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Degradation of Insect Humoral Immune Proteins by the Proteases Secreted from *Enterococcus faecalis*

Shin Yong Park, Koung Mi Kim, Iksoo Kim[1], Sang Dae Lee[2] and In Hee Lee*

Department of Bio-Technology, Hoseo University, 29-1 Sechuli, Baebang-Myun, Asan City, Chungnam-Do 336-795, South Korea.
[1]College of Agriculture & Life Sciences, Chonnam National University, Gwangju 500-757, Republic of Korea.
[2]Department of Biology, Seonam University, Namwon City, Chunbuk-Do 590-711, South Korea

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***Enterococcus faecalis* was isolated from the body fluid of dead *Galleria mellonella* larvae. Upon injection of *E. faecalis* into the hemocoel of *G. mellonella*, the bacteria destroyed parts of humoral defense systems in the hemolymph. In a test for the proteolytic activity of *E. faecalis* CS, it was confirmed that the enzyme degraded three well-known á-helical antimicrobial peptides, cecropin A, melittin and halocidin, and abolished their activities. We also determined putative cleavage sites on the primary sequences of three peptides through purification and mass analysis of peptide fragments digested by *E. faecalis* CS. Furthermore it was found that apolipophorin-III, recently known as a critical recognition protein for invading microbes in the hemolymph of *G. mellonella*, was also degraded by *E. faecalis* CS. Taken together, the present work shows that the protease in secretions from *E. faecalis* destroyed two critical humoral immune factors in the hemolymph of *G. mellonella* larvae. In addition, this paper demonstrates that the relationship between the host insect and the pathogenic bacteria might provide a valuable model system to study the enterococcal virulence mechanism, which may be relevant to mammalian pathogenesis.**

Key words: *Enterococcus faecalis*; *Galleria mellonella*; Apolipophorin III; Metalloprotease; Antimicrobial peptides; Extracellular proteases

*To whom correspondence should be addressed.
Department of Bio-Technology, Hoseo University, 29-1 Sechuli, Baebang, Asan City Chungnam 336-795, South Korea.
Tel: +82 41 540 5626; Fax: +82 41 548 6231;
E-mail: leeih@office.hoseo.ac.kr

Introduction

Insects are the most prosperous animals on Earth in terms of the numbers of species. They have developed rapid and effective defense systems against a wide range of microbial infections even though they lack the adaptive immune systems present in vertebrates. As such, insects have often been considered good model systems for the study of bacterial virulence. Over the last three decades, a number of studies have been performed to elucidate the molecular mechanism of insect immune systems (Vilmos and Kurucz, 1998; Hultmark, 2003). In particular, recent advances in the field of insect immunity have provided clues on how invading microbial pathogens are recognized, killed and eliminated during an infection.

The immune system of insects consists of humoral and cellular reactions, which cooperatively function as a defense mechanism against microbial infections. Antimicrobial peptides (or proteins) such as cecropins (Moore *et al.*, 1996; Bulet *et al.*, 1996), insect defensins (Hughes, 1999; Lee *et al.*, 2004), lysosyme (Yu *et al.*, 2002) have been known to be critical components in the insect humoral immune system. They are synthesized in the fat body upon microbial infection and secreted into the hemolymph. They then play a key role in clearing the invading microbes from the hemolymph at the initial phase of immune reactions. Owing to these inducible antimicrobial agents, the bacteriolytic activity of the insect hemolymph increases dramatically after a microbial infection. On the other hand, invading microbes into the hemocoel of insects must overcome or avoid the immune systems occurring in the hemolymph to successfully proliferate in their hosts. In this context, the relationship between the entomopathogenic microbes and the insect's immune system has recently attracted considerable interest.

Photorhabdus luminescens and *Xenorhabdus nematophilus* are well-known symbiotic bacteria of the entomopathogenic nematodes of the genera *Heterorhabditis* and *Steinernema*, respectively (Ffrench-Constant and Bowen, 2000). The *P. luminescens* cells secrete a variety of proteins including proteases, lipases, and toxins during growth in various complex culture media with no nematode association (Chen *et al.*, 1996; Jeffke *et al.*, 2000). It has been considered that these proteinaceous secretions might cause a lethal effect on host insects, although there is little information about the specific mechanisms by which the defense system in the insect hemolymph is affected.

For more than a century, enterococci have been recognized as a cause of infective endocarditis and the most common pathogens found in hospital-acquired infection (Chavers *et al.*, 2003; Patel, 2003). Furthermore, the enterococcal infections have been clinically problematic due to their multi-drug resistance or their potential to serve as a reservoir for antibiotic resistant genes (Dzidic and Bedekovic, 2003). In spite of their importance, their pathogenic mechanisms are not yet well understood. We have recently isolated an enterococcal bacterium, *E. faecalis*, from the body fluid of dead *Galleria mellonella* larvae. Injected into the hemocoel of *G. mellonella* larvae, the proteinaceous fraction of *E. faecalis* culture supernatant exhibited highly potent proteolytic activity. We have also endeavored to understand how infecting *E. faecalis* overcomes the immune system in the hemolymph of *G. mellonella*. This paper describes the characterization of proteases as an immunosuppressor in secretions from *E. faecalis*, and demonstrates one of the mechanisms by which the immune system in the *G. mellonella* hemolymph is neutralized. We also herein present a simple model host to elucidate the virulence mechanism of enterococcal infections.

Materials and Methods

Bacterial cultures and insect rearing

A bacterial strain was isolated from the larval cadaver of *G. mellonella*. To identify the bacteria, a GN2 MicroPlate™ (BIOLOG, Hayward, CA, USA) was used to characterize a bacterial strain based on substrate utilization profiling. A single colony grown on a trypticase soy agar plate was streaked onto Biolog universal growth agar medium containing 5% sheep blood and incubated overnight at 37°C. Cells were suspended in normal saline (0.15%) and inoculated into the GN2 MicroPlate™. After incubation for 20 hrs, the resulting pattern was read using the Biolog automated Micro-Station™ instrument. *Enterococcus faecalis* ATCC51299 used as a control bacterium was obtained from the Korea Research Institute of Bioscience and Biotechnology. Stocks were maintained on petri plates containing 1% bacto-agar (Difco, USA) in tryptic soy broth (TSB: Difco, USA) (TSA). The cultures were incubated in TSB for 18 hrs at 37°C on a rotary shaker. The *E. coli* K112 strain was used for the immunization of insects. *Galleria mellonella* larvae were reared on an artificial diet as described in Frobius *et al.* (2000) at 37°C in the dark. Only the last instar larvae with a body weight of more than 160 mg were used in this study.

Degradation of apoLp-III by *E. faecalis* infection

At the predetermined post-infection time (4, 8, 12 and 16 hrs), the hemolymph was collected from larvae infected by *E. faecalis* (2.5×10^5 cells per larva) and each sample was subjected to tricine SDS-PAGE analysis. Duplicate gel was used for immunoblotting analysis performed with an Ab to apolipophorin III (apoLp-III), which was previously purified from *G. mellonella* hemolymph (Park *et al.*, 2005). The immunization of New Zealand white rabbit to apoLp-III and the collection of antiserum were carried out in the conventional manner. Also, the immuno- blotting analysis was performed as described in Towbin *et al.* (1992).

Preparation of concentrated culture supernatants

Each culture of *E. faecalis* or *E. faecalis* ATCC strain grown for 18 hrs in TSB was centrifuged at 10,000 × g for 10 min. Each 3 liter sample of the cell-free supernatant was concentrated to ~ 20 ml by ultrafiltration using a 10 kDa cutoff membrane (Amicon Model 8200, USA).

Antimicrobial activity of hemolymph from the immunized insects

To immunize insects, the *G. mellonella* larvae were infected by live *E. coli* (5×10^3 cells/larva). After further rearing for 24 hrs, the immunized insects were injected with the concentrated CS (5 μl/larva). Hemolymph was then collected after predetermined incubation times (15 min, 30 min, 45 min and 60 min) and centrifuged at 10,000 g for 20 min to remove hemocytes and cell debris. To measure the antimicrobial activity of each sample, the cell-free hemolymph was acid-extracted according to the procedure described in Yu *et al.* (2002). Briefly, cell-free plasma was mixed with the same volume of 10% acetic acid and stirred overnight at room temperature. The mixture was centrifuged at 10,000 g for 20 min and the acid-extracted sample was obtained as a supernatant. Antimicrobial activities of acid extracts were tested in a radial diffusion assay (Lehrer *et al.*, 1991). Briefly, the acid extract samples were completely dried in the Speed-Vac system (Centra Evaporator, Bioneer, Daejon, South Korea) and the stock protein solutions (4 mg/ml) were prepared in 0.01% acetic acid. Then protein samples were twofold serially diluted to 125 μg/

ml. Five microliters of each sample were loaded into 3-mm diameter wells that had been punched in underlay gels. Underlay gels contained a uniform dispersion of washed, mid-logarithmic *E. coli* (4×10^6 cells/10 ml) immobilized in a 1% agarose matrix that contained a buffer (9 mM sodium phosphate, 1 mM sodium citrate, pH 7.4) and 0.3 mg/ml of TSB powder (Difco, USA). After incubation at 37°C for 3 hrs, a 10 ml nutrient-rich overlay gel consisting of 6% TSB and 1% agarose was poured. The plates were then incubated overnight to allow the surviving bacteria to form colonies; the diameters of clearing zones that indicate antimicrobial activity were measured to the nearest 0.1 mm. Zone diameters were expressed in units (0.1 mm = 1 unit) and graphed against the $\log_{10}$ of the protein concentration. The experiments were repeated three times and mean values were used to make a graph.

Effect of *E. faecalis* culture supernatant on three purified immune proteins in hemolymph

The concentrated CS of *E. faecalis* was incubated for 30 min at room temperature with 2 μg of cecropin A (Kim *et al.*, 2004), lysozyme (Yu *et al.*, 2002) or apoLp-III (Park *et al.*, 2005) which were previously purified from hemolymph of *G. mellonella* larvae. The mixture was then subjected to tricine SDS-PAGE analysis.

Degradation of antimicrobial peptides by *E. faecalis* CS

The proteolysis activities of *E. faecalis* CS were tested against three antimicrobial peptides: cecropin A, melittin, a bee venom toxin, and 18 mer of halocidin, an antimicrobial peptide purified from hemocytes of tunicate (Jang *et al.*, 2002). Ten μg of each peptide sample was incubated with about 50 ng of *E. faecalis* CS for 30 min at room temperature. The mixture was applied to C18 RP-HPLC column (Vydac 218TP54) and eluted in a linear gradient concentration of acetonitrile in 0.1% trifluoroacetic acid. The molecular mass of each peptide fragment included in some major peaks from RP-HPLC was measured by MALDI-mass spectrometric analysis.

Other methods

A Model Mini-Protean 3 Cell (Bio-Rad, USA) was used to perform tricine SDS-PAGE (Schagger and Jagow, 1987). Protein concentrations were measured with bicinchoninic acid (Sigma, USA).

Results

Effect of *E. faecalis* secretion on hemolymph proteins

In an initial experiment, it was observed by tricine SDS-PAGE analysis that one hemolymph protein of the *G. mellonella* larvae vanished after injection of *E. faecalis* concentrated CS (Fig. 1A). Considering their electrophoretic mobility, it was postulated that the protein was an apoLp-III (Kreft *et al.*, 1992; Park *et al.*, 2005), which is known as a critical immune mediator in the insect hemolymph. In our following experiment, it was tested whether or not the amount of apoLp-III in hemolymph decreased upon *E. faecalis* infection. As shown in Fig. 1B, the apoLp-III band dramatically diminished at post-infection time of 12 hr and disappeared from the gel at 16 hrs. This result strongly supported our assumption that apoLp-III might be degraded by secretion from *E. faecalis*. In addition, we tested if the purified apoLp-III was affected by the *E. faecalis* secretion. Two μg of apoLp-III purified from *G.*

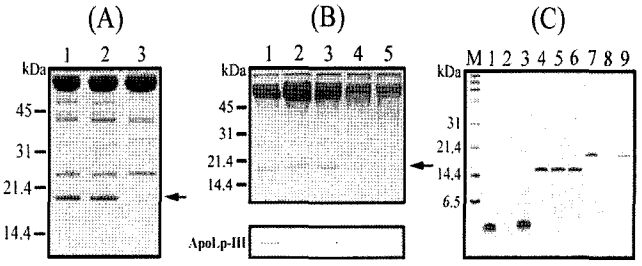

Fig. 1. Proteolytic activity of *E. faecalis* CS against cecropin A, Gm lysozyme and apoLp-III.

(A) The CS of *E. faecalis* or *E. faecalis* ATCC51299 was injected into hemocoel of naïve *G. melleonella* larvae. After 1 hr incubation, hemolymph of each group of insects was collected and subjected to SDS-PAGE analysis. Lane 1, hemolymph from normal larvae; lane 2, hemolymph from larvae injected by *E. faecalis* ATCC51299 CS; lane 3, hemolymph from larvae injected by *E. faecalis* CS. Arrow indicates an apoLp-III band. (B) Tricine SDS-PAGE and immunoblotting analyses performed with hemolymph from larvae infected by *E. faecalis*. Lane 1, normal hemolymph; lane 2, hemolymph from larvae at 4 hr after infection; lane 3, hemolymph from larvae at 8 hrs after infection; lane 4, hemolymph from larvae at 12 hrs after infection; lane 5, hemolymph from larvae at 16 hrs after infection. The equal amount of proteins (~18 μg) was loaded on each lane. Duplicate gel was subjected to an immunoblotting analysis using antibody to apoLp-III (lower panel). (C) After incubation of *E. faecalis* or *E. faecalis* ATCC51299 CS (~8 μg of proteins) with each peptide or protein (2 μg), the mixture was electrophoresed on tricine SDS-PAGE gel. Lane 1, purified cecropin A; lane 2, cecropin A incubated with *E. faecalis* CS; lane 3, cecropin A incubated with *E. faecalis* ATCC51299 CS; lane 4, purified Gm lysozyme; lane 5, Gm lysozyme incubated with *E. faecalis* CS; lane 6, Gm lysozyme incubated with *E. faecalis* ATCC51299 CS; lane 7, purified apoLp-III; lane 8, apoLp-III incubated with *E. faecalis* CS; lane 9, apoLp-III incubated with *E. faecalis* ATCC5129 CS.

mellonella hemolymph (Park *et al.*, 2005) was incubated with *E. faecalis* CS and subjected to SDS-PAGE analysis (Fig. 1C). As a result, the apoLp-III band disappeared from the gel, indicating it was degraded by a protease in the *E. faecalis* CS. Furthermore, we confirmed that the cecropin A (Kim *et al.*, 2004) was also destroyed by *E. faecalis* CS. In contrast, the insect lysozyme, another inducible antimicrobial protein previously purified from the hemolymph of *G. mellonella* larvae (Yu *et al.*, 2002), was not affected by *E. faecalis* CS. On the other hand, the *E. faecalis* ATCC51299 CS as a control sample did not exhibit a proteolytic activity toward three hemolymph proteins of *G. mellonella*.

Change of antibacterial activity of the immunized hemolymph after injection of *E. faecalis* CS

To assess the change of antibacterial activity of the immunized hemolymph after injection of *E. faecalis* CS, 5 μl of *E. faecalis* CS was injected into each of 40 larvae previously immunized against *E. coli*. Hemolymph was collected at a given post-injection time and acid-extracted by 10% acetic acid. When the antibacterial activity of each acid-extract sample was tested in radial diffusion assay (Fig. 2), it gradually decreased to the level of naïve hemolymph within 1 h. This observation allowed us to postulate that an inducible antibacterial peptide like

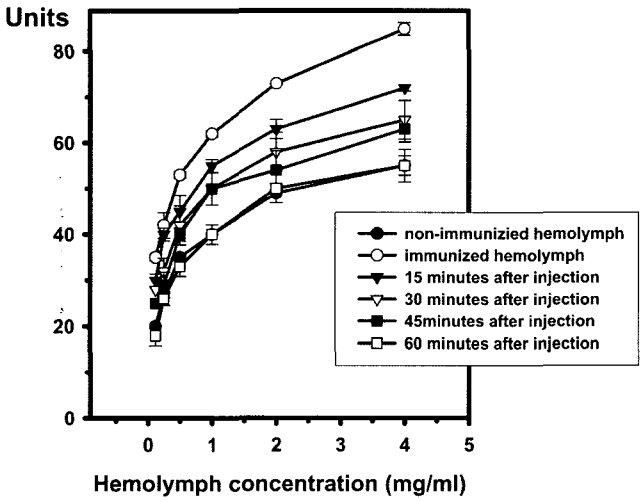

Fig. 2. Immunosuppression for the hemolymph of immunized *G. mellonella* larvae. Antimicrobial activity of *G. mellonella* hemolymph was reduced by the *E. faecalis* CS. After injection of *E. faecalis* CS into the *G. mellonella* hemoceol, hemolymph was collected from 10 insects at given post-injection times and then acid-extracted. Antimicrobial activities of the acid extracts of the hemolymph were assessed in a radial diffusion assay. Clearing zone diameter was expressed in units (10 units = 1 mm).

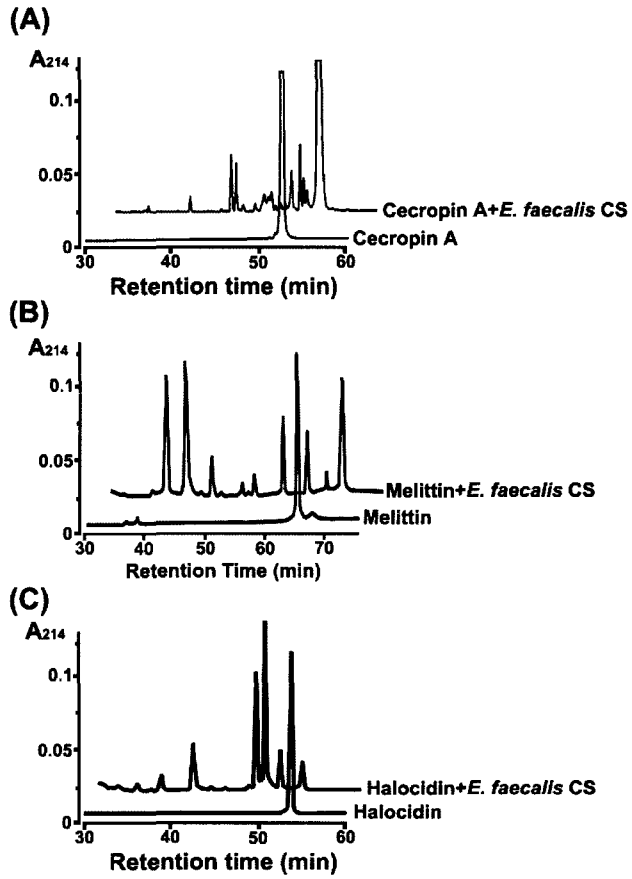

Fig. 3. RP-HPLC chromatograms of the digestion of cecropin A, melittin and halocidin by *E. faecalis* CS. (A) HPLC chromatograms of the digestion of purified cecropin A by *E. faecalis* CS. (B) HPLC chromatograms of the digestion of purified melittin by *E. faecalis* CS. (C) HPLC chromatograms of the digestion of purified 18 mer of halocidin by *E. faecalis* CS.

cecropin in the hemolymph might be attacked by *E. faecalis* CS.

Degradation of several antimicrobial peptides by *E. faecalis* CS

To examine the proteolysis effect of *E. faecalis* CS on antimicrobial peptides, three α-helical peptides (cecropin A, melittin and 18 mer of halocidin) were selected as test peptides. After incubation of each peptide with ~ 50 ng of *E. faecalis* CS for 30 min at room temperature, the mixture was applied to C18 RP-HPLC column to purify fragments generated by enzyme reaction (Fig. 3). Major fragments were tested for antimicrobial activities by radial diffusion assay and subjected to MALDI-TOF mass spectrometric analysis. All peptide fragments did not exhibit antibacterial activities (data not shown), and the part of each fragment was predicted by comparing the MALDI mass value with the calculated one (Table 1). In addition,

Table 1. MALDI mass values of fragments generated from digestion of antimicrobial peptide by *E. faecalis* CS, and putative fragments predicted by calculated mass.

Antimicrobial peptides	Putative fragments estimated from molecular mass	MALDI mass (Da)	Calculated mass (Da)
Cecropin A	KWKLFKKIEKVGQN	1747.15	1746.13
	GIIKAGPAVAVVGQA	1350.62	1347.67
Melittin	ISWIKRKREE	1341.80	1344.58
	TTGLPALISWIKR	1454.80	1455.76
	GIGAVLK	656.08	656.82
	GIGAVLKVLTTGLPAL	1522.86	1522.89
18mer of halocidin	GLNCAKG	659.50	661.77
	WLNALLHHG	1060.5	1060.2
	WLNALLHGLNCAKG	1646.94	1646.93

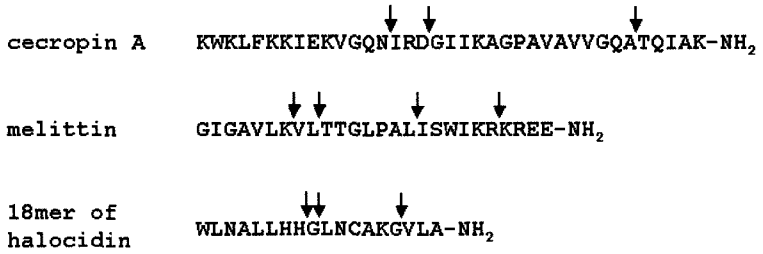

Fig. 4. Cleavage sites by *E. faecalis* CS are indicated as arrows on the sequence of antimicrobial peptides. They are determined by comparing MALDI mass values of peptide fragments with those calculated in Table 1.

the cleavage sites were determined and indicated (Fig. 4).

Discussion

The relationship between the virulence factors and the host immune system is critical to the understanding of bacterial pathogenic mechanisms. Some information about the bacterial virulent mechanism has recently come from studies on the insecticidal mechanism of several entomopathgenic bacteria. In the present work, we have shown that *E. faecalis* invading into the hemocoel of *G. mellonella* larvae destroys the humoral immune system. Although this effect was generated by an artificial injection of *E. faecalis* into the insect, our finding might make feasible the elucidation of the virulence mechanism of enterococcal infections that occur in mammals.

Antimicrobial peptides are considered pivotal components in the innate immune system of vertebrates and invertebrates. Recently, it has been demonstrated that common pathogenic bacteria, such as *E. faecalis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, use a variety of strategies to overcome the peptide-based innate defense system. Extracellular proteases from *P. aeruginosa*, *E. faecalis* and *Streptococcus pyogenes* have been shown to cause the release of dermatan sulfates, which bind to the antimicrobial peptide such as α-defensin and lead to its inactivation (Schmidtchen *et al.*, 2001). The pathogenic *S. aureus* modifies the membrane lipid phosphatidylglycerol with L-lysine. This modification leads to a reduced negative charge of the membrane surface and hinders the binding of cationic antimicrobial peptides to bacterial surface. As a result, the bacteria become resistant to several host defense peptides such as defensins and protegrins (Peschel *et al.*, 2001). Also, it has been reported that early in *Shigella* infections, expression of antimicrobial peptides is reduced or halted with bacterial DNA as a potential regulator (Islam *et al.*, 2001). Several modifications of the lipopolysaccharide (LPS) regulated by the PhoP-PhoQ two-component system contribute to the resistance of the pathogenic bacteria such as *Salmonella* spp. to antimicrobial peptides, and are necessary for their virulence (Ernst *et al.*, 1999). In addition, it has been shown that several extracellular proteases of pathogenic bacteria such as *P. aeruginosa*, *E. faecalis*, *Strptococcus pyogenes* and *Proteus mirabilis* degrade and inactivate an antimicrobial peptide LL-37 (Schmidtchen *et al.*, 2002). Taken together, bacterial evasion of the peptide-based immune system has to be considered as one of the key virulent mechanisms. To date, there have been conflicting

results on the insecticidal function of extracellular proteases from entomopathogenic bacteria. Toxin complexes purified from the culture supernatant of *P. luminescens* (Bowen and Ensign, 1998) have no correlation with proteolytic activities (Bowen *et al.*, 2000). In contrast, Jarosz (Jarosz, 1998) demonstrated that the activity of proteases plays a critical role in the active resistance of entomophagous rhabditid *Heterorhabditis bacteriophora* to insect immunity and is associated with its virulence. More recently, Caldas *et al.* (2002) also reported that an extracellular protease from *X. nematophilus* destroys cecropin-derived antibacterial activity in insect hemolymph although it has not been fully characterized.

ApoLp-III in the hemolymph of *G. mellonella* has been recently described as playing a role in the recognition of pathogen-associated molecular pattern and cellular immune reactions (Whitten *et al.*, 2004). Therefore, it was considered that the degradation of apoLp-III might result in neutralization of insect immune systems and invading microbes be protected from attacks of host immune systems. In the present work, we showed that the apoLp-III of *G. mellonella* disappeared from the hemolymph during *E. faecalis* infection and it was degraded by the *E. faecalis* CS. Accordingly, it was suggested that the immune functions of both apoLp-III and antimicrobial peptide were paralyzed during *E. faecalis* infection. On the other hand, the culture supernatant of *E. faecalis* ATCC strain used as a control bacterium in our works did not show proteolytic activities toward cecropin and apoLp-III.

In conclusion, this paper presents results that two humoral immune proteins including apoLp-III and cecropin, were attacked by the proteins secreted by *E. faecais*. Although we have not yet purified the protein that might be the protease, we believe that the present study provides an interesting insight into the virulence mechanism by the protease originated from the bacterium.

Acknowledgements

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References

Bowen, D., M. Blackburn, T. Rocheleau, C. Grutzmacher and R. H. Ffrench-Constant (2000) Secreted proteases from *Photorhabdus luminescens*: separation of the extracellular proteases from the insecticidal Tc toxin complexes. *Insect Biochem. Mol. Biol.* **30**, 69-74.

Bowen, D. J. and J. C. Ensign (1998) Purification and characterization of a high-molecular-weight insecticidal protein complex produced by the entomopathogenic bacterium *photorhabdus luminescens*. *Appl. Environ. Microbiol.* **64**, 3029-3035.

Bulet, P., Hetru C., Dimarcq, J.L. and Hoffmann, D. (1999) Antimicrobial peptides in insects; structure and function. *Dev. Comp. Immunol.* **23**, 329-344.

Caldas, C., A. Cherqui, A. Pereira and N. Simoes (2002) Purification and characterization of an extracellular protease from *Xenorhabdus nematophila* involved in insect immunosuppression. *Appl. Environ. Microbiol.* **68**, 1297-1304.

Chavers, L. S., S. A. Moser, W. H. Benjamin, S. E. Banks, J. R. Steinhauer, A. M. Smith, C. N. Johnson, E. Funkhouser, L. P. Chavers, A. M. Stamm and K. B. Waites (2003) Vancomycin-resistant enterococci: 15 years and counting. *J. Hosp. Infect.* **53**, 159-171.

Chen, G., Y. Zhang, J. Li, G. B. Dunphy, Z. K. Punja and J. M. Webster (1996) Chitinase activity of *Xenorhabdus* and *Photorhabdus* species, bacterial associates of entomopathogenic nematodes. *J. Invertebr. Pathol.* **68**, 101-108.

Dzidic, S. and V. Bedekovic (2003) Horizontal gene transfer-emerging multidrug resistance in hospital bacteria. *Acta. Pharmacol. Sin.* **24**, 519-526.

Ernst, R.K., T. Guina and S. I. Miller (1999) How intracellular bacteria survive: surface modifications that promote resistance to host innate immune responses. *J. Infect. Dis.* **2**, S326-S330.

Ffrench-Constant, R. H. and D. J. Bowen (2000) Novel insecticidal toxins from nematode-symbiotic bacteria. *Cell. Mol. Life. Sci.* **57**, 828-833.

Frobius, A.C., M. R. Kanost, P. Gotz and A. Vilcinskas (2000) Isolation and characterization of novel inducible serine protease inhibitors from larval hemolymph of the greater wax moth *Galleria mellonella*. *Eur. J. Biochem.* **267**, 2046-2053.

Hughes, A. L. (1999) Evolutionary diversification of the mammalian defensins. *Cell. Mol. Life. Sci.* **56**, 94-103.

Hultmark, D. (2003) *Drosophila* immunity: paths and patterns. *Curr. Opin. Immunol.* **15**, 12-19.

Islam, D., L. Bandholtz, J. Nilsson, H. Wigzell, B. Christensson, B. Agerberth and G. Gudmundsson (2001) Downregulation of bactericidal peptides in enteric infections: a novel immune escape mechanism with bacterial DNA as a potential regulator. *Nat. Med.* **7**, 180-185.

Jang, W.S., K. N. Kim, Y. S. Lee, M. H. Nam and I. H. Lee (2002) Halocidin: a new antimicrobial peptide from hemocytes of the solitary tunicate, *Halocynthia aurantium*. *FEBS Lett.* **19**, 81-86.

Jarosz, J. (1998) Active resistance of entomophagous rhabditid *Heterorhabditis bacteriophora* to insect immunity. *Parasitology* **117**, 201-208.

Jeffke, T., D. Jende, C. Matje, R. U. Ehlers and L. Berthe-Corti (2000) Growth of *Photorhabdus luminescens* in batch and glucose fed-batch culture. *Appl. Microbiol. Biotechnol.* **54**, 326-330.

Kim, C.H., J. H. Lee, I. Kim, S. J. Seo, S. M. Son, K. Y. Lee

and I. H. Lee (2004) Purification and cDNA cloning of a Cecropin-like Peptide from the Great Wax Moth, *Galleria mellonella*. *Mol. Cells* **17**, 262-266.

Kreft, B., R. Marre, U. Schramm and R. Wirth (1992) Aggregation substance of *Enterococcus faecalis* mediates adhesion to cultured renal tubular cells. *Infect. Immun.* **60**, 25-30.

Lee, Y. S., E. K. Yun, W. S. Jang, I. Kim, J. H. Lee, S. Y. Park, K. S. Ryu, S. J. Seo, C. H. Kim and I. H. Lee (2004) Purification, cDNA cloning and expression of an insect defensin from the great wax moth, *Galleria mellonella*. *Insect. Mol. Biol.* **13**, 65-72.

Lehrer, R. I., M. Rosenman, S. S. Harwig, R. Jackson and P. Eisenhauer (1991) Ultrasensitive assays for endogenous antimicrobial polypeptides. *J. Immunol. Methods* **137**, 167-173.

Moore, A. J., W. D. Beazley, M. C. Bibby and D. A. Devine (1996) Antimicrobial activity of cecropins. *J. Antimicrob. Chemother.* **37**, 1077-1089.

Park, S. Y., C. H. Kim, W. H. Jong, J. H. Lee, S. J. Seo, Y. S. Han and I. H. Lee (2005) Effects of two hemolymph proteins on humoral defense reaction in the wax moth, *Galleria mellonella*. *Dev. Comp. Immunol.* **29**, 43-51.

Patel, R. (2003) Clinical impact of vancomycin-resistant enterococci. *J. Antimicrob. Chemother.* **51**, 13-21.

Peschel, A., R. W. Jack, M. Otto, L. V. Collins, P. Staubitz, G. Nicholson, H. Kalbacher, W. F. Nieuwenhuizen, G. Jung, A. Tarkowski, K. P. van Kessel and J. A. van Strijp (2001) *Staphylococcus aureus* resistance to human defensins and evasion of neutrophil killing via the novel virulence factor MprF is based on modification of membrane lipids with l-lysine. *J. Exp. Med.* **193**, 1067-1076.

Schagger, H. and G. von Jagow (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **166**, 368-379.

Schmidtchen, A., I. M. Frick, E. Andersson, H. Tapper and L. Bjorck (2002) Proteinases of common pathogenic bacteria degrade and inactivate the antibacterial peptide LL-37. *Mol. Microbiol.* **46**, 157-168.

Schmidtchen, A., I. M. Frick and L. Bjorck (2001) Dermatan sulphate is released by proteinases of common pathogenic bacteria and inactivates antibacterial alpha-defensin. *Mol. Microbiol.* **39**, 708-713.

Towbin, H., T. Staehelin and J. Gordon (1992) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Biotechnology* **24**, 145-149.

Vilmos, P. and E. Kurucz (1998) Insect immunity: evolutionary roots of the mammalian innate immune system. *Immunol. Lett.* **62**, 59-66.

Whitten, M. M., I. F. Tew, B. L. Lee and N. A. Ratcliffe (2004) A novel role for an insect apolipoprotein (apolipophorin III) in beta-1,3-glucan pattern recognition and cellular encapsulation reactions. *J. Immunol.* **172**, 2177-2185.

Yu, K. H., K. N. Kim, J. H. Lee, H. S. Lee, S. H. Kim, K. Y. Cho, M. H. Nam and I. H. Lee (2002) Comparative study on characteristics of lysozymes from the hemolymph of three lepidopteran larvae, *Galleria mellonella*, *Bombyx mori*, *Agrius convolvuli*. *Dev. Comp. Immunol.* **26**, 707-713.