Recognition and regulation of metalloproteinase activity in the haemolymph of Galleria mellonella: a new pathway mediating induction of humoral immune responses

Jochen Griesch a, Marianne Wedde a, Andreas Vilcinskas b,*

a Institute of Zoology, Free University of Berlin, Königin-Luise-Strasse 1-3, 14195 Berlin, Germany
b Systematic Zoology and Evolutionary Biology, University of Potsdam, Villa Liegnitz, Lennéstrasse 7a, 14471 Potsdam, Germany

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Abstract

Proteolytic activity released within an organism by wounded tissues or invading pathogens can strongly impair the physiological homeostasis when it remains non-regulated. Thus, an efficient mechanism that enables recognition and inactivation of non-regulated proteolytic activity is essential to limit toxic effects. In larvae of the Greater wax moth Galleria mellonella we discovered that injection of bacterial thermolysin at a sublethal concentration mediates both acquired resistance against a subsequently injected lethal concentration of this metalloproteinase and stimulation of humoral immune response accompanied by the synthesis of an inducible metalloproteinase inhibitor (IMPI) which is released within the haemolymph. In search of a putative mechanism mediating recognition and regulation of released microbial metalloproteinases we determined that thermolysin-mediated hydrolysis of G. mellonella haemolymph proteins \textit{in vitro} yields small (\textlessthan3 kDa), heat-stable molecules which were discovered to represent potent elicitors of humoral immune responses when injected into untreated larvae. Obtained results allowed to design a model explaining for the first time regulation of released metalloproteinases within the haemolymph of insects. The determined coherence between regulation of released metalloproteinases by IMPI and the simultaneous induction of antimicrobial proteins provides a new insight into the mechanisms leading to expression of genes in course of humoral immune responses. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Insect immunity; Proteases; Protease inhibitor; Protease regulation; Galleria mellonella

1. Introduction

Insects respond to challenge with microbial agents or injury by the rapid and transient synthesis of potent antimicrobial compounds. Numerous immune proteins from insect sources which inhibit microorganisms in vitro have been identified, characterized and cloned. Most of them exhibit antibacterial activity, whereas others were demonstrated to affect fungi (Cociancich et al., 1994; Hoffmann, 1995; Hoffmann et al., 1996; Gillespie et al., 1997). However, evidence is available that the insect humoral immune responses includes the release of protease inhibitors within the haemolymph (Boucias and Pendland, 1987; Vilcinskas and Wedde, 1997). Protease inhibitors are suggested to play multifaceted roles in the insect defense system. Besides regulation of endogenous proteases which are, for example, involved in the activation of cascades mediating coagulation and phenoloxidase activation, they could be released during humoral immune response to inactivate proteases which are released by invading pathogens (Kanost and Jiang, 1996).

Among the proteolytic enzymes produced by human pathogens, particularly metalloproteinases are reported to play a predominant role as key factors determining virulence of micro-organisms. Microbial metalloproteinases cause inflammatory response and multiple organ dysfunction in infected patients. They can strongly affect host cells and degrade endogenous inhibitors which normally control proteolytic cascades (Maeda, 1996). Metalloproteinases have also been determined to be associa-
tated with different groups of entomopathogens and attributed to facilitate their development within the infected insect host or to interfere with its immune system. For example, enhancin, a metalloproteinase associated with granulosis viruses, is reported to promote the fusion of the virus particles with the cell membrane of epithelia in susceptible host insects (Lepore et al., 1996) Bacillus thuringiensis produces a metalloproteinase which degrades cecropin of the infected host (Dalhammar and Steiner, 1984). A thermolysin-like metalloproteinase has been identified among the enzymes released by the entomopathogenic fungus Metarhizium anisopliae (St Leger et al., 1994). Recently, it was shown that proteinases released by this fungus affect immune competent host cells in vitro. At sublethal concentrations, they inhibited phagocytic activity, attachment and spreading of plasmatocytes isolated from larvae of the Greater wax moth G. mellonella (Griesch and Vilcinskas, 1998). In addition, metalloproteinases such as thermolysin were found to be extremely toxic when injected into G. mellonella. Untreated larvae challenged with thermolysin exhibited 100% mortality within 24 h, whereas larvae pre-injected with soluble microbial elicitors such as LPS or a zymosan preparation survived this treatment (Wedde et al., 1998).

The acquired resistance of immunized G. mellonella larvae against the injected metalloproteinase thermolysin is mediated by an inducible metalloproteinase inhibitor that was not detectable in untreated larvae (Wedde et al., 1998). This molecule was successfully purified and characterized at molecular level. It represents the first specific metalloproteinase inhibitor identified in invertebrates. The inducible insect metalloprotease inhibitor (IMPI) molecule is glycosylated, contains ten cysteine residues, which are presumed to form five disulfide bridges, and has a molecular mass of 8360 Da. Its partially determined amino-acid sequence exhibits no homology with other known proteins (Wedde et al., 1998).

In this paper we report that acquired resistance of G. mellonella against injected thermolysin and IMPI induction can be mediated by pre-injection with a sublethal dose of this metalloproteinase. Injected thermolysin is sufficient to induce the IMPI which is, surprisingly, simultaneously synthesized in G. mellonella with lysozyme and cecropin-like molecules. This observation erected the question how can the insect immune system recognize injected metalloproteinase molecules which could in principle digest all proteins involved in non-self recognition and mediating the activation of humoral immune responses? Our experiments were designed to elucidate the unknown mechanisms behind the recognition and inactivation of non-regulated metalloproteinases in the haemolymph of insects. They were initiated by the idea that non-regulated proteases may be recognized by the products of haemolymph protein degradation.

2. Material and methods

2.1. Insects

Last instar G. mellonella larvae, each weighing between 250–350 mg, were used in the mortality studies and as a source of haemolymph samples. The larvae were reared on an artificial diet (22% maize meal, 22% wheat germ, 11% dry yeast, 17.5% bee wax, 11% honey and 11% glycerine) at 31°C in darkness.

2.2. Injection and bleeding

Soluble (zymosan and LPS) or cellular (yeast cells, bacteria) elicitors of immune responses were dissolved or suspended within bidistilled water which was also used for control injections. Provocator solutions or suspensions were injected dorsolaterally into the haemocoel of G. mellonella larvae using 1 ml disposable syringes and 0.4×20 needles (Luer) mounted on a microapplicator. The injected volume was always 10 μl. A total of 10 larvae were injected per provocator, and each treatment was replicated twice. Haemolymph samples were obtained as follows: last instar larvae were precooled at 4°C for 15 min, one of each larva’s prolegs was pierced with a needle and haemolymph outflow was collected in sterile glass capillaries that were prepared with a few crystals of phenylthiourea to prevent melanization. Prior to use in assays for antibacterial activity or thermolysin inhibitory activity, haemolymph was rendered cell-free by centrifugation (2×10000 g, 5 min, 4°C).

2.3. Mortality studies

In order to determine whether pre-injection of G. mellonella larvae with either thermolysin (0.5 μg/larva) or peptidic fragments obtained after in vitro hydrolysis of haemolymph proteins is sufficient to mediate immunity against this metalloproteinase we injected 2 μg/larva into untreated and immunized larvae. The mortality of untreated and pre-injected larvae was monitored daily. The experiments were performed with groups of 10 individual larvae and repeated twice. The percentage of mortality was calculated from the total of all three experiments (30 larvae per group).

2.4. Preparation of inducers

Lipopolysaccharide (LPS) from bacterial cell walls is a known soluble microbial agent that induces high levels of antibacterial proteins in G. mellonella and other insects (De Verno et al., 1984). Five milligrams LPS (Sigma, purity 93%) was dissolved in 1 ml saline, incubated for 30 min at 55°C and ultrasonicated (2 s). The soluble fraction (β 1–3 glucans) of a zymosan suspension (preparation of freeze dried yeast cells) was used.
to provoke a humoral immune response. Twenty milligrams of zymosan A (Sigma) were suspended in 1 ml sterile saline, homogenized with a whirlmixer and centrifuged (10,000 g, 5 min). The supernatant that resulted from a second centrifugation was injected. The injected volume of both solutions was always 10 μl/larva.

2.5. Assaying of antibacterial activity

Lysozyme-activity was monitored with a lytic zone assay against freeze-dried Micrococcus luteus (Sigma) in accordance with Mohrig and Messner (1968). 5 ml of sterile M. luteus agar (1% Agar L 11, Oxoid; 5 mg/ml freeze-dried M. luteus, Sigma; 0.1 mg/ml streptomycin sulphate, Serva and 67 mM potassium phosphate buffer, pH 6.4) was placed into Petri dishes (9 cm). Holes with a diameter of 3 mm were punched into the agar and filled with 4 μl of cell-free haemolymph. The diameters of the lytic zones were measured after 24 h of incubation in the Petri dishes maintained at 37°C. Hen egg white lysozyme (Sigma) was used for calibration.

Activity against Escherichia coli was measured by inhibition zone assay with a lipopolysaccharide-defective, streptomycin- and ampicillin-resistant mutant of E. coli K12 strain D31 (Boman et al., 1974) in accordance with Faye and Wyatt (1980). Petri dishes (9 cm) were filled with 5 ml agar suspension (2.5% nutrient broth I, pH 6.4) was placed into Petri dishes (9 cm). Holes with a diameter of 3 mm were punched into the agar and filled with 4 μl of cell-free haemolymph. The diameters of the lytic zones were measured after 24 h of incubation in accordance with Mohrig and Messner (1968). 5 ml of cell-free haemolymph and thereby diluted 1:1. The final protease activity was measured by diluting only haemolymph or protease stock solutions in bidistilled water. All samples were transferred into sterile Eppendorf tubes and then incubated for 1 h on a rotary shaker at 36°C.

To remove proteases and other large molecules after incubation, all samples were subsequently ultrafiltered by using Centricron centrifugal concentrators (Centricon-30, Centricon-10 and Centricon-3: 30,000, 10,000 and 3000 molecular mass cut-off, Amicon, Inc.). Molecules with a molecular weight above the membrane cut-off are retained in the sample reservoir. Samples were centrifuged by using a fixed-angle rotor for 4–6 h (7000 g, 4°C). Filtrates containing solvent and proteins were replaced by buffer A. The microtiter plates were incubated on a rotary shaker at 37°C for 2 h. Suspended azocoll was pelleted (1200 g, 2 min) using a centrifuge equipped with an adapter for microtiter plates and the clear supernatant (150 μl) was transferred with a multipipette into another microtiter plate. The absorbance of azo-dye liberated by the proteolytic activity of thermolysin was measured with an Microtiterplate reader at 492 nm. The difference of absorbances between positive and negative controls (mean value of six measurements) was taken as representing 100% of thermolysin activity. For each sample assayed, a plot of absorbance vs dilution was constructed to calculate the dilution factor corresponding to 50% inhibition of thermolysin activity.

2.7. Preparation of hydrolysed haemolymph protein fragments

Cell-free haemolymph was obtained as described above. Protease stock solutions were prepared using sterile bidistilled water as solvent. Protease stock solutions (500 μl) were added to the same volume of cell-free haemolymph and thereby diluted 1:1. The final thermolysin concentration was 1 mg, 100 μg or 10 μg/larva. Control samples were made by diluting only haemolymph or protease stock solutions in bidistilled water. All samples were transferred into sterile Eppendorf tubes and then incubated for 1 h on a rotary shaker at 36°C.

Thermolysin and thermolysin-inhibiting activity were measured with the azocoll assay according to Chavira et al. (1984). The method was modified and adapted for measurements with a microtiterplate photometer (Wedde et al., 1998). One hundred and fifty milligrams of azocoll (Sigma) were suspended in 10 ml of buffer A (50 mM Tris/HCl, pH 7.5) and washed for 1 h by whirling. The suspension was centrifuged (10,000 g, 5 min); the azocoll was then resuspended in 10 ml of fresh buffer A and agitated for another two hours. The assayed sample (100 μl) was pipetted into a flat-bottomed microtiter plate and serially diluted in buffer A. Subsequently, 100 μl thermolysin solution (1 μg/ml) and 100 μl of a thoroughly homogenized azocoll suspension (15 mg/ml) were added into each well. In negative controls (no proteolytic activity) and positive controls (no inhibitory activity) thermolysin and sample aliquots, respectively, were replaced by buffer A. The microtiter plates were incubated on a rotary shaker at 37°C for 2 h. Suspended azocoll was pelleted (1200 g, 2 min) using a centrifuge equipped with an adapter for microtiter plates and the clear supernatant (150 μl) was transferred with a multipipette into another microtiter plate. The absorbance of azo-dye liberated by the proteolytic activity of thermolysin was measured with an Microtiterplate reader at 492 nm. The difference of absorbances between positive and negative controls (mean value of six measurements) was taken as representing 100% of thermolysin activity. For each sample assayed, a plot of absorbance vs dilution was constructed to calculate the dilution factor corresponding to 50% inhibition of thermolysin activity.

2.6. Assay for measurement of thermolysin-inhibitory activity

Thermolysin and thermolysin-inhibiting activity were measured with the azocoll assay according to Chavira et al. (1984). The method was modified and adapted for measurements with a microtiterplate photometer (Wedde et al., 1998). One hundred and fifty milligrams of azocoll (Sigma) were suspended in 10 ml of buffer A (50 mM Tris/HCl, pH 7.5) and washed for 1 h by whirling. The suspension was centrifuged (10,000 g, 5 min); the azocoll was then resuspended in 10 ml of fresh buffer A and agitated for another two hours. The assayed sample (100 μl) was pipetted into a flat-bottomed microtiter plate and serially diluted in buffer A. Subsequently, 100 μl thermolysin solution (1 μg/ml) and 100 μl of a thoroughly homogenized azocoll suspension (15 mg/ml) were added into each well. In negative controls (no proteolytic activity) and positive controls (no inhibitory activity) thermolysin and sample aliquots, respectively, were replaced by buffer A. The microtiter plates were incubated on a rotary shaker at 37°C for 2 h. Suspended azocoll was pelleted (1200 g, 2 min) using a centrifuge equipped with an adapter for microtiter plates and the clear supernatant (150 μl) was transferred with a multipipette into another microtiter plate. The absorbance of azo-dye liberated by the proteolytic activity of thermolysin was measured with an Microtiterplate reader at 492 nm. The difference of absorbances between positive and negative controls (mean value of six measurements) was taken as representing 100% of thermolysin activity. For each sample assayed, a plot of absorbance vs dilution was constructed to calculate the dilution factor corresponding to 50% inhibition of thermolysin activity.

3. SDS–PAGE

Electrophoresis was performed on Polyacrylamide gels (80×70×0.75 mm) using Mighty Small II mini-sys-

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prepared under reducing or non-reducing conditions according to Laemmli (1970). Running conditions were 10–15 min at 100 V and 45–60 min at 200 V. The gels were stained with Coomassie Brilliant Blue R-250 (CBB) or overstained with silver nitrate as described by Görg et al. (1985). The markers were obtained from Pharmacia (LMW calibration kit: 94, 67, 43, 30, 20.1, 14, 4 kDa).

3.1. FPLC and HPLC analysis

Fast protein liquid chromatography (FPLC) served for separation of small haemolymph protein fragments obtained after ultrafiltration and subsequent concentration of 1 ml samples using a vacuum centrifuge (speed-vat). The pellet containing fragments <3 kDa was dissolved in 200 µl sterile filtered, bidistilled water and injected onto the Superose 12 HR 10/30 FPLC column (Pharmacia, Uppsala, Sweden) and eluted with NaCl (1 mol/l) dissolved in sterile filtered, bidistilled water. Twenty microlitres of this preparation was dissolved in 200 µl sterile filtered, bidistilled water a flow rate of 0.5 ml/min.

Reverse-phase HPLC was performed on a Spherisorb RP-18.5 micron column (Spherisorb Co.) using a complete system (Kontron Instruments) with a flow rate of 1 ml/min. One millilitre of the <3 kDa fraction was concentrated with a speed-vac and reconstituted in 200 µl min. One millilitre of the complete system (Kontron Instruments) with a flow rate of 5/RP-18.5 micron column (Spherisorb Co.) using a common (Pharmacia, Uppsala, Sweden) and eluted with NaCl (1 mol/l) dissolved in sterile filtered, bidistilled water. Twenty microlitres of this preparation was dissolved in 200 µl sterile filtered, bidistilled water a flow rate of 0.5 ml/min.

For elution, a 30-min linear gradient from 5 to 50% B was used. Fractions were collected manually according to their absorption at 215 nm monitored by a Waters model 486 UV-detector. One milliliter fractions were obtained after FPLC or reverse phase HPLC separation of samples containing haemolymph fragments of size smaller than 3 kDa.

3.2. Detection and characterization of proteolytically digested haemolymph proteins

Cell-free haemolymph from untreated larvae was diluted 1:2 in a thermolysin solution (2 mg/ml) and incubated for 1 h at room temperature. Control samples were correspondingly diluted with bidistilled water. All samples were subsequently ultrafiltered through 100, 30, 10 and 3 kDa membranes as described above. Obtained fractions were injected into G. mellonella larvae to test their immune stimulatory activity. Heat sensitivity of haemolymph protein fragments was tested by incubation of obtained fractions for 15 min in a water bath at a temperature of 50, 70 or 100°C, respectively. In addition, we compared the immune stimulatory activity of fresh preparations with fractions which were stored at −20°C until use.

3.3. Statistics

Data are presented as means±standard deviation. Significance of differences was calculated with the Student’s t-test (p=0.001).

4. Results

4.1. Mortality studies

Injection of thermolysin at a sublethal concentration (0.5 µg/larva) increased resistance of treated G. mellonella larvae against a second injection with a toxic concentration of this metalloproteinase. Injection of 2 µg/larva resulted in 70% mortality within 4 days in untreated larvae, whereas pre-injected larvae survived this treatment. Similar results were obtained after injection of in vitro produced small-sized peptides isolated by ultrafiltration of samples containing thermolysin-hydrolysed haemolymph proteins. Untreated larvae exhibited 80% mortality within 2 days post injection whereas only 10% of larvae pre-injected with immune stimulatory peptides died in this period (Fig. 1). Cadavers of killed larvae became black, suggesting that injected thermolysin elicits activation of the prophenoloxidase which mediates melanization.

4.2. Induction of immune responses by injected proteases

Injection of thermolysin, pronase or trypsin at sublethal concentrations resulted in significantly enhanced lysozyme and cecropin-like activity within the cell-free haemolymph of injected G. mellonella larvae. Induced levels measured 24 h after injection of the metalloproteinases thermolysin (0.5 µg/larva) or pronase (0.5 µg/larva) were comparable to those monitored after injection of microbial elicitors of humoral immune responses such as the soluble components of LPS (10 µg/larva) or zymosan (200 µg/larva) (Fig. 2).

The results obtained from the lytic zone assay and the inhibition zone assay against living E. coli were confirmed by electrophoretic analysis of cell-free haemolymph samples from untreated and injected larvae. The same new or enhanced bands occurred in haemolymph samples in response to injected microbial elicitors (zymosan, LPS), proteases (thermolysin, pronase or trypsin) or thermolysin-processed haemolymph protein fragments <3 kDa with immune stimulatory activity. For example, the lysozyme band around 15 kDa was enhanced in all haemolymph samples obtained from pre-injected larvae. Similar changes in protein pattern confirm the potency of injected metalloproteinases to stimulate humoral immune responses which are equivalent to
Fig. 1. Survival of untreated and pre-injected *G. mellonella* larvae after injection of bacterial thermolysin (2 μg/larva). Last instar larvae which were pre-injected with thermolysin at a sublethal concentration (0.5 μg/larva) or with peptidic fragments obtained after thermolysin-mediated haemolymph protein hydrolysis in vitro exhibited remarkably higher survival than untreated larvae (*n*=30 larvae per group).

After injection of microbial elicitors such as LPS or β-1,3 glucans (Fig. 3).

4.3. **Immune stimulatory activity of proteolytically digested haemolymph proteins**

Injection of fractions obtained after ultrafiltration of thermolysin-digested haemolymph samples enhanced lysozyme activity in the cell-free haemolymph of injected larvae to a higher degree than control injections with non-hydrolysed haemolymph samples. Obtained fractions containing haemolymph protein fragments smaller than 3 kDa were the most potent elicitors of lysozyme. Such peptides were only obtained after in vitro hydrolysis of cell-free haemolymph with the metalloproteinases thermolysin or pronase. Addition of trypsin, even in high amounts, did not result in production of immune stimulatory peptides which could be due to trypsin inhibitors present in the cell-free haemolymph (Fig. 4).

To rule out the possibility that the immune stimulatory activity of proteolytically digested haemolymph samples is due to products of thermolysin self-digestion or impurities in our thermolysin preparation, we repeated the experiments using haemolymph samples from pre-injected *G. mellonella* larvae containing IMPI.
lysin was added to haemolymph samples from untreated larvae in a concentration which is reportedly inactivated by IMPI present in the haemolymph of pre-injected larvae (Wedde et al., 1998). Addition of thermolysin to haemolymph samples from injected larvae containing IMPI at a concentration sufficient to completely inhibit the thermolysin activity did not result in production of haemolymph proteins smaller than 3 kDa with immune stimulatory activity (Fig. 5). Only injection of thermolysin was added to haemolymph samples from untreated larvae in a concentration which is reportedly inactivated by IMPI present in the haemolymph of pre-injected larvae (Wedde et al., 1998). Addition of thermolysin to haemolymph samples from injected larvae containing IMPI at a concentration sufficient to completely inhibit the thermolysin activity did not result in production of haemolymph proteins smaller than 3 kDa with immune stimulatory activity (Fig. 5). Only injection of thermolysin was added to haemolymph samples from untreated larvae in a concentration which is reportedly inactivated by IMPI present in the haemolymph of pre-injected larvae (Wedde et al., 1998). Addition of thermolysin to haemolymph samples from injected larvae containing IMPI at a concentration sufficient to completely inhibit the thermolysin activity did not result in production of haemolymph proteins smaller than 3 kDa with immune stimulatory activity (Fig. 5). Only injection of thermolysin was added to haemolymph samples from untreated larvae in a concentration which is reportedly inactivated by IMPI present in the haemolymph of pre-injected larvae (Wedde et al., 1998). Addition of thermolysin to haemolymph samples from injected larvae containing IMPI at a concentration sufficient to completely inhibit the thermolysin activity did not result in production of haemolymph proteins smaller than 3 kDa with immune stimulatory activity (Fig. 5). Only injection of thermolysin was added to haemolymph samples from untreated larvae in a concentration which is reportedly inactivated by IMPI present in the haemolymph of pre-injected larvae (Wedde et al., 1998). Addition of thermolysin to haemolymph samples from injected larvae containing IMPI at a concentration sufficient to completely inhibit the thermolysin activity did not result in production of haemolymph proteins smaller than 3 kDa with immune stimulatory activity (Fig. 5). Only injection of thermolysin was added to haemolymph samples from untreated larvae in a concentration which is reportedly inactivated by IMPI present in the haemolymph of pre-injected larvae (Wedde et al., 1998). Addition of thermolysin to haemolymph samples from injected larvae containing IMPI at a concentration sufficient to completely inhibit the thermolysin activity did not result in production of haemolymph proteins smaller than 3 kDa with immune stimulatory activity (Fig. 5). Only injection of thermolysin was added to haemolymph samples from untreated larvae in a concentration which is reportedly inactivated by IMPI present in the haemolymph of pre-injected larvae (Wedde et al., 1998). Addition of thermolysin to haemolymph samples from injected larvae containing IMPI at a concentration sufficient to completely inhibit the thermolysin activity did not result in production of haemolymph proteins smaller than 3 kDa with immune stimulatory activity (Fig. 5).
sin-hydrolysed and ultrafiltered haemolymph samples from untreated larvae, which did not contain detectable inhibitory activity against thermolysin, elicited an remarkable increase of lysozyme (Fig. 5). Similar results were obtained when the induced IMPI activity was measured instead of lysozyme activity. Small sized products of proteolytically processed haemolymph proteins elicited a tremendous increase in inhibitory activity against thermolysin, whereas control injections with bidistilled alone water or thermolysin-treated or non-treated haemolymph samples from pre-injected larvae resulted in significantly lower or not detectable IMPI activity in pre-injected larvae (Fig. 5). These results lead to the conclusion that small peptides produced by thermolysin-mediated hydrolysis of haemolymph protein represent potent stimuli of humoral immune responses, that include the release of IMPI.

4.4. Induction of humoral immune responses by injected haemolymph protein fragments

Neosynthesis of lysozyme, cecropin-like molecules and IMPI in response to injected peptides obtained after haemolymph protein hydrolysis was determined using specific inhibitors of protein synthesis such as actinomycin D and cycloheximide. Simultaneous injection of one of these compounds at a sublethal concentration along with the fraction containing <3 kDa haemolymph protein fragments significantly inhibited the induced synthesis of lysozyme, cecropin-like molecules and IMPI (Fig. 6). These results clearly indicate that proteolytic digestion of haemolymph proteins yields peptide fragments which induce the synthesis of immune proteins in *G. mellonella*. These products of haemolymph protein hydrolysis were found to be highly heat stable and did not lose activity after long term storage at -20°C (data not shown).

Before we attempted to further purify and characterize these peptide fragments, we intended to clarify the question whether protein fragments smaller than 3 kDa are generally potent elicitors of humoral immune responses in *G. mellonella*. Therefore, we added thermolysin to dissolved bovine serum albumin (BSA) or to commercially available purified proteins which are commonly used as low molecular weight markers in electrophoretic analysis. The protein content in the solutions was adjusted to that measured in cell-free in *G. mellonella* haemolymph, and the production of smaller sized products of hydrolysis was confirmed by electrophoretic analysis (data not shown). Injection of thermolysin-processed fractions containing <3 kDa fragments of hydrolysed BSA or low molecular weight (LMW) marker proteins caused no or significantly lower increases of lysozyme activity than those obtained from hydrolysed haemolymph proteins. This observation ruled out the possibility that small-sized protein fragments in general
are potent elicitors of humoral immune responses (Fig. 7).

4.5. Separation of peptides obtained after thermolysin-mediated haemolymph protein hydrolysis

FPLC-separation of <3 kDa filtrates containing thermolysin-processed haemolymph protein fragments using a Superose 12 column yielded fractions which exhibited more or less humoral immune responses when injected into *G. mellonella* larvae suggesting insufficient separation of peptidic compounds. Reverse-phase HPLC separation of <3 kDa filtrates containing immune stimulatory haemolymph protein fragments yielded fractions containing a number of distinct products which were separated according to their molecular mass and hydrophobic properties. Haemolymph protein fragments were detected by comparison of untreated cell-free haemolymph samples with thermolysin-processed haemolymph samples. The elution profile of the latter consisted of a number of peaks that were not detectable in <3 kDa filtrate of untreated haemolymph or thermolysin alone. Interestingly, not all of the separated haemolymph protein fragments exhibited immune stimulatory activity when injected. A number of peaks, detectable at the elution profile after 20 min, was separated, and pooled. This fraction was determined to contain compounds with high immune stimulatory activity (Fig. 8). FPLC and HPLC separation confirmed that thermolysin-mediated hydrolysis of haemolymph proteins yields a number of fragments with immune stimulatory activity which was not due to impurities in the thermolysin preparation or to products of its self digestion.

Combined FPLC- and reverse phase HPLC separation

**Fig. 7.** Lysozyme activity within the cell-free haemolymph of *G. mellonella* larvae 24 h after injection of <3 kDa protein fragments obtained after thermolysin-mediated in vitro hydrolysis of BSA, low molecular weight marker proteins or haemolymph proteins which were adjusted to equivalent protein contents by dilution in bidistilled water. Only ultrafiltrates containing <3 kDa haemolymph protein fragments exhibited a strong immune stimulatory activity as detected by lysozyme activity. Data are given as mean±standard deviation of at least eight measurements (*n*=10 larvae per group).

**Fig. 8.** Reverse phase HPLC separation of the <3 kDa filtrate obtained after ultrafiltration of thermolysin (A), cell-free haemolymph of untreated larvae (B) and cell-free haemolymph hydrolysed with thermolysin for 1 h (C). Thermolysin-mediated processing of haemolymph proteins in vitro yielded a number of small sized compounds (labelled with fat arrows) which exhibited immune stimulatory effects when injected whereas others did not (arrows).
techniques enabled the preparation of a number of fractions exhibiting a single peak on the elution profile. However, their individual peptidic content was too low to allow aminoacid sequencing.

5. Discussion

Injection of thermolysin at a sublethal concentration into untreated G. mellonella larvae, lacking inhibitor molecules against metalloproteinases within the haemolymph, elicited humoral immune responses which were comparable to those observed after challenge with microbial elicitors such as LPS or a zymosan preparation. Pre-injected larvae exhibited enhanced levels of lysozyme and cecropin-like molecules and increased IMPI activity within their haemolymph. The enhanced lysozyme, cecropin-like or IMPI activity in the cell-free haemolymph of injected larvae reached similar levels although thermolysin was injected at a 20-times lower concentration than LPS. This observation suggests that the capacity of metalloproteinase to elicit humoral immune responses in G. mellonella is remarkably higher compared to LPS which is commonly used as a soluble inducer of immune responses in insects (Gillespie et al., 1997). In this context it is noteworthy that we detected metalloproteinase activity in dissolved samples of commercially available LPS (93% purity) which is probably due to unidentified impurities. This observation is important because the same material is commonly used to stimulate immune responses under experimental conditions. We did not attempt to discriminate to which extent the immune stimulatory activity of LPS could be mediated by metalloproteinase impurities detected in commercially available preparations.

Endogenous proteinases are normally regulated by endogenous inhibitors (Kanost et al., 1990; Kanost and Jiang, 1996). Non-regulated proteolytic activity can be released within the haemolymph by wounded tissues or invading pathogens. The latter employ hydrolytic enzymes suited to provide nutrients from the host during infection. Entomopathogenic viruses, bacteria and fungi produce metalloproteinases, which have been recognized to play key roles in pathogenesis. In contrast, specific metalloproteinase inhibitors have not been previously reported from invertebrates (Kanost and Jiang, 1996). The discovery of the IMPI within the haemolymph of immunized G. melonella larvae (Wedde et al., 1998) and its determined induction after injection of thermolysin raised the question about the recognition and inactivation of non-regulated proteolytic activity within the haemolymph of insects. Regarding the physiological impact of non-regulated proteolysis within the haemolymph, we postulated an efficient mechanism which provides rapid control of protein degradation in order to maintain the homeostatic balance.

The results of our experiments clearly demonstrate that particular <3 kDa products of haemolymph protein hydrolysis are potent elicitors of humoral immune responses when injected into G. mellonella larvae. In vitro incubation of cell-free haemolymph samples from untreated larvae with thermolysin yielded protein fragments which induced remarkable stronger immune responses than injection of non-hydrolysed haemolymph samples. The highest inducing capacity was in the fraction containing haemolymph protein fragments smaller than 3 kDa. Such molecules were not detectable when thermolysin was added at the same concentration to cell-free haemolymph from immunized larvae that contained the IMPI.

The results of our study allow us to postulate a novel pathway for the induction of immune responses, which can be explained by the following model (Fig. 9). Proteases released by wounded tissues or invading pathogens hydrolyse haemolymph proteins. Particular small sized products of haemolymph protein hydrolysis activate a novel pathway leading to expression of inducible proteins such as lysozyme, cecropins and IMPI. The latter inactivates released metalloproteinases, thereby restricting further production of haemolymph protein fragments and regulating the induction of humoral immune responses. Induced expression of the genes encoding for lysozyme, cecropins and IMPI was demonstrated using specific inhibitors of protein syntheses such as actinomycin D and cycloheximide.

The protein concentration in the fraction with the small sized products of haemolymph protein hydrolysis was below the detection limit of the Bradford assay. Even electrophoretic separation of this fractions and subsequent staining with silver was insufficient for detection and visualization of the immune stimulatory molecules. From these results we concluded that the protein concentration in the fraction with fragments of size <3 kDa was below 50 ng, the detection limit of silver staining in combination with SDS–gel electrophoresis. Injection of such fractions induced levels of lysozyme and metalloproteinase inhibitory activity within the haemolymph of G. mellonella larvae which were comparable to those observed in response to injected LPS at a concentration of 50 μg/larva. In conclusion, the immune stimulatory activity of these haemolymph protein fragments is several orders higher than that of LPS. According to our knowledge, they probably represent the most potent soluble elicitors of humoral immune responses in insects.

The intense immune stimulatory potency of proteolytically processed haemolymph protein fragments give evidence for an efficient physiologic mechanism which provides rapid inactivation of non-regulated proteases. In agreement, increasing IMPI concentrations were detected in G. mellonella larvae during natural infection with the entomopathogenic fungus M. anisopliae (Wedde et al., 1998) which is known to produce a
Fig. 9. Summarizing obtained results we designed this model describing recognition and regulation of proteolytic activity in the haemolymph of the greater wax moth *G. mellonella*: 1 Endogenous or microbial metalloproteinases such as thermolysin released within the haemolymph, 2, hydrolyse haemolymph proteins, 3, thereby producing <3 kDa fragments, 4, which are recognized by presently unidentified receptor molecules. The latter activate a signaling pathway, 5, leading to transcription, 6, and cytosolic translation of lysozyme, cecropin like molecules and IMPI, 7. Among the induced proteins which are secreted into the haemolymph, 8. IMPI regulates the released metalloproteinases thereby inhibiting the production of further immune stimulatory haemolymph fragments.

thermolysin-like metalloproteinase (St Leger et al., 1994). The activity of such pathogen-derived metalloproteinase is probably sufficient to produce haemolymph protein fragments which induce the observed synthesis of the metalloproteinase inhibitor in *M. anisoyliae* infected *G. mellonella* larvae. If the release of small-sized haemolymph protein fragments represents a sensitive novel pathway for the induction of humoral immune responses in insects, then we can postulate that entomopathogens limit the synthesis of extracellular proteases during infection. Indeed, the synthesis of *M. anisupliae* proteases is strongly regulated by carbon and nitrogen repression (Clarkson and Charnley, 1996). The regulation of protease production plausibly helps to avoid activation of immune responses. This explains, for example, why only limited or no haemolymph protein degradation is detectable during early stages of fungal development within the living host (Vilcinskas and Matha, 1997a). Excessive proteolytic degradation of host proteins probably occurs after overcoming of the immune system of the host or its death (Vilcinskas and Matha, 1997b). Suppression of immune responses in *G. mellonella* by *M. anisopliae* has been attributed to its secondary metabolites. For example, destruxins at sub-lethal concentrations were shown to inhibit in phagocytosis, attachment, spreading and cytoskeleton formation of isolated plasmatocytes (Vilcinskas et al., 1997a,b). The latter represent an immune competent cell type which contribute to antifungal cellular defense reactions in this insect. Evidence is available that both *M. anisopliae* infection in vivo and destruxin-treatment of isolated *G. mellonella* plasmatocytes in vitro induces
programmed cell death in this haemocyte type (Vilcinskas and Götz, 1999). Induction of apoptosis in immune competent haemocytes by released destruxins appears as a convincing strategy of *M. anisopliae* to overcome the immune system of the infected insect and to avoid the synthesis of inducible inhibitors against its proteolytic enzymes.

FPLC and reverse phase HPLC separation of fractions containing <3 kDa compounds independently exclude that thermolysin-mediated hydrolysis of haemolymph yields a single particular fragment with immune stimulatory activity. Furthermore, comparative analysis of obtained chromatograms ruled out the possibility that the release of immune stimulatory fragments is due to self digestion of thermolysin. However, a number of immune stimulatory haemolymph protein fragments were separated and determined to be highly heat stable.

The induction of humoral immune responses in insects (or other invertebrates) by proteolytically processed haemolymph proteins has not been previously reported. We speculate that the expression of inducible proteins such as lysozyme and IMPI is activated after interaction of such fragments with cell membrane associated receptors molecules of immune competent cells. This hypothesis correlates well with a model for induction of antifungal immune responses in insects which was recently introduced by Lemaitre et al. (1996). The authors demonstrated that the dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the antifungal response in *Drosophila* adults and provided evidence for the existence of at least two distinct pathways controlling the expression of antimicrobial genes. One pathway is postulated to be activated during humoral immune responses by as yet unidentified endogenous proteases which cleave the spätzle protein in the haemolymph to its active form. The latter has a cytokine-like role. Our results indicate that cleavage of haemolymph proteins by thermolysin results in a number of different fragments with strong immune stimulatory activity. Such haemolymph protein fragments can be released by pathogen-derived metalloproteinases and do not necessarily require endogenous proteases or proteolytic cascades as postulated in the model of Lemaitre et al. (1996). The recognition of distinct haemolymph protein fragments requires corresponding receptors with distinctive ligand-binding properties. We suggest the Toll-receptor family as suitable candidates. These receptors are coupled to signal transduction pathways that control expression of a variety of inducible immune response genes (Kopp and Medzhitov, 1999). The discovered immune stimulatory activity of metalloproteinases opens a new insight into the interactions between pathogen-derived proteases and inducible protease inhibitors of the host. This aspect has been neglected in the research concerning host-defense-related processes.

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**References**


Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.-M., Hoffmann, J.A., 1996. The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the antifungal response in *Drosophila* adults and provided evidence for the existence of at least two distinct pathways controlling the expression of antimicrobial genes. One pathway is postulated to be activated during humoral immune responses by as yet unidentified endogenous proteases which cleave the spätzle protein in the haemolymph to its active form. The latter has a cytokine-like role. Our results indicate that cleavage of haemolymph proteins by thermolysin results in a number of different fragments with strong immune stimulatory activity. Such haemolymph protein fragments can be released by pathogen-derived metalloproteinases and do not necessarily require endogenous proteases or proteolytic cascades as postulated in the model of Lemaitre et al. (1996). The recognition of distinct haemolymph protein fragments requires corresponding receptors with distinctive ligand-binding properties. We suggest the Toll-receptor family as suitable candidates. These receptors are coupled to signal transduction pathways that control expression of a variety of inducible immune response genes (Kopp and Medzhitov, 1999). The discovered immune stimulatory activity of metalloproteinases opens a new insight into the interactions between pathogen-derived proteases and inducible protease inhibitors of the host. This aspect has been neglected in the research concerning host-defense-related processes.

Lepore, L.S., Roelvink, P.R., Granados, R.R., 1996. Enhancin, the granulosis virus protein that facilitates nucleopolyhedrovirus (NPV) infections, is a metalloproteinase. J. Invertebr. Pathol. 68, 131–140.


