Active resistance of entomophagous rhabditid *Heterorhabditis bacteriophora* to insect immunity

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SUMMARY

A specific extracellular proteinase, degrading selectively the cecropin-based defence system of insects, is secreted into the larval body during parasitism of the greater wax moth by the *Heterorhabditis bacteriophora/Photorhabdus luminescens* complex and by phase 1 of *P. luminescens*. The proteolytic digestion of insect inducible cecropin-like immune molecules was demonstrated by the disappearance of the *Galleria mellonella* cecropins and purified *Hyalophora* cecropin B peptide PAGE bands upon exposure to infected extracts, and a similar abrogation of antibacterial activity using an agar diffusion assay. Proteolytic activity of infected extracts produced by nematode/bacterial complex and phase 1 variant of *P. luminescens* was shown to be correlated with cecropin-inhibitory activity, suggesting that this anti-cecropin agent may be responsible for the ability of bacteria to establish infection and the insecticidal nature of *H. bacteriophora*. Antibacterial activity of *Galleria* lysozyme and that of chicken egg-white lysozyme to which *P. luminescens* is insensitive, was unaffected by *H. bacteriophora* proteinase.

Key words: insect immunity, *Heterorhabditis bacteriophora*, *Photorhabdus luminescens*, cecropin-like peptides, haemolymph lysozyme, *Heterorhabditis bacteriophora* proteinase.

INTRODUCTION

The insect immune system reacts against foreign intruders of the body cavity with the recruitment of blood cells and with a cell-free response. The cellular defence reactions of insects against microbial infections comprise phagocytosis and nodule formation around bacteria, conidia and fungal hyphae, while parasitic eggs and parasites are encapsulated (Salt, 1970; Poinar, 1974; Ratcliffe, 1982). The cellular defence by haemocytes takes place immediately after contact with bacteria, but inducible humoral defence follows after a short delay, because time is needed for synthesis of antibacterial immune proteins. If invading micro-organisms recognized as foreign cannot rapidly be phagocytosed or nodulated by the circulating blood cells, they induce a humoral (cell-free) immune response. Insect cell-free immunity depends on the production of a number of antibacterial peptides and proteins and is attributable to innate compounds such as lysozyme (Mohrig & Messner, 1968; Jarosz & Śpiewak, 1979), and lectins (Olafsen, 1996), and to inducible immune proteins absent in non-immunized insects. The insect inducible immune proteins belong to 1 of the 5 following groups: lysozyme-type, short α-helical peptides, peptides with intramolecular disulphide bridges, short-Pro-rich peptides and long multi-domain proteins (glycine-rich peptides) (Bulet *et al*. 1993; Casteels-Josson *et al*. 1994; Cociancich, Bulet & Hoffmann, 1994). These antibacterial proteins include, for example, cecropins (Boman & Hultmark, 1981) and attacins (Hultmark *et al*. 1983) in Lepidoptera, diptericins in Diptera (Keppi *et al*. 1986), apidaecins in bees (Casteels *et al*. 1989, 1990, 1994), and insect defensins in certain flies, hymenopterans and coleopterans (Bulet *et al*. 1991; Cocianchich *et al*. 1993).

Insect lysozymes are the naturally occurring proteins found in the haemolymph of most groups of insects and other invertebrates (Mohrig & Messner, 1968). Lysozyme tends to increase drastically in infected insects, but persists well beyond the duration of protective immunity and bactericidal activity of the haemolymph (Chadwick, 1970). It works synergistically with cecropins, enzymatically destroying the murein sacculus of bacterial cells left after cecropin action (Boman & Hultmark, 1987).

Cecropins and cecropin-like substances represent a closely related family of basic proteins with molecular weight of about 4 kDa. Most bacterial species, both Gram negative and Gram positive, are killed by lepidopteran cecropins at concentrations in the micromolar range. The cecropins affect the integrity of the cell membrane, causing leakage of potassium ions and interferring with the generation of ATP (Durrell, Raghunathan & Guy, 1992). It is believed that attacins, a class of inducible proteins of molecular weight about 23 kDa with activity against Gram-negative bacteria, notably *Escherichia coli* is

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the most sensitive (Hultmark *et al*. 1983; Cociancich *et al*. 1994), facilitate the activity of cecropins and lysozyme.

Not all invaders are killed in the insect body cavity. Many will survive and develop, unaffected by the host immune system. Bacterial pathogens and some parasites of insects, including entomogenous rhabditid nematodes of the families *Steinernematide* and *Heterorhabditidae*, have developed reactions by which they may counteract insect immunity. This phenomenon is realized either by escaping the immune response (the passive resistance to immunity) or by degrading antimicrobial factors in an active process (the active resistance to insect immunity) (Boman & Hultmark, 1987). How the parasites survive, or escape, the immunological control of an insect is a complex subject not yet fully understood. In general, the passive resistance to immunity is a result of a strong evolutionary pressure on pathogens to develop mechanisms which enable them to escape the insect immune response or to minimize its effectiveness through changes in the pathogen itself. The immune inhibitors of the type A (InA) (Edlund, Sidén & Boman, 1976) elaborated during parasitism by some bacterial pathogens of insects (Sidén *et al*. 1979; Jarosz & Gliński, 1990; Jarosz, 1996) and by the insect pathogenic nematodes *Steinernema carpocapsae* (Götz, Boman & Boman, 1981) and *Heterorhabditis bacteriophora* (Jarosz, Boemare & Gaj, 1994) are most important in immune protein degradation by an active process. The type A inhibitor from *Bacillus thuringiensis* (Sidén *et al*. 1979) is a heat-sensitive metalloproteinase with a molecular weight of about 70 kDa that degrades proteolytically cecropins and attacins (Dalhammar & Steiner, 1984).

A specific association between the rhabditid *H. bacteriophora* and its bacterial associates *Photorhabdus luminescens* contributes to virulence of this obligatory parasite of insects, and is believed to be responsible for host mortality (Poinar, 1975; Thomas & Poinar, 1979; Akhurst, 1980, 1983; Boemare, Akhurst & Mourant, 1993). The bacteria released into the body cavity multiply rapidly in the host haemolymph and the insects die due to *P. luminescens* septicaemia. This investigation supports a hypothesis that a specific type of extracellular proteinase secreted by *H. bacteriophora* in parasitized hosts degrades selectively the antibacterial proteins of insects, predisposing the host to septicaemia during natural parasitism. Evidence is provided that the proteolytically active phase 1 variant of *P. luminescens* is primarily responsible for immune inhibitor production, and the symbiosis with nematode enhances the secretion of the anti-cecropin agent.

MATERIALS AND METHODS

Nematode and isolation of bacterial variants

The Polish indigenous rhabditid *Heterorhabditis bacteriophora* (Strain PLHb81) was isolated originally by Dr Henryk Skrzypek (Skrzypek, 1987), Department of Environmental Biology, Catholic University of Lublin (Poland) from a soil sample using the *Galleria* trap technique of Bedding & Akhurst (1975), and is maintained in the Culture Collection of Entomogenous Nematodes of this Department. Infectives were reared on larvae of the greater wax moth, *Galleria mellonella* (Lepidoptera: Pyralidae), at 23 °C according to the method of Dutky, Thompson & Cantwell (1964), and juveniles harvested from traps 2 days after initial emergence from insect carcasses were routinely stored in a thin layer of 0·005 % formaldehyde solution. Before use they were checked microscopically for activity and tested for pathogenicity against last-instar *G. mellonella*, a highly susceptible host to *H. bacteriophora* invasion.

Seventh instar larvae of *G. mellonella* were selected from a stock culture propagated on dark honeycombs at 29 °C in total darkness. Only healthy and vigorous caterpillars were selected for experimentation.

Phase 1 cultures were isolated by homogenizing surface-sterilized infective nematodes, and phase 2 cultures from larval plasma of the greater wax moth parasitized with *H. bacteriophora* (Akhurst, 1980). Using a glass tissue grinder, about 100 juveniles, suspended in 1·0 ml of saline W (Weevers, 1966), were macerated thoroughly in order to release bacterial symbionts. The homogenates were streaked (10 μl/plate) onto Tergitol-7-agar (Poinar & Thomas, 1978) and incubated at 23 °C until phase 1 colonies of *Photorhabdus luminescens* could be differentiated morphologically. On Tergitol-7-agar, phase 1 variant grows slowly (colony diameter 3·0–3·5 mm after cultivation for 2 weeks at 23 °C) as irregular debris-like in shape, green colony surrounded by decolourized zone after 12 days of incubation (Akhurst, 1980, 1983). Inoculated into larval *G. mellonella* body cavity it produced a deep purple colouration and gummy consistency in the animals (Jarosz & Skrzypek, 1992). In order to isolate the phase 2 variant, dilutions of larval plasma were spread on Tergitol-7-agar and incubated for 3 days. Isolates of both phase 1 and phase 2 were checked for purity by the determination of antibiotic production (Akhurst, 1983), pathogenicity for *G. mellonella* and symptoms of *P. luminescens* septicaemia, and re-isolation from moribund larvae.

Samples and assay of proteolytic activity

Invasive-stage nematodes on filter paper in a Petri dish were used to colonize 7th instars of the greater

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wax moth. Other groups of larvae were inoculated via the proleg with about 30 living cells of *Photorhabdus*, phase 1 or phase 2 variant. The number of bacteria was established by colony counts on agar plates. Larval cadavers (weighing about 210 mg) with typical symptoms of parasitism were used for preparation of infected extracts. Ten insects were blended in 10 ml of distilled water, centrifuged at 10000 ***g*** to remove the larval debris, and then cold-sterilized by passage through a Schot G5 filter. The control extracts were prepared by homogenizing healthy larvae in the same manner. Sterility of samples was checked by spreading 10 μl of each sample on nutrient agar and incubating at 28 °C for 48 h.

Cultures of *P. luminescens*, phase 1 or phase 2 variant, were grown in nutrient broth at 26 °C for 5 days. Bacterial pellets were removed by centrifugation at 8000 ***g*** for 20 min. Broth cultures, sterilized by passage through a Schöt G-5 filter, were used for experimentation.

Proteolytic activity of larval extracts and broth cultures was determined by the agar-diffusion assay technique and expressed in the terms of trypsin activity (EC 3.4.4.4). The medium in each 10 cm Petri dish was 10 ml of Sörensen's buffer, pH 6·8; 5 mg of Hide powder azure (Sigma), a sensitive general proteolytic substrate; 30 μg of streptomycin sulphate, and 100 mg of agarose (Serva, Heidelberg). Wells (2·7 mm in diameter) in the assay medium each contained 7·5 μl of the test sample. The plates were incubated at 28 °C for 24 h. Then, the diameter of proteolysis around the wells was measured to the nearest 0·5 mm. The concentration of proteinase in the infected extracts (or spent broth) to be assayed was calculated from a standard curve of known trypsin (Serva, twice crystallized) concentrations (20, 10, 5, 2·5, 1·25 μg/ml) plotted on millimetre graph paper and expressed as μg/ml of sample. Activity was multiplied by the dilution factor.

Immunization and antibacterial activity of immune proteins

Immune haemolymph containing cecropin-like antibacterial polypeptides and other immune proteins, was obtained from several holometabolous insect species: from larvae of *G. mellonella* and *Arge berberidis*, as well as from diapausing pupae of *Pieris brassicae*, *Sphinx pinastri* and *Celerio euphorbiae*. Antibacterial activity was generated by inoculation into haemocoel of *Enterobacter cloacae*, a potent inductor of immune response in insects (Boman & Hultmark, 1987). The insects were maintained at 26 °C, and immune haemolymph was collected 2 days after immunization.

Antibacterial activity of inducible immune proteins was recorded as the diameter of inhibition zones around wells (diameter 2·7 mm) in a thin layer of soft (0·7 %) nutrient agar inoculated with viable log phase cells of *Escherichia coli* D31 (Hoffmann, Hultmark & Boman, 1981), a bacterium sensitive to cecropins (Boman *et al*. 1978). Nutrient broth contained streptomycin sulphate (70 μg/ml) to inhibit the growth of contaminating bacteria. Lysozyme activity in samples was also detected in an inhibition zone assay using freeze-dried *Micrococcus luteus* (Sigma) incorporated into Sörensen's buffer (pH 6·4) solidified with agarose (0·7 %), according to Mohrig & Messner (1968).

Assay for inhibition of antibacterial activity

As an *in vitro* test for the presence of an immune inhibitor which inactivates the bactericidal activity of inducible immune proteins, the ability of proteinase to inhibit the lysis of *E. coli* D31 by immune haemolymph was determined. The reaction mixture contained 20 μl of immune haemolymph and 5, 2·5, 1·25, 0·612 or 0·306 μl of an infected extract, and sterile water up to 100 μl in an Eppendorf tube. Another mixture contained 6·0 μg of cecropin B (Sigma) of *Hyalophora cecropia* and various volumes of infected extract in 100 μl of an assay mixture. The remaining activity of immune proteins in the mixture was bioassayed against *E. coli* after pre-incubation at 23 °C for 0 and 30 min. The inhibitory effect of proteinase which suppressed or totally inactivated the activity of antibacterial proteins was indicated by the reduction or total disappearance of the lysis zone around the well (diameter 2·7 mm). Maximal inhibitory dilution (MID) was defined as the greatest dilution of infected extract (percentage of the sample) that completely destroyed antibacterial activity of cecropin-like immune proteins in 20 μl of insect haemolymph (or 6·0 μg of cecropin B peptide) exposed to proteinase for 30 min.

Electrophoretic analysis and proteolytic digestion of immune proteins

Acidic gel electrophoresis of insect antibacterial proteins, or standards pre-treated with the proteinase, was run on 15 % polyacrylamide gels at pH 4·3, using discontinuous non-denaturing buffer systems by the method of Reisfeld, Lewis & Williams (1962). Assay mixtures containing 20 μl of immune haemolymph (in the case of standards, 6 μg of cecropin B or 9 μg of chicken egg white lysozyme in 20 μl of aqueous solution) and 10 μl of infected extract were pre-incubated at 28 °C for 30 min before electrophoresis. Gels were stained with amido black for protein. To localize bands with lysozyme or cecropin-like antibacterial activity, the gels were first washed twice with phosphate buffer (pH 7·4) and then overlaid with a layer of soft nutrient agar inoculated with an exponential phase of *E. coli* (detection for cecropins and attacin-like activity) or

Table 1. Inhibition of antibacterial activity of cecropin-like immune proteins of *Galleria mellonella* larvae by a virulence proteinase secreted from *Heterorhabditis bacteriophora* or its bacterial associate *Photorhabdus luminescens*

Infected extract	Concentration of proteolytic activity (μg/ml)*			Cecropin-like activity (*E. coli* D31 zone lysis diameter, mm)
	Lowest	Highest	Mean ± S.D.	
Larvae parasitized with:				
H. bacteriophora				
Hb 0 (moribund larvae)	4·5	18·0	12·7 ± 4·3	0
Hb 24 (24 h after larval death)	49·0	79·0	63·3 ± 10·5	0
P. luminescens				
Phase 1				
Pl 0	3·0	6·5	5·1 ± 1·4	T‡
Pl 24	10·5	19·0	14·4 ± 3·1	0
Phase 2				
Pl 24			T†	7·5
Broth cultures:				
Phase 1 variant	5·9	10·1	7·6 ± 2·2	0
Phase 2 variant			0	7·5
Control (from healthy larvae)			T†	7·5

* Activity expressed in the terms of trypsin activity (EC 3.4.4.4). Mean concentration of proteolytic activity based on 7 replicates. T†, indicates trace (< 1·5 μg/ml) of proteolytic activity. Antibacterial activity determined by a conventional agar diffusion assay technique. T‡, trace antibacterial activity (zone of *E. coli* D31 lysis less than 4·0 mm).

with a dense suspension of freeze-dried *Micrococcus luteus* (for lysozyme activity) in Sörensen's buffer (pH 6·4) solidified by 0·7% agarose. Antibacterial activity was registered by the lysis zone of bacterial species used as target cells.

RESULTS

Proteolytic activity and interaction with immune antibacterial proteins of insects

During parasitism of the greater wax moth, an extracellular proteinase was secreted into the larval body by the *H. bacteriophora*/*P. luminescens* complex and by phase 1 of *P. luminescens* but phase 2 variant of *P. luminescens* was proteolytically inactive (Table 1). The titre of proteinase in larvae parasitized with the nematode bacterium complex is remarkably higher than in animals infected with bacterial associate alone. This extracellular proteinase is produced by phase 1 variant of *P. luminescens*, and bacterial symbiosis enhanced the production of the enzyme within the parasitized host. The proteinase appeared in moribund larvae, and the production increased during parasitism and coincided with the progress of *P. luminescens* bacteriaemia. No proteolytic activity was detected in haemolymph and larval tissues from unparasitized insects.

The increased activity of proteinase in dead insects coincided with the ability to inactivate cecropin-like antibacterial activity of immune blood (Table 1). Only slight inhibitory activity against cecropin-based defence system of *G. mellonella* became evident in extracts from moribund larvae infected with phase 1 of *P. luminescens*. The *in vitro* assay system demonstrated that cecropins of *Celerio euphorbiae*, *Pieris brassicae*, *Sphinx pinastri* and *Arge berberidis* were more susceptible to an anti-cecropin agent than were cecropin B of *Hyalophora cecropia* or *Galleria* cecropins (Table 2). Infected extracts at a dilution of 1 × MID for cecropin-family peptides of *G. mellonella* depressed totally the antibacterial activity of 6·0 μg synthetic cecropin B peptide of *H. cecropia* against *E. coli*. In immune haemolymph not exposed to proteinase action, the activity of cecropin-like antibacterial peptides remained unchanged.

Lack of inhibitory activity in larval body homogenates from healthy animals killed by either decapitation or freezing and processed up to 24 h after death, confirms a specific ability of the proteinase present in infected extracts to destroy cecropin-like activity in immune haemolymph of the greater wax moth and other lepidopterans. The proteolytically inactive larval extract from *Galleria* killed by the phase 2 bacterial symbiont failed to induce any inhibitory effect (Table 1). The anti-cecropin agent of proteolytic nature is secreted during parasitism by the phase 1 of *P. luminescens* and by the *H. bacteriophora*/*P. luminescens* complex. It must be clearly stated that the phase 2 variant of *P. luminescens* was unable to produce any proteinases inhibiting the cecropin-based defence system and toxic for insects, both during parasitism and *in vitro* in broth culture.

Identification of immune antibacterial peptides

A typical pattern of proteins, both in immune and non-immune *Galleria* haemolymph, and the

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Table 2. The *in vitro* inhibition test for sensitivity of cecropin-like antibacterial proteins to an immune inhibitor from *Heterorhabditis bacteriophora*

(Immunity was elicited by inoculation of *Enterobacter cloacae* into the insect body cavity.)

Cecropin source	Order/stage	Maximal Inhibitory Dilution (% of infected extract)
Cecropin B (Sigma) of *Hyalophora cecropia*	*Lepidoptera*/pupa	2·34
Galleria mellonella	*Lepidoptera*/larva	2·34
Pieris brassicae	*Lepidoptera*/pupa	0·78
Celerio euphorbiae	*Lepidoptera*/pupa	1·95
Sphinx pinastri	*Lepidoptera*/pupa	1·56
Arge berberidis	*Hymenoptera*/larva	1·56

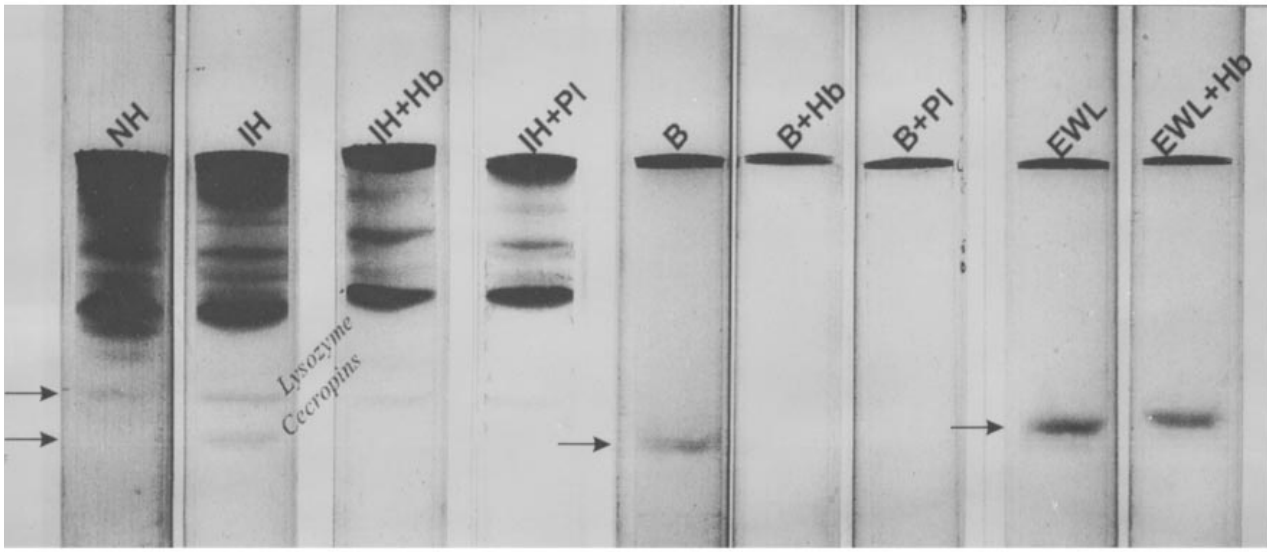

Fig. 1. Electrophoretic patterns of immune antibacterial proteins visualized by staining for proteins with amido black after electrophoresis in 15% acid polyacrylamide gels. The gels were loaded with 7 μl samples of native haemolymph from *Galleria mellonella* larvae (NH), from larvae immunized with *Enterobacter cloacae* (IH), with immune haemolymph of *G. mellonella* exposed to proteolytic digestion by proteinase from *Heterorhabditis bacteriophora* (IH+Hb) or *Photorhabdus luminescens* (IH+Pl), with 6·0 μl of cecropin B (Sigma) from *Hyalophora cecropia* (B), with cecropin B (Sigma) exposed to proteinase from *H. bacteriophora* (B+Hb) or *P. luminescens* (B+Pl), with 9 μg of chicken egg white lysozyme (EWL) and egg white lysozyme pre-treated with *H. bacteriophora* proteinase (EWL+Hb).

standards (cecropin B peptide of *Hyalophora cecropia* and chicken egg white lysozyme) used to localize the insect immune proteins on polyacrylamide gel, are presented in Fig. 1. Acidic gel electrophoresis under non-denaturing buffer system conditions combined with bacterial overlaying of the gels showed that immune haemolymph contained antibacterial activity against freeze-dried *M. luteus* with the mobility expected for the hen egg white lysozyme (Fig. 3). and somewhat faster running activity against *E. coli* (Fig. 2). A comparison of the antibacterial assays and the protein staining indicated that the mobilities of insect immune proteins correspond to the egg white lysozyme and to cecropin B peptide (Sigma) from *H. cecropia*. Therefore, the insect lysozyme and cecropin-like antibacterial peptides were identified in immune haemolymph from *G. mellonella* larvae.

Activity of proteinases against antibacterial peptides

By acidic gel electrophoresis and bioassays for antibacterial activity, it was shown that cecropin-like antibacterial peptides of *Galleria* and cecropin B peptide of *H. cecropia*, each exposed to the action of proteinase from *H. bacteriophora*/*P. luminescens* complex, quickly lost their antibacterial activity against *E. coli* (Fig. 2). A similar digestion of purified cecropin B (Sigma) peptide and cecropin-family polypeptides in immune blood of *Galleria* was demonstrated using a virulence proteinase from larvae killed with phase 1 variant of *P. luminescens*. As can be seen by protein staining (Fig. 1), cecropin-like proteins of *Galleria*, like cecropin B peptide of *H. cecropia*, were highly sensitive to proteolytic digestion.

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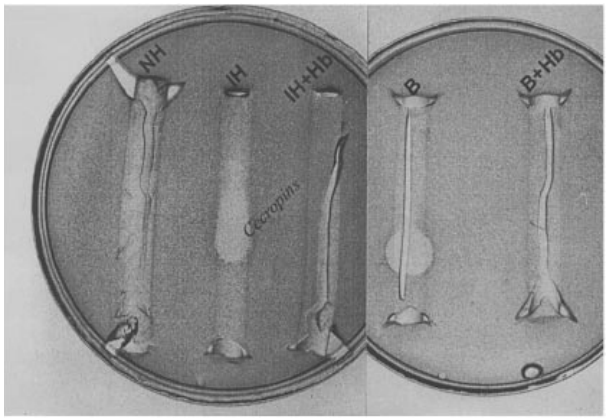

Fig. 2. Cecropin antibacterial peptides visualized by overlaying with viable *Escherichia coli* after acidic electrophoresis in 15% polyacrylamide gels. The gels were loaded with 12 μl samples of native haemolymph from *Galleria mellonella* larvae (NH), from larvae immunized with live *Enterobacter cloacae* (IH), with immune haemolymph exposed to proteolytic degradation by *Heterorhabditis bacteriophora* protease (IH + Hb), and with samples of purified cecropin B (Sigma); non-exposed (B) and exposed to proteinase of *H. bacteriophora* (B + Hb).

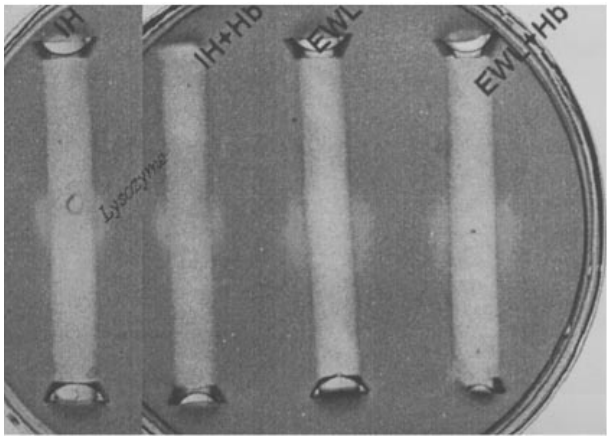

Fig. 3. Antibacterial assays of lysozyme activity pre-treated with *Heterorhabditis bacteriophora* proteinase on polyacrylamide gels overlaid with freeze-dried *Micrococcus luteus* cells. Assay mixtures containing 20 μl of immune haemolymph (or 9 μg of chicken egg white lysozyme in 20 μl of solution) and 10 μl of infected extract were pre-incubated for 1 h at 28 °C before electrophoresis. The samples were: immune haemolymph from *Galleria mellonella* larvae (IH), immune haemolymph of *Galleria* treated with *H. bacteriophora* proteinase (IH + Hb), chicken egg white lysozyme (EWL), egg white lysozyme pre-treated with proteinase from *H. bacteriophora* (EWL + Hb).

Lysozyme proteins, both insect lysozyme and chicken egg white lysozyme, appeared to be insensitive to enzymatic degradation, and the gels overlaid with freeze-dried *M. luteus* cells showed lytic zones corresponding to those obtained with the untreated samples of the lysozymes (Fig. 3). The proteinase did not digest the lysozyme bands even when the concentration of proteinase was doubled and the time of exposure prolonged to 1 h.

DISCUSSION

During parasitism of *G. mellonella* larvae, the entomogenous rhabditid *H. bacteriophora* and phase 1 of its bacterial symbiont *P. luminescens* secreted an extracellular proteinase that specifically degraded the inducible antibacterial proteins of the host insect. The inhibitory effect on antibacterial activity is caused by proteolytic digestion of the cecropin-like immune peptides, produced in *G. mellonella* following bacterial infection. These antibacterial molecules seem to be the primary immune proteins responsible for elimination of bacterial invaders from insect haemocoelomic cavity (Jarosz, 1995). Evidence from the results with cecropin B of *H. cecropia* exposed to proteinase action, shows that this immune inhibitor selectively degraded the insect response proteins in an active process. Neither the induction kinetics nor bactericidal activity of *Galleria* lysozyme to which *P. luminescens* is insensitive were affected by proteinase. The apparent decrease or total disappearance of lysozyme activity in insects parasitized by *H. bacteriophora* is due rather to fat body destruction by this insect obligatory parasite.

It is well documented that induction of immunity offers a significant protection against the saprophytic bacteria given alone (Boman & Hultmark, 1987), but not when they were inoculated together with an inhibitor (Jarosz, 1995). The anti-cecropin agent from *H. bacteriophora* destroyed the antibacterial immunity of the insect, and thus could determine the fate of an infection. Larvae of *G. mellonella*, injected with *Enterobacter cloacae* alone, produced antibacterial proteins that protected them effectively against bacteriaemia of *E. cloacae*. By injecting an immune inhibitor, that selectively depressed the induction of a cell-free immune response, the bacterium multiplied in haemolymph and ultimately killed the insects (Jarosz, 1995). Other avoidance or resistance mechanisms could also be involved in the fate of bacterial infection, since *Steinernema carpocapsae* and/or *Xenorhabdus nematophilus* suppress the phenoloxidase cascade at an early step (Yokoo, Tojo & Ishibashi, 1992) and interfere with haemocytes in insect defence (Dunphy & Webster, 1984, 1986).

Preliminary identification of a secreted virulence proteinase from *H. bacteriophora*, responsible for inactivation of the antibacterial activity of insect blood, revealed similarities, if not functional identity, to the type A immune inhibitor from *B. thuringiensis* (Edlund, Sidén & Boman, 1976). Structurally, the inhibitor of the type A (InA) from *B. thuringiensis*

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represents a specific type of heat-sensitive metalloproteinase (Sidén *et al*. 1979) with a molecular weight of approximately 70 kDa that degrades specifically the cecropin-like antibacterial proteins (Dalhammar & Steiner, 1984) of *Lepidoptera* and other holometabolous insects. The structure of InA resembles thermolysin from *Bacillus thermoaquaticus*, but functionally it is rather similar to *Pseudomonas aeruginosa* elastase (Morihara & Tsuzuki, 1975). Like insecticidal effects produced by InA from *B. thuringiensis*, inoculation of *H. bacteriophora* proteinase into larval body cavity intoxicated the *Galleria* with symptoms of paralysis and muscular contraction (Jarosz & Skrzypek, 1992), probably because of muropeptide degradation, since insects die with similar symptoms when muropeptide is lacking (Lovgren *et al*. 1990).

Finally, it is reasonable to conclude that bacterial invaders provoke the insect immune system to generate antibacterial activity in order to eliminate the infection, but obligate insect parasites such as *H. bacteriophora*, release the anti-cecropin agent that inactivates inducible cell-free antibacterial immunity of the host during parasitism. In this way the colonization of insect body cavity by *P. luminescens* is made easier. The extracellular proteinase, by degrading the cecropin-like immune proteins in an active process could, therefore, contribute to the insecticidal nature of *H. bacteriophora*.

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