Effects of Cecropin Peptides on Aquatic Animal Pathogens and Shrimp Hemocytes

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ABSTRACT—In this paper, the effects of the well-characterized antimicrobial amphipathic peptides (cecropins A and P1) on pathogens of aquatic organisms were assessed. Results showed that cecropins inhibited growth of 15 strains of bacteria, including Gram-negative bacteria: *Aeromonas hydrophila*, *A. salmonicida*, *Photobacterium damsela* subsp. *damsela*, *Pseudomonas anguilliseptica*, *Vibrio alginolyticus*, *V. anguillarum*, *V. carchariae*, *V. harveyi*, *V. ordalii*, *V. parahaemolyticus*, *V. tubiashii* and *V. vulnificus* biogroup I. Cecropins also inhibited growth of two strains of the yeast *Debaryomyces hansenii*, pathogenic to freshwater prawn *Macrobrachium rosenbergii*. Minimum inhibitory concentration (MIC) of cecropin A ranged from 0.98 to 7.81 μ M and minimum bactericidal concentration (MBC) ranged from 3.90 to 15.59 μ M against the most prevalent vibrios pathogenic to cultured shrimp. A higher level of cecropin was required in a high salt environment to exhibit the effect against the pathogens. Influence of NaCl on bactericidal activity of cecropin A was also assayed. ⁵¹Cr-release assay showed a non-cytotoxic effect of cecropin A on shrimp (*Penaeus monodon*) hemocytes at MBC, but cytotoxic effect at 50 μ M (3.2-fold MBC) or higher concentration. This suggests that cecropin peptides may be difficult to be applied *in vivo* to treat infected marine shrimp.

Key words: cecropin, antimicrobial activity, *Penaeus monodon*, MIC, MBC, cytotoxic effect, hemocyte

Shrimp culture industry faces a crisis of production due to diseases all over the world. Nearly 20 distinct viruses, many *Vibrio* species and filamentous bacteria, several fungal and peritrich protozoan agents have been identified from diseased shrimp (Song *et al.*, 1990; Song and Lee, 1993; Bower *et al.*, 1994; Karunasagar *et al.*, 1998; Lavilla-Pitogo and de la Peña, 1998; Lightner and Redman, 1998; Takahashi *et al.*, 1998). It is common for a shrimp to be infected simultaneously with multiple pathogens. Recent documents show that some shrimp diseases resulted from co-infection by several viruses (Munday and Owens, 1998; Wang and Chang, 2000). Increasing the non-specific immunity to provide shrimp with a broad-spectrum defensive ability may effectively and practically protect them from infection.

Animal peptide antibiotics are widespread in nature, occurring in mammals (Lee *et al.*, 1989), amphibians (Zasloff, 1987), fish (Cole *et al.*, 1997), insects (Boman,

1991) and crustaceans (Destoumieux *et al.*, 1997). These peptides are major constituents of the innate immune system for non-specific defense of the host against microbial invaders. This type of defense can be rapidly activated after injury or invasion of the host by microbial agents. This is done by combating parasitic growth immediately after infection. Antimicrobial peptides thus provide an important defense mechanism in animals and the first line of host defense during the time required for mobilization of specific immunity in vertebrates.

Cecropins, the highly basic and amphipathic small peptides, have been induced and isolated from insects (Hultmark *et al.*, 1980; Hoffmann *et al.*, 1981; Flyg *et al.*, 1987; Matsumoto *et al.*, 1986; Dickinson *et al.*, 1988) and porcine (Lee *et al.*, 1989). They have broad-spectrum bactericidal activity. In the past, many researchers reported that cecropins could inhibit the growth of bacteria, both Gram positive and Gram negative (Qu *et al.*, 1982; Lee *et al.*, 1989). Synthesized cecropins have also been reported active against bacteria that are

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pathogenic to plants (Nordeen *et al.*, 1992) and insects (Hultmark *et al.*, 1982). At lethal concentrations, they are active against protozoa, fungi, enveloped viral pathogens and cancer cells without apparent deleterious effect on host cells. Chalk *et al.* (1995) reported that cecropin could attenuate the motility of microfilariae, *Brugia pahangi*, a human pathogenic nematode. However, only one paper has been documented in relation to the antimicrobial activities of cecropin peptides on fish pathogens including four bacterial species (Kjuul *et al.*, 1999). Neither the antimicrobial activity of the peptides on the pathogens of the other aquatic organisms such as bivalves and crustaceans nor the cytotoxic effect has been assessed for therapeutic purpose.

It will be of interest to see whether such peptides, when expressed or given passively *in vivo*, will play safely a protective role in cultured shrimp. In the present study, cecropins were assessed in terms of its *in vitro* antimicrobial activity against some prevalent aquatic pathogens using a radial diffusion assay. Effect of salinity on the bactericidal activity of cecropin was also evaluated using a plate count method. Minimal inhibitory concentration and minimal bactericidal concentration of cecropin on *Vibrio* and cytotoxic concentration of cecropin on shrimp hemocytes using a ^{51}Cr -release assay were also measured.

Materials and Methods

Bacterial and yeast culture

Twenty-three bacterial strains and two yeast strains were collected for this study. It comprised of 18 species and 8 genera. The bacterial strains were grown in tryptone soya broth (TSB, Oxoid), except *Aeromonas salmonicida* and *Vibrio* strains which were cultured in the same medium supplemented with 2.5% NaCl. *Escherichia coli* strains were maintained in Lennox L broth (LB, Acumedia). *Debaryomyces hansenii* was grown in yeast malt broth (YMB, Difco). All strains were incubated with shaking at 28°C but *E. coli* at 37°C. Cells were prepared for testing when they grew to mid-log phase, i.e. absorption value equals to 0.55 at 595 nm in wavelength using a spectrophotometer (DigiSpec, Helena Laboratories). The concentration of the culture was then measured to be 10^8 CFU/mL using a plate count method.

Radial diffusion method

The diffusion method was done mainly according to Hultmark *et al.* (1982, 1983). Culture plates were made on a leveling table from 6.8 mL of the melting LB agarose (LBA, 1%) and the final concentration of cultured cells was 10^5 CFU/mL. *E. coli* strains such as D21, D31 and JM107, the cecropin-susceptible strains, were used as the positive bacterial control. *Micrococcus lysodeikticus* (Sigma, ATCC4698), the cecropin-non-

susceptible strain, was used as the negative bacterial control. Lysozyme (purified from chicken egg white, Sigma) cleaving β -(1,4)-glycosidic bonds in the peptidoglycan layer of Gram positive bacterial cell wall was used as antimicrobial peptide control. Wells were punched 3 mm in diameter on the hardened LBA plate. Four μL sample of cecropin A (62 μM , synthetic silkworm peptide, Sigma), cecropin P1 (75 μM , synthetic porcine peptide, Sigma) or lysozyme (280 μM) was loaded into wells. Plates were read following overnight incubation at 28/37°C using a dissecting microscope. Two diameter readings at 90° angles were taken. Positive inhibitory response was defined as the area of the clear zone being bigger than the area of the well.

Broth microdilution assay of antimicrobial activity

Doubling dilutions of cecropin A stock solution (1000 $\mu\text{g/mL}$) were made in LB or TSB broth. Bacterial suspension of each strain was diluted to a final concentration of 10^5 – 10^6 CFU/mL in LB or TSB. Cecropin solution in each dilution was mixed with an equal volume of bacterial suspension and each strain was made in triplicates. Turbidity of bacterial suspension was observed by naked eyes after incubation for 16–20 h at 28°C. Minimal inhibitory concentration (MIC) was defined as the lowest concentration (the highest dilution) of cecropin that inhibited bacterial growth in the mixture. Minimal bactericidal concentration (MBC) was further determined by culturing the clear broth on TSA plates in triplicates. MBC was defined as the lowest concentration of cecropin that reduced bacterial colonies by 99.9%.

NaCl susceptibility testing

Each one strain of *V. harveyi* (W36G) and *V. alginolyticus* (Val) were selected and cells were grown in aerated TSB supplemented with different concentrations of NaCl at 0.1 M, 0.3 M and 0.5 M. The peptide concentration was chosen as about three-fold the MIC value of cecropin A when tested against the respective pathogen and was the same for all the killing studies conducted. The killing rate tests were started by adding peptide to a final concentration of 5.0 μM for *V. harveyi* and 2.5 μM for *V. alginolyticus*, in a volume of 1 mL diluted culture (10^5 cells/mL). The cells were incubated with shaking at 25°C. At intervals, 50 μL samples were withdrawn and directly, or after dilution in TSB (3% NaCl), plated on TSA agar plates. Colony forming units (CFUs) were counted after overnight incubation at 28°C.

Experimental shrimp

Apparently healthy tiger shrimp (*Penaeus monodon*) weighing 10–20 g each were purchased from a local hatchery. They were kept in a plastic tank (75 × 45 × 45 cm³) supplied with 100-L constant flow of recirculating brackishwater at 20 ppt salinity, 25°C temperature, 80–

90 ppb ammonia-N and 10–30 ppb nitrite. Shrimp were stocked at a density of 20–25 individuals per square meter. Synthetic feed pellets equivalent to 5% of the body weight were fed twice a day and shrimp were conditioned for more than one month before experiment.

Chromium release assay

Eight tenth mL of hemolymph was collected using a 1-mL syringe containing 0.2 mL anticoagulant (0.1 M sodium citrate, 0.4 M sucrose, 0.01 M Tris-HCl, pH 7.6, 750 mOsm/kg) from each of the twenty shrimp. After centrifugation at $300 \times g$ for 10 min, hemocytes were harvested and suspended in 0.5 mL anticoagulant solution. Chromium release assay was conducted following that of Hogan *et al.* (1996) with slight modification. Briefly, 0.2 mL sodium ^{51}Cr chromate (1 mCi/mL, Amersham) was added to 10^7 hemocytes and incubated at room temperature for 2 h. Isotope-labeled hemocytes were washed 3 times at $300 \times g$ centrifugation for 10 min and then suspended in the anticoagulant solution. Hemocytes were stained with trypan-blue dye and counted using a hemacytometer. The viability of hemocytes was estimated to be 77%. The final concentration of viable hemocytes was adjusted to 10^7 cells/mL in anticoagulant.

Completed Hanks' balanced salt solution (CHBSS, Gibco) was supplemented with 10 mM CaCl_2 , 3 mM MgCl_2 and 5 mM MgSO_4 as the modified CHBSS (MCHBSS) to maintain osmolarity to 750 mOsm/kg. Cecropin A was diluted to 50 μM , 100 μM and 150 μM with MCHBSS. Fifty μL of labeled hemocytes was mixed with an equal volume of cecropin solution, or MCHBSS as control. The mixture was incubated at room temperature for 1 h. After centrifugation, 50 μL of the supernatant was transferred to a scintillation vial and measured to determine counts per minute (CPM) using a gamma counter (1282 Compugamma, LKB). Fifty μL of MCHBSS or 2% IGEPAL CA-630 (octylphenoxy) polyethoxyethanol, Sigma) in replacement of cecropin solution was added to the labeled hemocytes, respectively, to serve as the controls of spontaneous (minimum) and complete (maximum) releases. Percent specific release is defined as $100\% \times (\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})$.

Results and Discussion

Radial diffusion assay showed that cecropins A and P1 could inhibit growth of 15 strains of Gram-negative bacteria. They are *Aeromonas hydrophila*, *A. salmonicida*, *Photobacterium damsela* subsp. *damsela*, *Pseudomonas anguilliseptica*, *Vibrio alginolyticus*, *V. anguillarum*, *V. carchariae*, *V. harveyi*, *V. ordalii*, *V. parahaemolyticus*, *V. tubiashii* and *V. vulnificus* biogroup I. Noticeably these Gram-negative bacteria have al-

ready been characterized as the etiological agents of many infectious diseases such as motile aeromonad septicemia, furunculosis, gastroenteritis, red spot disease and vibriosis that cause high mortalities in cultured fishes. Cecropins also inhibited growth of yeast, *D. hansenii*, which is pathogenic to freshwater prawn *Macrobrachium rosenbergii* (Lu *et al.*, 1998). These results indicate that cecropin peptides provide antimicrobial activity with a broad spectrum, although the growth inhibitory activity was not found against *Edwardsiella tarda*, *Flavobacterium columnare*, *Flexibacter maritimus* and *V. vulnificus* biogroup II (Table 1). Kjuul *et al.* (1999) reported that *Edwardsiella ictaluri* was less sensitive to cecropin, which could be attributed to the structure differences in the bacterial cell wall. Zaldivar (1985) found that the outer membrane of *F. columnare* was surrounded by galactosamine-rich secretion in TEM preparations treated with ruthenium red. Whether the high-molecular-weight amino sugars interfere the channel formation and/or blebbing of the outer membrane that are known to be essential steps in the mechanism used by cecropins to kill live bacteria is not clear. Apparently, lysozyme does inhibit growth of Gram-positive bacteria such as *Bacillus subtilis* ATCC6051 and ATCC6633 (Song and Lin, unpublished data), *Micrococcus lysodeikticus* and pathogenic yeast strains. Growth inhibitory activity of lysozyme was not found on Gram-negative bacteria although a high dose was applied in the present study. Since most bacterial diseases of cultured aquatic organisms are caused by Gram-negative group, a limited potential will be expected on lysozyme in the treatment of infectious diseases of aquatic organisms.

Although a slight high dose of cecropin P1 was applied in the inhibition zone assay, the results indicated that cecropin A appeared equal or more potent for the 12 among 17 cecropin-susceptible strains tested (Table 1). Consistent conclusions were noted from the previous studies that cecropin A proved more potent than the B form (Samakovlis *et al.*, 1990) and the killing rate was higher for cecropin B than for cecropin P1 (Kjuul *et al.*, 1999). Therefore, we selected cecropin A in the following MIC, MBC, NaCl tolerance and cytotoxic assays. The range of MIC was determined from 0.98 to 7.81 μM and MBC from 3.90 to 15.59 μM (Table 2) against selected *Vibrio* strains which are prevalent and pathogenic to cultured shrimp (Song *et al.*, 1990; Song and Lee, 1993; Mohny *et al.*, 1994).

Since lethal concentration value of cecropin B1 measured on *E. coli* using inhibition zone assay in the previous investigation (Morishima *et al.*, 1990) is close to MIC value of cecropin A measured on *E. coli* using broth microdilution assay in this study, we examined the effect of NaCl. As a result we found that a higher level of cecropin A was required to inhibit bacterial growth in a salt-supplemented TSB (3% NaCl) in comparison with

Table 1. Effect of antimicrobial peptides on the growth of some pathogens of aquatic organisms using radial diffusion assay

Species	Strain	Source	Area of inhibition zone (mm ²)		
			Cecropin A ^a	Cecropin P1 ^b	Lysozyme ^c
<i>Escherichia coli</i>	D21		184	183	0
<i>E. coli</i>	D31		170	157	0
<i>E. coli</i>	JM107	—	184	190	0
<i>Micrococcus lysodeikticus</i>	—	—	0	0	393
Gram negative bacteria					
<i>Aeromonas hydrophila</i>	ATCC 7966	Tin of milk with a fishy odour	32	51	0
<i>A. salmonicida</i>	—	—	85	85	0
<i>Edwardsiella tarda</i>	820429-1L	Eel (<i>Anguilla japonica</i>)	0	0	0
<i>Flavobacterium columnare</i>	DD3	Spring Chinook salmon (<i>Oncorhynchus tshawytscha</i>)	0	0	0
<i>Flexibacter maritimus</i>	NCMB2154 ^T	red sea bream (<i>Pagrus major</i>)	0	0	0
<i>Photobacterium damsela</i> subsp. <i>damsela</i>	ATCC 33539	Damselfish (<i>Chromis punctipinnis</i>)	103	44	0
<i>Pseudomonas anguilliseptica</i>	ATCC 33660	Japanese eel (<i>Anguilla japonica</i>)	112	128	0
<i>Vibrio alginolyticus</i>	ATCC 17749	Spoil horse mackerel (<i>Sarda sarda</i>)	81	79	0
<i>V. alginolyticus</i>	Val	Diseased shrimp (<i>Penaeus monodon</i>)	132	92	ND
<i>V. anguillarum</i>	ATCC 19264	Cod (<i>Gadus callaris</i>)	61	92	0
<i>V. carchariae</i>	ATCC 35084	Brown shark (<i>Carcharhinus plumbeus</i>)	31	27	0
<i>V. harveyi</i>	ATCC 14126	Amphipod (<i>Talorchestia</i> sp.)	33	46	0
<i>V. harveyi</i>	13b	Diseased shrimp (<i>P. monodon</i>)	209	142	ND
<i>V. harveyi</i>	W36G	Diseased shrimp (<i>P. monodon</i>)	140	140	ND
<i>V. ordalii</i>	ATCC 33509	Coho salmon (<i>Oncorhynchus kisutch</i>)	140	61	0
<i>V. parahaemolyticus</i>	ATCC 17802	Shirasu food poisoning	93	80	0
<i>V. tubiashii</i>	ATCC 19106	Hard clam larvae	125	117	0
<i>V. vulnificus</i> biogroup I	TG617	Diseased shrimp (<i>P. monodon</i>)	84	26	ND
<i>V. vulnificus</i> biogroup II	ATCC 33148	Diseased eel (<i>Anguilla japonica</i>)	0	0	0
Yeast					
<i>Debaryomyces hansenii</i>	QF82-334	Giant freshwater prawn (<i>Macrobrachium rosenbergii</i>)	77	78	79
<i>D. hansenii</i>	QF83-80	Giant freshwater prawn (<i>M. rosenbergii</i>)	90	86	73

Area of inhibition zone = πr^2 – area of the well. r is the measured radius.

^asilkmoth synthetic cecropin, 1 μ g/well; ^bporcine synthetic cecropin, 1 μ g/well; ^cpurified from chicken egg white, 16 μ g/well.

ND: not determined

Table 2. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of cecropin A for the bacteria pathogenic to crustaceans

Bacterial strain	MIC (μ M)	MBC (μ M)
<i>Vibrio vulnificus</i> TG617 (biogroup I)	7.81	15.59
<i>V. harveyi</i> ATCC14126	1.95	3.90
<i>V. harveyi</i> 13b	1.95	7.81
<i>V. harveyi</i> W36G	1.95	3.90
<i>V. alginolyticus</i> Val	0.98	7.81
<i>Escherichia coli</i> D21	0.39	0.98

Tabulated values presented are average of two or more independent experiments.

those in LB (1% NaCl), although cecropin A appears to be more potent than the B form. In NaCl susceptibility test (Fig. 1), the killing activity of cecropin performed well in a low concentration (0.1–0.3 M NaCl) but was decreased in a high concentration (0.5 M NaCl). Kjuul *et al.* (1999) reported that the antibacterial effect of cecropin peptides was strongly reduced with increased concentration of NaCl in the growth medium. Kjuul *et al.* (1999) and Yomogida *et al.* (1997) concluded that the

antibacterial effect of cecropin, and defensin, too, was strongly sensitive to salt. It is most probable that the higher levels of salt interfere with the charge interactions by which the cecropin bind to the bacterial membrane thus inhibiting the activity of these peptides depending on the amount of salt. Because of osmoregulation, the salt content in a marine fish is almost the same as that of freshwater fish hence the peptide might work well in marine fish except in sites such as gill, skin surfaces and the gastro-intestinal tract. However, the aquatic invertebrates with an open circulatory system such as shrimp change their hemolymph osmosis depending upon the external environment. Although cecropin does have anti-*Vibrio* activity at salt concentrations found in the NaCl-supplemented TSB, the prospect of using cecropins in the treatment of infectious diseases for marine invertebrates needs careful consideration.

Lethal concentrations ranged from 4.5 μ M for tomato protoplasts to 41 μ M for sugar beet protoplasts (Nordeen *et al.*, 1992). However, lethal concentration of cecropin for human embryonic lung fibroblastoid cells was reported to be greater than 4 μ M (Wachinger *et al.*,

Fig. 1. Influence of NaCl on bactericidal activity of cecropin A against (A) *Vibrio alginolyticus* Val and (B) *V. harveyi* W36G grown aerobically in tryptic soy broth (TSB). Cecropin was added in experimental groups (5 μ M for *V. harveyi* and 2.5 μ M for *V. alginolyticus*) (empty symbol); not added in control groups (solid symbol) but TSB was supplemented with the respective NaCl concentration. NaCl concentrations were of 0.1 M ($\diamond$, $\blacklozenge$), 0.3 M ($\square$, $\blacksquare$), 0.5 M ($\triangle$, $\blacktriangle$).

1998) for human liver cells greater than 20 μ M and for sheep red blood cells greater than 200 μ M (Steiner *et al.*, 1981). ^{51}Cr -release assay showed a cytotoxic effect of cecropin on shrimp hemocytes at concentrations equal to or more than 50 μ M in this study (Fig. 2). These cytotoxic data indicated that resistance of animal cells to cecropin varied greatly among cell types and species. Nevertheless, there was smaller, but still considerable, difference between the lethal concentrations of cecropin A for shrimp hemocytes and *Vibrio* pathogens. The lethal concentration of cecropin A for shrimp hemocytes (i.e. 50 μ M) is 3.2– to 12.8-fold MBC for the respective bacterial pathogens (Table 2) such as *V. vulnificus* biogroup I (15.59 μ M), *V. alginolyticus* (7.81 μ M) and *V. harveyi* (3.90–7.81 μ M). Only is the difference big enough between the lethal concentrations of a peptide for the host and the respective pathogens, the treatment of peptide will be considered safe in transgenic organisms or given passively *in vivo* to enhance disease resistance against pathogens. Therefore, it may be difficult to engineer a systemic resistance against infection in shrimp using cecropin or cecropin-based genes.

Fig. 2. Cytotoxic effect of cecropin A on shrimp (*Penaeus monodon*) hemocytes using a ^{51}Cr -release assay. Error bar represents standard error of percent specific release values of twenty samples. **Percent specific release was significantly different between cecropin-treated and non-treated hemocytes analyzed statistically using a t-test ($p < 0.01$).

The strong and broad-spectrum antimicrobial activity of cecropin peptides against aquatic pathogens is put into question because their killing activity is sensitive to salt and a small difference between the lethal concentrations of peptide for hemocytes and pathogens suggested that cecropin peptide may be difficult to express or be given passively *in vivo* for the purpose of treatment of infectious diseases in marine shrimp.

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