat (mg protein)⁻¹] for the five substrates were, respectively: 13381 (±1103), 2706 (±172), 46405 (±1200), 7500 (±442) and 1888 (±95). Results for *B. thuringiensis* subsp. *israelensis* were very similar.

Table 1. Effect of chitinase and allosamidin on the mortality levels of *C. nubeculosus* and *S. littoralis* treated with *B. thuringiensis* strains IPS78 and HD133

Suspensions were prepared as described in Methods. LD₅₀ values are expressed as µg lyophilized culture used per bioassay (means ± 95% confidence limits).

Bioassay treatment	LD ₅₀
<i>C. nubeculosus</i> larvae/strain IPS78	
Water	587 ± 80
Allosamidin (100 µM)	743 ± 98
Chitinase (50 µg protein ml ⁻¹)	439 ± 59
Chitinase (125 µg protein ml ⁻¹)	143 ± 25
Chitinase (250 µg protein ml ⁻¹)	103 ± 32
<i>S. littoralis</i> larvae/strain HD133	
Water	716 ± 96
Allosamidin (100 µM)	1006 ± 99
Chitinase (50 µg protein ml ⁻¹)	271 ± 60
Chitinase (100 µg protein ml ⁻¹)	139 ± 25

against all substrates tested, suggesting that they produce a mixture of enzymes with different types of activities (Fig. 1 shows results for HD133). Allosamidin inhibited the chitinolytic activities of both extracts in a very similar fashion (Fig. 1 shows results for HD133). IC₅₀ values for *B. thuringiensis* subsp. *israelensis* IPS78 and *B. thuringiensis* subsp. *aizawai* HD133 supernatant extracts using the Calcofluor assay were 43 and 63 µM allosamidin, respectively, whereas IC₅₀ values for 4-MeUmb-(GlcNAc)₂ and 4-MeUmb-(GlcNAc)₃ were in the 1–10 µM range. Negligible inhibition of activity was

Fig. 2. Effect of chitinase A on the killing rate of *B. thuringiensis*: (a) subsp. *israelensis* IPS78 against *C. nubeculosus* larvae and (b) subsp. *aizawai* HD133 against *S. littoralis* larvae. Mortality levels are of larvae treated with (a) IPS78 at concentrations of 1.5 (■) or 0.8 mg (●) lyophilized culture ml⁻¹ and chitinase A (125 µg protein ml⁻¹), or IPS78 at concentrations of 1.5 (□) or 0.8 mg (○) lyophilized culture ml⁻¹ and distilled water; and (b) HD133 at concentrations of 1.2 (■) or 0.6 mg (●) lyophilized culture ml⁻¹ and chitinase A (100 µg protein ml⁻¹), or HD133 at concentrations of 1.2 (□) or 0.6 mg (○) lyophilized culture ml⁻¹ and distilled water. Bioassays were performed as described in Methods. Bars represent standard error values for four replicates.

observed using 4-MeUmb-(GlcNAc)₄ or 4-MeUmb-GlcNAc as substrates.

Effect of allosamidin on toxicity

Allosamidin (100 µM) had a strong inhibitory effect on the toxicity of both *B. thuringiensis* subsp. *israelensis* IPS78 against larvae of *C. nubeculosus* and *B. thuringiensis* subsp. *aizawai* HD133 against larvae of *S. littoralis* (Table 1). The allosamidin solution by itself had no discernible effect on any larvae in these experiments.

Effect of chitinase A on toxicity

The presence of exogenous chitinase A greatly enhanced both the insecticidal activity and the rate of kill of *B. thuringiensis* subsp. *israelensis* IPS78 against larvae of *C. nubeculosus* and of *B. thuringiensis* subsp. *aizawai* HD133 against larvae of *S. littoralis* (Table 1, Figs 2 and 3). Controls using chitinase alone had no effect on any larvae used and the *E. coli* A992 supernatant fraction

Fig. 3. Influence of chitinase A on the insecticidal activities of *B. thuringiensis* subsp. *israelensis* IPS78 and subsp. *aizawai* HD133 against (a) *C. nubeculosus* larvae, after 3 d, and (b) *S. littoralis* larvae, after 4 d. Mortality levels are of larvae treated with IPS78 plus chitinase A (125 µg protein ml⁻¹) (●) or distilled water (○), and HD133 plus chitinase A (100 µg protein ml⁻¹) (■) or distilled water (□). All bioassays were performed as described in Methods. Bars represent standard error values for four replicates.

lacking the chitinase A plasmid produced no enhancement of insecticidal activity of either bacterial strain. In the cross-infection experiments, addition of exogenous chitinase had no effect on the weak entomocidal activity of *B. thuringiensis* subsp. *israelensis* IPS78 against larvae of *S. littoralis* or of *B. thuringiensis* subsp. *aizawai* HD133 against larvae of *C. nubeculosus* (Fig. 3).

DISCUSSION

Both strains of *B. thuringiensis* used in this study showed high chitinase activities, particularly for exochitinase. A range of other strains all showed lower activities, but a range of insecticidal strains of *Bacillus sphaericus* had no detectable activities (M. N. Sampson & G. W. Gooday, unpublished).

Evidence for the importance of endogenous chitinase in the pathogenesis of *B. thuringiensis* is provided here by the observation of a decrease in insecticidal activity in the presence of the specific chitinase inhibitor allosamidin for both bacterial strains tested.

Inhibition of chitinase activities from the bacterial strains by allosamidin varied depending on the substrate used. Differences may be due to *B. thuringiensis* pro-

ducing a mixture of chitinases with different affinities for allosamidin. *Bacillus circulans* WL-12, for example, produces at least nine chitinase molecules (Alam *et al.*, 1996). Previous work has shown that allosamidin has a diverse spectrum of activities against chitinases, with very strong inhibition of activity of some fish and nematode chitinases, less strong inhibition of those of insects and fungi and the weakest effect on those of bacteria (Gooday, 1990). The inhibition of *B. thuringiensis* chitinase by allosamidin is comparatively weak, with IC_{50} values in the micromolar range; based on these results a high concentration of allosamidin (100 μ M) was used for the bioassay experiments.

In our experiments, allosamidin had no discernible effect on the larval stages of growth of either *C. nubeculosus* or *S. littoralis*. This is in contrast to reports of its insecticidal activity on developing insects. Thus it inhibited moulting and led to death of *Bombyx mori* and *Leucania separata* after being injected into larvae at different growth stages immediately after ecdysis (Sakuda *et al.*, 1987a). When administered orally, allosamidin (synonym A82516) stopped *Musca domestica* larvae from forming pupae (Somers *et al.*, 1987).

We have shown that addition of *S. marcescens* chitinase A enhances the activity of two strains of *B. thuringiensis* against hosts from different orders of insects. These results reinforce observations that the addition of commercial chitinase preparations enhances insecticidal activity of *B. thuringiensis* (e.g. Smirnoff, 1974). Studies on the mortality rate of baculovirus against its host also showed an increase in toxicity in the presence of added commercial chitinase preparation (Shapiro *et al.*, 1987). Wiwat *et al.* (1996) transformed *B. thuringiensis* subsp. *israelensis* with chitinase-encoding genes from *Aeromonas hydrophila* and *Pseudomonas maltophilia* with the aim of potentiating insecticidal activity, but only low levels of expression were obtained.

The observations that the addition of chitinase had no effect on death rates in the cross-infection experiments, i.e. strain IPS78 versus caterpillars, strain HD133 versus midge larvae (Fig. 3), required experiments with very high concentrations of bacteria, which would have led to high endogenous chitinase activities. These observations thus underline the host specificities of the two bacterial strains.

Our results are consistent with previous studies which have demonstrated a link between chitinase activity and pathogenicity of a range of microbial insect pathogens (Huber *et al.*, 1991; Schlein *et al.*, 1991; Fuhrman *et al.*, 1995; Hawtin *et al.*, 1997), and with those of Shahabuddin *et al.* (1993), who observed a blocking of transmission of the malarial parasite *P. gallinaceum* in the presence of allosamidin. The precise mechanism through which chitinase exerts its effect on insect pathogenesis in these different systems is unclear. One suggestion is that microbial chitinases partially digest the peritrophic membrane, aiding the microbes and/or their toxins to penetrate the peritrophic membrane (Shahabuddin & Kaslow, 1993). Evidence for this

hypothesis includes the observation that exogenous chitinase is capable of digesting the peritrophic membrane of the mosquito *Aedes aegypti* (Huber *et al.*, 1991). By contrast, studies on the transmission of *Trypanosoma brucei rhodesiense* suggested that chitinolytic activity may not be required for the parasite to traverse the peritrophic membrane in tsetse flies, and a model was proposed whereby products of the action of chitinolytic endosymbiotic bacteria may inhibit the antiparasitic action of host defence lectins (Welburn *et al.*, 1993).

The data presented here show a clear link between chitinase production and enhancement of virulence in *B. thuringiensis*, but further research is required to elucidate the functional role of chitinase activity.

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