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Full Length Research Paper

Antibacterial activity from Siamese crocodile (*Crocodylus siamensis*) serum

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Antibacterial agents were purified from Siamese crocodile serum by anion exchange, gel filtration and reversed phase HPLC. Six antibacterial agents designed as Hp14, Hp15, Hp17, Hp31, Hp36 and Hp51 were purified and proved to carry activity against *Salmonella typhi*, *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Vibrio chorelae*. The mass analysis of MALDI-TOF for antibacterial agent of Hp14, Hp15 and Hp51 revealed that they are small molecule with a molecular mass less than 1 kDa. The scanning electron microscopy demonstrated that these agents targeted the bacterial membrane and they act like as antimicrobial peptides. The antibacterial agent in the serum may represent the first line of an immune system in a freshwater crocodile.

Key words: Antibacterial agent, crocodile, ***Crocodylus siamensis***, reptile.

INTRODUCTION

Bacteria and fungi coexist with other living organisms. However, in spite of the fact that such microorganisms are always in contact with other organisms, microbial invasion into hosts hardly takes place, probably because of their unique defense systems. In the insect and animal, one such system is non-specific immunity which comprises a wide variety of antimicrobial peptides (Steiner et al., 1981) and factors (Ourth and Chung, 2004) with potent antimicrobial activities.

Crocodylus siamensis, the Siamese crocodile, is one of the critically endangered species of freshwater crocodile, which are originally distributed throughout much of South East Asia. Crocodilians are known to live with opportunistic bacterial infection but exhibit no physiological effects. While crocodilians have no complete immunity to microbial infection, these species do exhibit remarkable resistance. This biological activity of crocodilians has not been well characterized. However, some reports have described the efficacy of alligator's serum in fighting against bacteria (Merchant et al., 2003), viruses (Merchant et al., 2005a) and amoeba (Merchant et al., 2004). Recently, Merchant et al. (2005b) proposed that the complement systems of alligator are effective in killing bacteria. More recently, leukocyte extract of American alligator has shown a broad spectrum of antibiotic effects on bacteria, fungi and viruses (Merchant et al., 2005a; Merchant et al., 2006a). The studies by Merchant et al. (2006b) also confirmed the effects of bacterial lipopolysaccharide on peripheral leukocytes in the American alligator (***Alligator mississippiensis***). However, for freshwater crocodile, antibacterial activities components have not been characterized yet.

In this study, we purified the antibacterial agents from crocodile serum. These antibacterial agents have mole-

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Abbreviations: CFU, Colony forming unit; **DDW**, sterile distilled water; **HCCA**, α-cyano-4-hydroxy-cinnamic acid; **HPLC**, High Performance Liquid Chromatography; and **TFA**, Trifluoro acetic acid.

cular weight around 1 kDa. They exert potent activity with remarkable broad spectrum, suggesting applicability for use in therapeutics.

MATERIALS AND METHODS

Chemicals and reagent

Trifluoroacetic acid (TFA) for protein sequencing was purchased from Fluka Biochemika, Switzerland and acetronitile of HPLC grade was purchased from Lab-Scan Asia Ltd., Thailand. The other chemicals used were of the highest grade commercially available.

Crocodile serum

Crocodiles (***Crocodylus siamensis***) were captured and housed at the local Sriracha Moda Farm Chon Buri, Thailand. The crocodiles (age ranging from 1 - 3 years) were housed in a single tank, and treated with electric shock. Then, the crocodile blood was obtained by drawing blood from the dorsal vein using a heparinized needle and transferred into heparinized vacuum tubes. The crocodile blood in heparinized vacuum tubes was kept at 4°C for overnight, and then centrifuged to obtain the serum and kept at -70°C until required.

Bacterial culture

Bacterial strains tested in this study were ***Salmonella typhi*** ATCC 11778, ***Escherichia coli*** O157:H7, ***Staphylococcus aureus*** ATCC 25923, ***Staphylococcus epidermidis*** (clinical isolates), ***Klebsiella pneumoniae*** ATCC 27736, ***Pseudomonas aeruginosa*** (clinical isolates) and ***Vibrio chorelae*** (clinical isolates). All bacterial strains were maintained in nutrient agar slants at 4°C.

Purification of antimicrobial component

Crocodile blood in heparinized vacuum tubes was kept at 4°C for overnight, and then centrifuged to obtain the serum. The serum was diluted (1:3) in 25 mM Tris-HCl, pH 8.1 and filtrated with 0.45 µm membrane filter. Separation of serum was performed by FPLC (BioLogic DuoFlow Systems, Bio-Rad, USA) over Q Sepharose fast flow column previously equilibrated with 25 mM Tris-HCl, pH 8.1. Elution was achieved with a linear NaCl gradient in 25 mM Tris-HCl, pH 8.1, and the eluate was monitored at 280 nm. The antimicrobial activities of fractions were determined as indicated below. The protein peak containing antimicrobial activity was pooled and then lyophilized. The lyophilized sample was re-suspended in 1 ml water and applied to a Sephadex G-50 (Superfine, Amersham Bio-sciences, 2.6 × 100 cm) gel filtration column equilibrated with 0.1% TFA. Elution was performed with the same solvent, collecting fractions of 3.0 ml. The absorbance of the eluate was monitored at 220 nm. The pooled fraction with the antimicrobial activity was lyophilized and resolved in 0.1% TFA before purified further by C_{18} reverse-phase high-performance liquid chromatography (RP-HPLC) (Alltech, Japan, 4.6 × 250 mm) equilibrated with solvent A (0.1% TFA/water) at flow rate of 1 ml/min. First, the linear gradient elution from solvent A to solvent B (60% acetonitrile in solvent A) was carried out to attain 20% solvent B at a flow rate of 1 ml/min for 20 min. The next gradient elution was further carried out from 20 to 100% solvent B at a flow rate of 1 ml/min for 30 min. All of these procedures were done at room temperature on a Shimadzu LC-10A HPLC system with a Shimadzu photo diode array detector (SPD-M6A). The column effluent was monitored for absorbance at 220 nm and 280 nm. The fractions corresponding to absorbance peaks were collected and then dried by a speed vacuum concentrator (Savant Instruments, Inc. USA).

Antibacterial assay

Antibacterial activity was monitored by a liquid growth inhibition assay as described by Charles and Philippe (1997). The dried aliquots of chromatoghaphic effluent in sterile water (50 µl) were incubated with inoculums (100 µl of 10^6 CFU/ml) from log phase cultures of each bacterium in a sterile 96 well plate at 37°C. After incubation for 16 h, at a given time interval, the optical density at 550 nm of each well was determined using a Benchmark microtiter plate reader (BIO-RAD, USA). In order to monitor the validity of the assays, incubations with bacteria were carried out in parallel with sterile water and streptomycin (0.4 mg/ml) as control. Antimicrobial activity is expressed as no growth observed when tested with the above procedure (Nakamura et al., 1988; Casteels et al., 1989; Charles and Phillippe, 1997). The minimal inhibitory concen-tration (MIC) is defined as the minimal peptide concentration that inhibits bacteria growth (Zhu et al., 2007).

Molecular mass determination

The molecular mass of antibacterial peptide was determined by mass spectrometry at the BioService Unit (BSU), National Science and Technology Development Agency (NSTDA) Thailand. Antimi-crobial peptides were analyzed by matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) mass spectrometry. The dried peptide was dissolved in acetronitile: TFA (1:1) and then was mixed with equal volumes of α-cyano-4-hydroxycinnamic acid (HCCA, 10 mg/ml of acetonitile: TFA; 1:1). One microliter of mixed sample was spotted on the plate. The mass spectra were recorded on a reflector Bruker reflex V delayed extraction MALDI-TOF mass spectrometer equipped with a 2GHz LeCroy digitizer and 337 nm N_2 laser. Instrumental parameters were: positive polarity, accele-ration voltage 20kV; IS/2 17 kV; focusing lens voltage 8.90 kV; extraction delay 400 ns. The detector was gated. Typically 100 shot were accumulated from three to five different positions within a sample spot.

Scanning electron microscopy technique

Scanning electron microscopy was performed according to Lau et al. (2004) with slight modifications. ***S. typhi*** and ***S. aureus*** were grown in nutrient broth and harvested at the logarithmic phase of growth by centrifugation at 3,000 g for 5 min. The bacterial cells were then washed twice with PBS pH 7.0 and resuspended to final concentration of 10^6 CFU/ml. Aliquots of suspension of ***S. typhi*** and ***S. aureus*** (100 µl) were individually incubated with crude serum (300 µg/50 µl) and the active HPLC fraction (3 µg/50 µl) at 37°C for 2 h. The 150 µl of incubated bacteria were fixed with equal volumes of 5% w/v of glutaraldehyde (Sigma, USA) for 1 h. The fixed cells were carefully pipetted and settled onto a 0.2 µm polycarbonate membrane filter (Whatman, Germany) for 5 min and then washed twice with PBS buffer. The fixed material was dehydrated by rinsing (for 15 min) repeatedly with ethanol solutions, of which the concentrations are elevated stepwise from 30, 50, 70, 90, and finally 100% ethanol. The dehydrated materials in the abso- lute ethanol were dried in a critical point drier (CPD7510; Thermo VG Scientific, England) with carbon dioxide as the drying agent. Dry

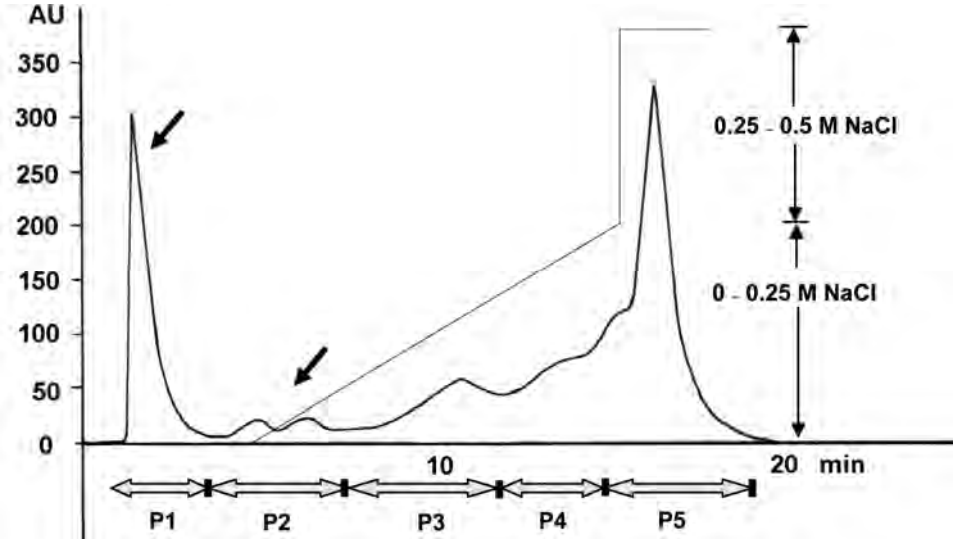

Figure 1. Chromatograph of crude serum on Q Sepharose fast flow column. Pooled fraction of P1 - P5 were examined the antibacterial activity against ***S. typhi***, ***K. pneumoniae, S. aureus*** and ***S. epidermidis***. The active pooled fractions were indicated as arrows.

materials were coated by sputter Coater (SC7620; Polaron, England) with gold palladium and examined by scanning electron microscopy (LEO1450VP; LEO Electron Microscopy Ltd, England) operating at 12-20 KV. The negative control was performed in a similar manner except that the bacterial cells were incubated with PBS buffer instead of the antibacterial peptide.

RESULTS

Purification of antimicrobial from crocodile blood

The serum of Siamese crocodile was separated into five pooled fractions by Q Sepharose fast flow namely P1 - P5 (Figure 1) and the antimicrobial activity occurred in the pooled fraction P1 and P2 marked by an arrow. The pooled fractions with antibacterial activity were lyophilized and re-suspended prior applied to a Sephadex G-50 gel filtration column, and two peaks, designed as Gp1/1 and Gp1/2 or Gp2/1 and Gp2/2, were obtained as indicated in Figure 2. The Gp1/2 and Gp2/2, the small molecule as well as the peptide were further purified by C_{18} RP-HPLC (Figure 3). The HPLC separation yielded at least 6 peaks. Six peaks with eluted at retention time 14.1, 15.9, 17.9, 31.0, 36.1 and 51.2 min were exhibited the antimicrobial activity against represent bacterial test, ***S. aureus*** and ***S. typhi*** (Figure 4). The peaks were named Hp14, Hp15, Hp17, Hp31, Hp36 and Hp51.

Antibacterial activity

Crocodile serum was isolated to five peaks by anion exchange column chromatography. P1 and P2 which was unbound on low ionic strength revealed the cationic property (Figure 1). By the liquid growth inhibition assay, they exhibited the antibacterial activity against all seven tested bacteria (data not show). In order to purify the small molecules as well as antibacterial peptide, both peaks were then subjected on G-50 column to obtain Gp1/1, Gp1/2, Gp2/1 and Gp2/2 fraction (Figure 2). The activity of peaks achieved by Sephadex G-50 was shown in Figure 5. The pooled fractions of Gp1/2 and Gp2/2 inhibited growth of all seven selected bacterial strains whereas Gp2/1 exhibited antimicrobial activity against only ***S. typhi***, ***S. aureus***, ***S. epidermidis*** and ***P. aeruginosa***. The antimicrobial activity was not found with Gp2/1. Because we are interested in studying the antibacterial peptide in crocodile serum, the large molecule Gp1/1 which was eluted with the void volume of G-50 was excluded in further studies even though it had antibacterial activity. The Gp1/2 and Gp2/2 were further purified on the C18 HPLC.

We next examined the minimal inhibitory concentration against bacterial strains (Table 1). The Gp1/2 has a high activity against Gram negative and Gram positive bacteria which showed the geometric mean (GM) of MIC of 15.46 μg/ml. For the Gp2/2, it had moderate good broad spect- rum activity which showed geometric mean of MIC of 33.97 μg/ml.

Molecular mass determination

Antibacterial agents purified by RP-HPLC (Figure 3) were analyzed for mass by MALDI-TOF. Three peaks fraction were observed and mass spectrometry profile showed

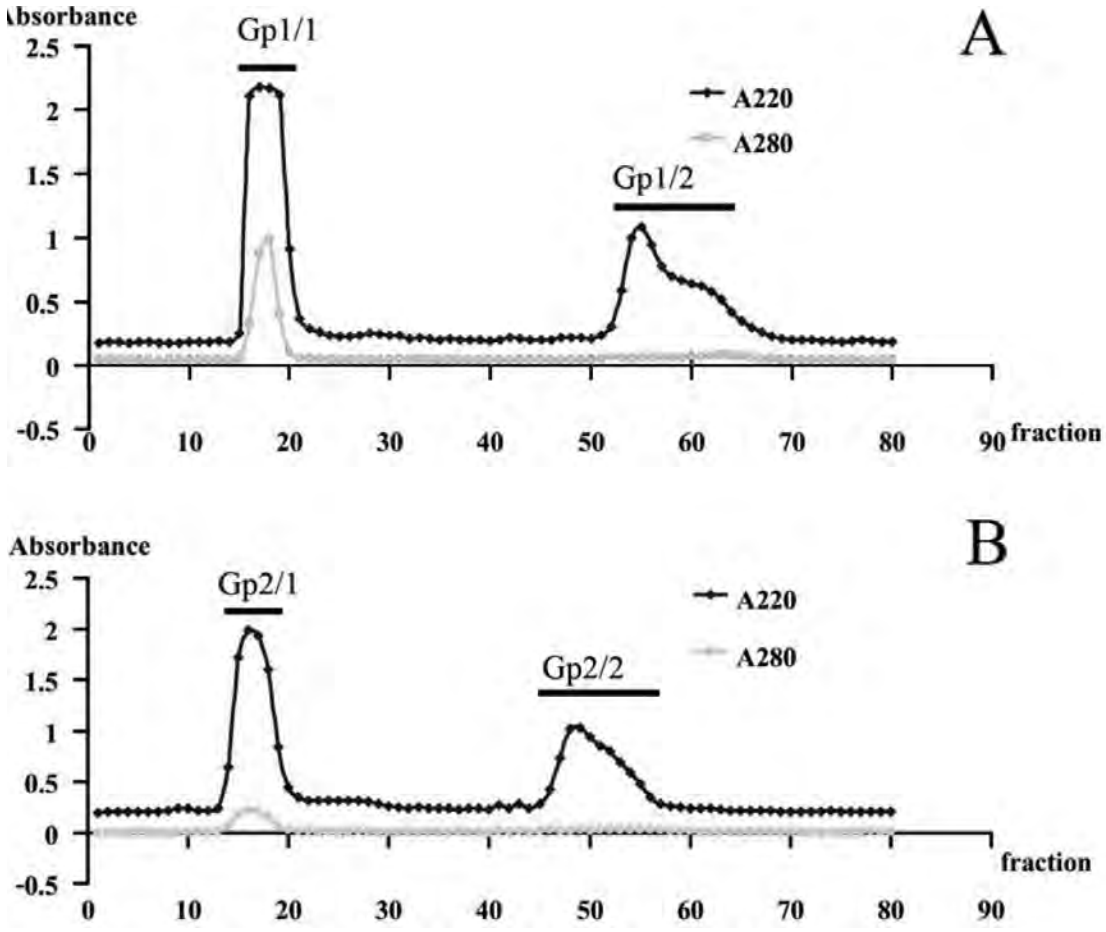

Figure 2. Elution profiles of active fraction on Sephadex G-50 column. A: Elution of P1 and B: Elution of P2. The elution was performed with the flow rate of 0.5 ml/min and the fraction was collected 3 ml/tube.

that Hp14, Hp16 and Hp51 had a molecular mass less than 1 kDa (Figure 6).

Scanning electron microscopy

The effects of crude serum and the purified fraction of HPLC on the cell membranes of ***S. typhi*** and ***S. aureus*** were examined by scanning electron microscopy (SEM). From SEM observation of ***S. typhi*** and ***S. aureus*** cells, crude serum and antibacterial fractions were found to deform of the cell membranes, causing formation of blebs on the cell surface. Whereas the untreated cells exhibited normal, smooth surfaces (Figure 7).

DISCUSSION

Little information has been accumulated on the basic innate immunity of crocodilians, especially the freshwater crocodile (***Crocodylus siamensis***). However, strong evidence of antimicrobial activity from American alligator (***Alligator mississippiensis***), saltwater (***C. porusus***) and freshwater (***C. johnstoni***) crocodiles has been reported (Merchant et al., 2005b; Merchant and Britton, 2006). Further, the leukocyte extract from American alligator exhibits antimicrobial activity (Merchant et al., 2006a). The present data is the first report that the blood circulation system of the freshwater crocodile exhibits antibacterial activity.

In this study we have purified the antibacterial agent from the serum of freshwater crocodile, ***C. siamensis***. The antibacterial agents were fractionated by anion exchange chromatography. The elution position of the active fraction from anion exchange column indicated that the nature of active component is cationic because the fraction is not significantly retained in the Q Sepharose column in the condition used. This fraction showed strong inhibition to growth of ***S. typhi, S. aureus, S. epidermidis*** and ***K. pneumoniae.*** Active fractions of ion exchange chromatography were fractionated further by Sephadex G-50 gel filtration column and reversed phase HPLC.

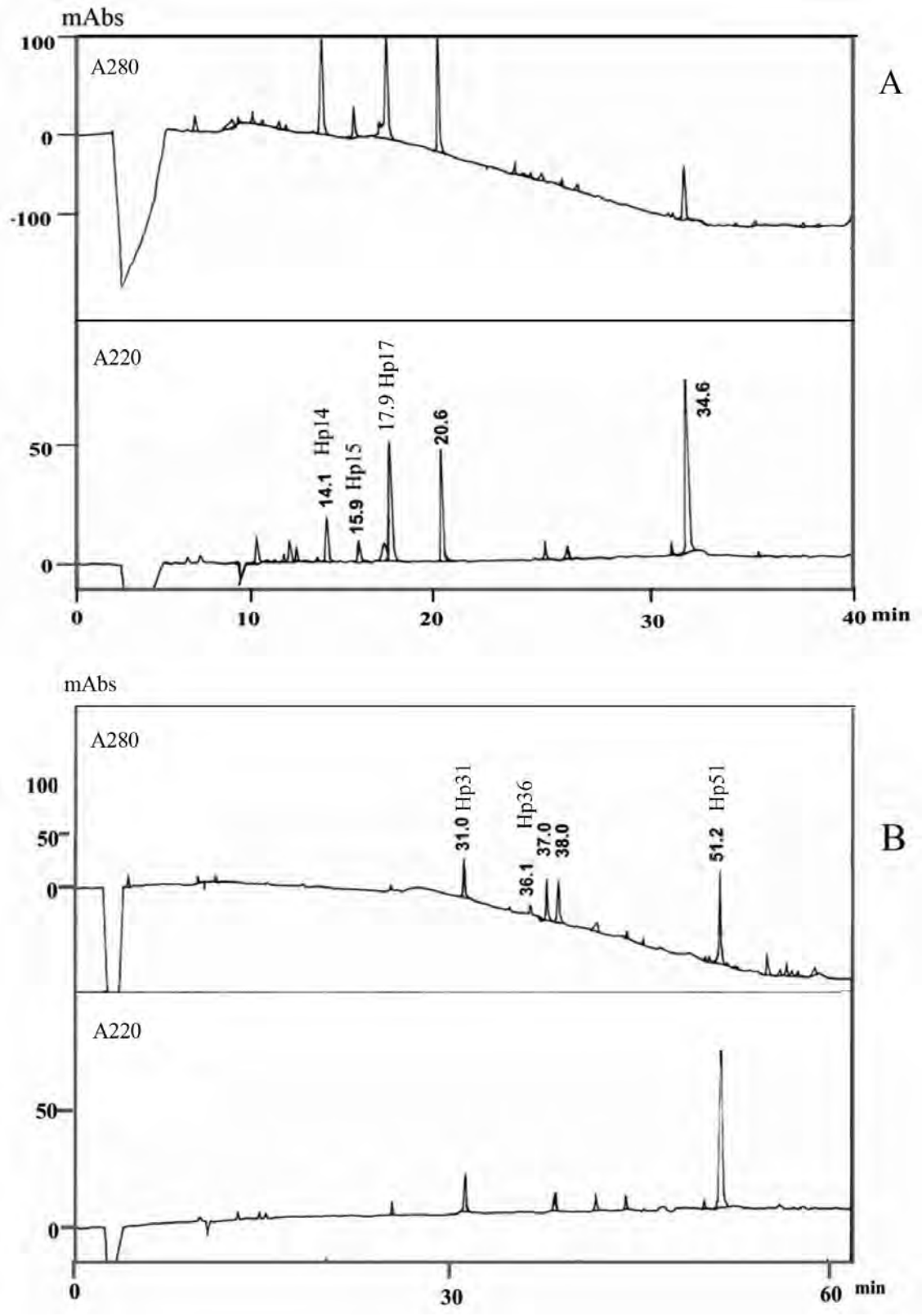

Figure 3. Purification of antibacterial agent on reversed phase HPLC. A: Purification of Gp1/2 and B: Purification of Gp2/2. The peaks with antimicrobial activity were indicated as Hp14, Hp15, Hp17, Hp31, Hp36 and Hp51.

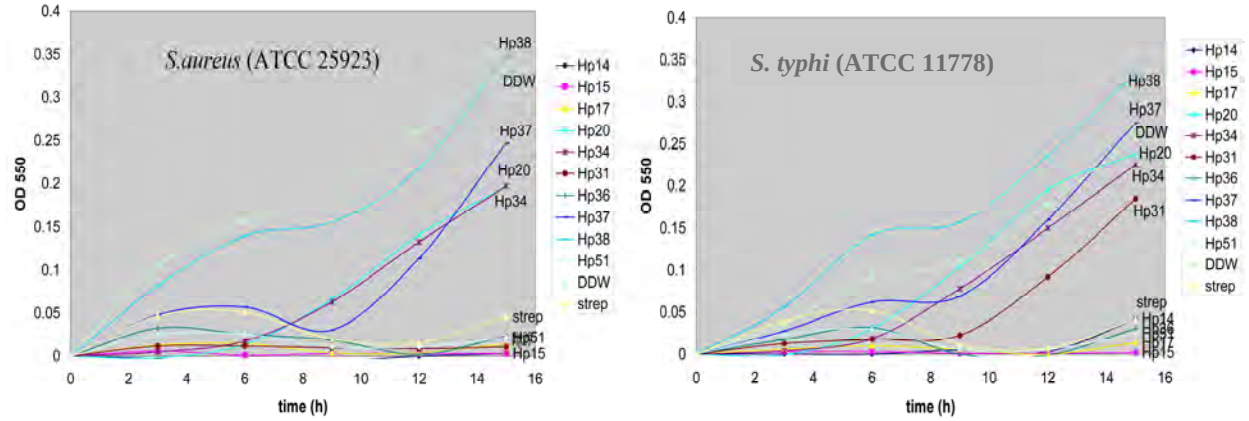

Figure 4. Antibacterial activity of HPLC fraction. The activity of pooled fractions achieved by C_{18} HPLC were examined for 15 h.

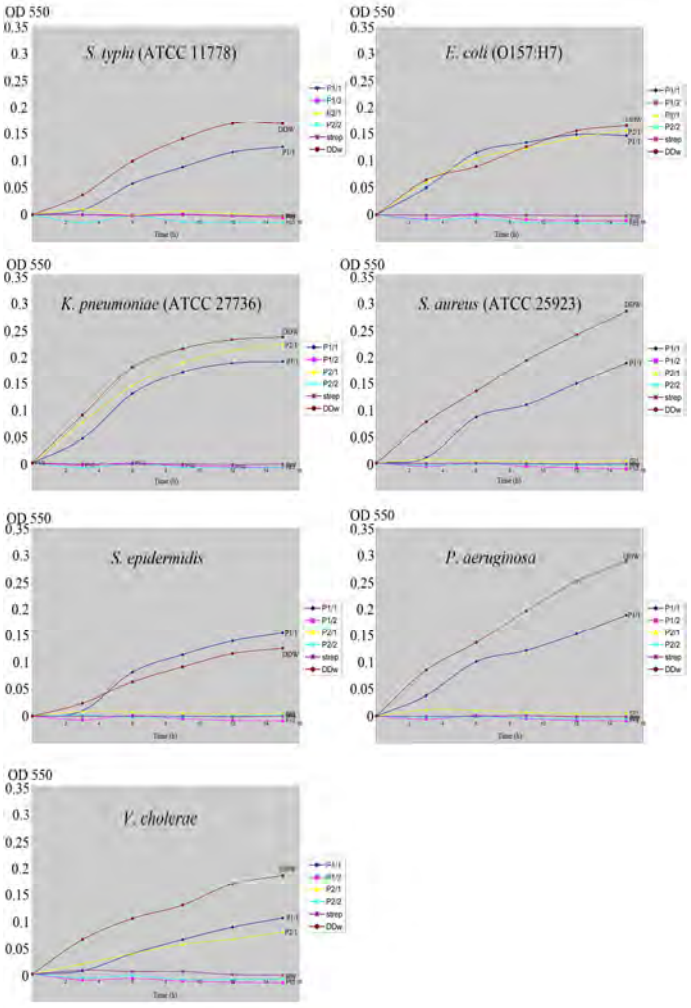

Figure 5. Antibacterial activity assayed by a liquid growth inhibition assay. The activity of pooled fractions achieved by gel filtration column were examined for 16 h against seven bacterial tested.

Either pooled fraction, Gp1/2 and Gp2/2, obtained by gel filtration column exhibits antibacterial activity against seven selected bacterial. They are eluted at the last position indicating that they should be a small molecule and possibly be a peptide because they can absorb absorbance of 220 nm and 280 nm. Moreover, almost all reported antimicrobial agents in animal are also peptide. Although the antibacterial agents purified from the fresh water crocodile serum are rather small, they can inhibit growth of bacteria just like β-alanyl-tyrosine (β-AY, 252 Da), one of such low molecular mass compounds isolated from the fleshfly ***Neobellieria bullata*** (Meylaers et al., 2003). The scanning electron microscopic images of antibacterial peptides suggest that the bacterial membrane is an important target of this for the agent. ***S. aureus*** treated with antimicrobial agents showed that the cells were irregular in shape, with less defined cell walls, outer membranous blebs and with ultrastructural damage. In the same manner, S***. typhi*** treated with antimicrobial agents showed rough surfaces containing extracellular debris and outer membranous blebs or thickened cell walls. A cardinal feature was the extensive blebbing in the cell wall. These results correspond with the actions of the antimicrobial peptides such as bactenecin 5, bactenecin 7, poly-L-lysine, cecropin B, LL-37, PGYa, melittin, hecate-1 and SMAP-29 (Henk et al., 1995; Tiozzo et al., 1998; Freer et al., 1999; Oren et al., 1999a; Oren et al., 1999b; Arzese et al., 2003). Most antimicrobial peptides found thus far, such as those from amphibian skin, magaingnin (Matsuzaki, 1998) and dermaseptin (Mor et al., 1991; Feder et al., 2000; Krugliak et al., 2000) are known to exert their antimicrobial activity by permeabilizing the membrane as seen in Figure 7.

The results from this study provide the first evidence that the freshwater crocodile has an active antibacterial system. This system may form the potency of indepen-

Table 1. The minimal inhibitory concentrations (MICs) of the peptide against bacterial strains.

Bacterial strains	MIC (µg/ml)	
	Gp1/2	Gp2/2
Salmonella typhi ATCC 11778	10.40	41.41
Escherichia coli O157:H7	20.81	41.41
Klebsiella pneumoniae ATCC 27736	20.81	41.41
Staphylococcus aureus ATCC 25923	20.81	41.41
Staphylococcus epidermidis (clinical isolate)	10.40	20.71
Psudomonas aeruginosa (clinical isolate)	20.81	41.41
Vibrio cholerae (clinical isolate)	10.40	20.71
Geometric mean (GM)*	15.46	33.97

* The geometric mean (GM) of the MIC values from all seven bacterial strains in this table.

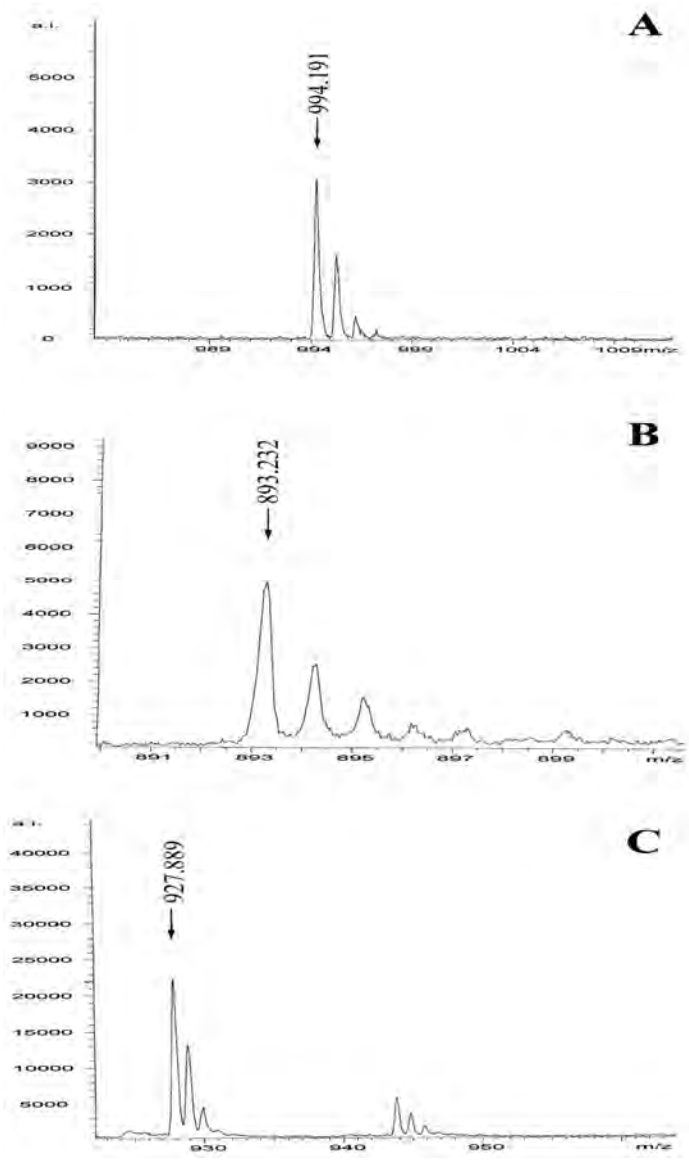

Figure 6. The mass of antibacterial agents determined by MALDI-TOF. A: Hp14; B: Hp15; C: Hp51.

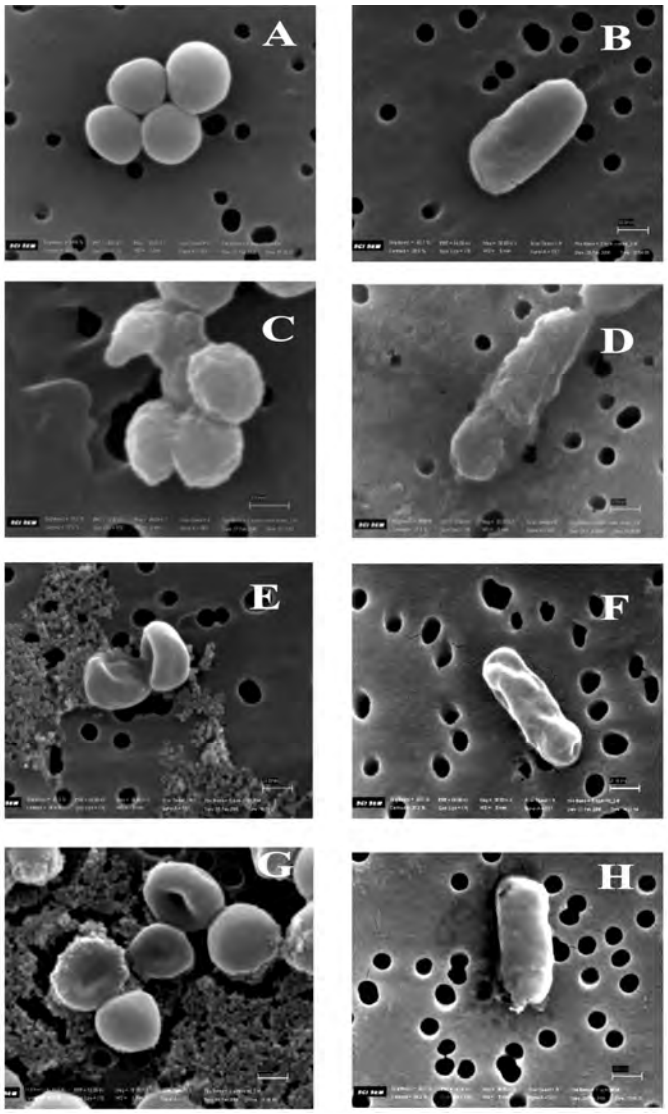

Figure 7. Scanning electron microscopy. Left panel: ***S. aureus***; Right panel: ***S. typhi***; A and B: bacterial treated with PBS (control); C and D: bacterial treated with crude serum; E and F: bacterial treated with Hp18; G and H: bacterial treated with Hp31.

dent innate immunity to fight against pathogens as the first line of antibodies which causes survival of crocodile from ancient period to the present time.

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