Characterization of the novel antibacterial peptide Leucrocin from crocodile (Crocodylus siamensis) white blood cell extracts

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ABSTRACT

Four novel antibacterial peptides, Leucrocin I–IV from Siamese crocodile white blood cell extracts were purified by reverse phase high performance liquid chromatography (RP-HPLC). Leucrocins exhibit strong antibacterial activity towards Staphylococcus epidermidis, Salmonella typhi and Vibrio cholerae. The peptides were 7–10 residues in length with different primary structure. The amino acid sequence of Leucrocin I is NGVQPKY with molecular mass around 806.99 Da and Leucrocin II is NAGSLLSWG with molecular mass around 956.3 Da. Further, the interaction between peptides and bacterial membranes as part of their killing mechanism was studied by fluorescence and electron microscopy. The outer membrane and cytoplasmic membrane was the target of action of Leucrocins as assayed in model membrane by release of β-galactosidase due to the membrane permeabilization. Finally, the hemolytic effect was tested against human red blood cell. Leucrocin I, III and IV showed less toxicity against human red blood cells than Leucrocin II.

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1. Introduction

The rapid emergence of antibiotic-resistant bacterial pathogens is a serious problem, and extensive effort has been focused on the development of new classes of antimicrobial agents (Neu, 1992). Antimicrobial proteins and peptides are one group of antimicrobial agents that are phylogenetically ancient components of innate host defense mechanisms and are expressed by immune and non-immune cells of both invertebrates and vertebrates (Beck and Habiicht, 1996). These antimicrobial agents have been isolated from numerous types of organisms, ranging from bacteria to plants and animals (Treffers et al., 2005). The biological activity of these agents demonstrates potency against a broad spectrum of microorganisms. Many antimicrobial molecules appear to act via specific, but not receptor-mediated, permeabilization of microbial membranes and have selective targets in their specificity of killing (Yeaman and Yount, 2003). All of them cause lysis by two-step mechanisms and consist of (i) binding to a negatively charged membrane with a cationic nature and then (ii) permeabilization of the microbial membrane (Dathe and Wieprecht, 1999). Nevertheless, these proteins and peptides use many structures and mechanisms for destroying microorganisms, including binding to an intracellular target such as DNA, RNA and/or protein and interfering with enzymes causing inhibition of metabolic processes (Brogden, 2005). These properties confer considerable potential for the development of these agents as novel therapeutic agents to overcome the resistance problem (Tossi et al., 2000).

Mammalian white blood cells (neutrophils, macrophages, eosinophils, basophils and natural killer cells) are a good source of antimicrobial substances (Treffers et al., 2005). These cells express a variety of proteins and peptides that act as effector molecules that are able to kill or inactivate microbial pathogens. Mammalian white blood cells produce many different species of antimicrobial substances such as peptidoglycan recognition proteins (Tydell et al., 2006), α-defensin (Lehrer et al., 1993), β-defensin (Selsted et al., 1993), LL-37 (Gudmundsson and Agerberth, 1999), lysozyme (Pellegrini et al., 1991), cathelicidins (Zanetti, 2004) and chemokines (Becerra et al., 2007). Many antimicrobial peptides display activity against Gram-positive and Gram-negative bacteria, yeasts and fungi, and even certain enveloped viruses and protozoa. Other peptides are more restricted in their spectrum of activity, and even minor variations in peptide structure can influence activity. A systematic understanding of the relationship between peptide structure and activity is an important area.
for future investigation. Accumulating evidence has shown that many peptides act synergistically with larger polypeptides whose antimicrobial activity is enzymatic (e.g., lysozyme) or is dependent on the specific recognition of bacterial macromolecules (e.g., the bactericidal permeability-inducing protein, BPI) (Levy et al., 1994). Synergistic interactions between two antimicrobial peptides in frog skin, magainin 2 and PGLa (antibiotic peptide in the magainin family), have also been reported (Westerhoff et al., 1995). In addition to their action towards microbes, some antimicrobial peptides can function as regulatory molecules in the host. For example, in vitro studies suggest that defensins attract phagocytes and lymphocytes to sites of infection, inhibit the release of cortisol from adrenal cells, induce the proliferation of fibroblasts and modify ionic flux in epithelial cells (Ganz and Lehrer, 1995). However, most research to date has focused on searching for peptides which possess antimicrobial activity without causing resistance in the targeted microorganisms. Crocodilians are known to live with opportunistic bacteria without overt physiological effects. The immune system of crocodilians has not been well characterized, but several reports have described the efficacy of alligator serum in fighting bacteria, viruses and amoeba (Merchant et al., 2003, 2005a). Recently, Merchant et al. (2005b) proposed that the complement systems of alligators are effective in killing bacteria. In addition, white blood cell extracts from the American alligator have been shown to have a broad spectrum of antibiotic effects on bacteria, fungi and viruses (Merchant et al., 2006). More recently, Preecharram et al. (2008, 2010) have reported that antimicrobial substances were found in the serum and plasma of Siamese crocodiles (Crocodylus siamensis). Similar to the American alligator, white blood cell extracts from Siamese crocodile might possess antimicrobial compounds to kill unwanted microbes.

2. Materials and methods

2.1. Microorganisms

Gram-positive bacteria: Bacillus amyloliquefaciens TISTR 1045, Bacillus cereus ATCC 11778, Bacillus licheniformis TISTR 1010, Bacillus megaterium (clinical isolate), Bacillus pumilus TISTR 905, Bacillus sphaericus TISTR 678, Bacillus subtilis TISTR 008, Micrococcus luteus ATCC 4618, Staphylococcus aureus ATCC 25923, S. aureus TISTR 5049, S. aureus (clinical isolate), Staphylococcus epidermidis (clinical isolate), Xanthomonas sp. Streptococcus pneumoniae DMS 5851.

Gram-negative bacteria: Aeromonas hydrophila ATCC 7966, Escherichia coli (clinical isolate), E. coli 0157:H7, Klebsiella pneumoniae ATCC 27736, Pseudomonas aeruginosa (clinical isolate), P. aeruginosa ATCC 27853, Salmonella typhi (clinical isolate), S. typhi ATCC 5784, S. typhi B (clinical isolate), S. paratyphi B (clinical isolate), S. paratyphi A (clinical isolate), Vibrio cholerae (clinical isolate).

Yeasts: Candida albicans TISTR 5779, Saccharomyces cerevisiae TISTR 5539, S. cerevisiae TISTR 5596.

2.2. Blood of crocodiles

The crocodile blood was obtained from slaughterhouse after the animals were stunned by electric blow and then blood samples were transferred immediately to sterile 15 ml centrifuge tubes containing 1 ml of 0.5 M EDTA. The tubes were kept on ice. The volume of blood collected from each crocodile depended on the size of the animal.

2.3. Isolation of white blood cells

Whole blood samples were put on ice overnight, during which time the erythrocytes settled to the bottom of the tubes. The interphase layer containing the white blood cells were collected and centrifuged at 800 x g for 20 min (25 °C). The contaminating red blood cells were lysed by addition of 0.83% ammonium chloride solution to the white blood cells suspension at a ratio of 3:1 (Treffers et al., 2005). The white blood cells were collected by centrifugation at 800 x g for 20 min (25 °C). The cell pellet was gently resuspended in a normal saline solution (0.89% NaCl) and centrifuged again at 800 x g for 20 min (25 °C). Pellets composed of white blood cells were then kept in centrifuge tubes at −70 °C prior to use.

2.4. White Blood Cells extraction

The crude extract from the white blood cells of the crocodiles was obtained using a modified method previously described by Treffers et al. (2005). Briefly, white blood cells were frozen at −70 °C overnight. For lysis, white blood cells were thawed, resuspended in 0.01 M acetic acid and sonicated to release the antimicrobial substances. The solution containing the antimicrobial substances was collected by centrifugation (12,000 x g, 20 min, 4 °C). The supernatant was kept and centrifuged again at 12,000 x g for 20 min (4 °C). The crude white blood cell extracts were lyophilized, dissolved in 0.01% acetic acid and stored at −70 °C until needed.

2.5. Antimicrobial activity assays

Antimicrobial activity was assayed using the disc diffusion technique. The disc diffusion assay was performed using nutrient agar, as described previously by Anderson and Yu (2003). Antibacterial activity was tested with both Gram-negative and Gram-positive bacteria and yeast. The bacteria (OD600 0.5–0.6) were diluted in nutrient broth (Yeast used YM medium) to a final optical density (OD 405) of around 0.1, and 200 µl of the diluted bacteria was pipetted and swabbed onto a 20 ml nutrient agar plate. Sterile paper discs (6 mm) were placed on the nutrient agar plate and a test sample such as white blood cell extract (30–60 µg/30 µl) was added and allowed to diffuse into each paper disc. Plates were incubated for 12 h at 37 °C. Antimicrobial activity was assessed by the size of the clearing zone around the paper disc. The broad-spectrum antibiotic, streptomycin at 10 µg/disc, and disc containing 0.01% sterile acetic acid were used as positive and negative controls, respectively.

2.6. Purification of antimicrobial substances from crocodile white blood cell extracts

The crude white blood cell extracts were filtered through 0.45 µm citrate filter membranes. The filtered solution (containing a total protein concentration of 46.25 µg) was subjected to chromatography on a UNO Q1 column (Bio-Rad, USA) which contains Q sepharose fast flow resin. Chromatography was performed using a biologic duo flow program (Bio-Rad, USA) for fast performance liquid chromatography (FPLC). The column was washed by the gradient solution composed of 25 mM Tris–HCl pH 8.1 (solvent A) and 0.5 M NaCl in solvent B (solvent B). The solutions were pumped through the column at a rate of 60 ml/h and the absorbance was monitored at a wavelength of 280 nm using a UV detector. The fractions (3 ml/fraction) corresponding to the peaks were collected, concentrated by freeze drying, dissolved in 0.01% sterile acetic acid and assayed for antimicrobial activity using the disc diffusion method. Antimicrobial substances from the active fraction were further purified by gel filtration on a 0.5 cm x 25 cm Superdex-30 prep column. The column was eluted with 0.1% trifluoroacetic acid (TFA) with a flow rate of 1.0 ml/min. Every protein and peptide fraction was saved and lyophilized. The fractions from gel filtration were dissolved in 0.01% acetic acid and stored at −70 °C until needed.
isolated by gel filtration were passed through a 0.45 μm citrate filter membrane. Samples were loaded onto a 250 mm × 4.6 mm Apollo C18 5U reverse phase HPLC column equilibrated with 0.1% trifluoroacetic acid (0.1%TFA, solvent A) at a flow rate of 1 ml/min. The column was eluted with a linear gradient of 0–60% acetonitrile (ACN) in 0.1% trifluoroacetic acid (solvent B). Elution was performed at a rate of 1% min⁻¹ for 0–20% ACN, 1.7% min⁻¹ for 20% to 50% ACN, and 10% min⁻¹ for 50–100% ACN. Fractions of purified peptides were collected, lyophilized and re-suspended in 0.01% sterile acetic acid. The purified peptides were then assayed for antimicrobial activity using the disc diffusion technique.

2.7. Minimal inhibitory concentration (MIC) of antimicrobial peptides

The minimal inhibitory concentrations (MICs) of the peptides Leucrocin I, II, III and IV were determined by a microdilution susceptibility test in 96-well microtiter plates, according to a modified version of Hancock’s “Modified MIC method for cationic antimicrobial peptides” (Falla et al., 1996). The antibacterial activity was tested on the reference strains S. epidermidis, S. typhi and V. cholerae. The antimicrobial activity assay was conducted with different concentrations of these peptides to compare their effects on bacterial growth. Antibacterial activity was examined using log phase culture bacteria in nutrient broth at 37 °C. The cultures were diluted with 10 mM phosphate buffer (PB) pH 7.4 to give approximately 1 × 10⁶ CFU/ml. One hundred microliters of the diluted test strain was transferred to a 96-well plate and 100 μl of the peptide diluted to different concentrations in 10 mM PB buffer pH 7.4 was added to each well. The plates were incubated at 37 °C for 16 h. After incubation for 16 h, the OD at 550 nm of each well was determined using a Benmark microtiter plate reader (Bio-Rad, USA). Sterile water and streptomycin were used as negative and positive controls, respectively. Antimicrobial activity is expressed in terms of the lack of growth observed when testing with the above experiment (Nakamura et al., 1988; Casteels et al., 1989; Hetru and Bulet, 1997). The MIC is defined as the minimal agent concentration that inhibits bacterial growth (Zhu et al., 2007).

2.8. Microbicidal activity assay of antimicrobial agents

S. epidermidis, S. typhi and V. cholerae were used as target organisms in the microbicidal assay as previously described by Selsted et al. (1985, 1993). Bacteria (OD₆₀₀ 0.5–0.6) was centrifuged at 800 × g for 5 min, then washed with 5 mM glucose in 0.1 M PB buffer pH 7.4 three times. Bacterial cells were suspended in the same buffer. The suspended bacterial cells (OD₆₀₀ around 0.5) were mixed with 100 μl of antimicrobial peptides; S. epidermidis (clinical isolate) and S. typhi (clinical isolate) were mixed with Leucrocin I and Leucrocin II; V. cholerae (clinical isolate) was mixed with Leucrocin III and Leucrocin IV. As positive and negative controls, 70% ethanol and buffer were used, respectively. The mixtures for each test were incubated at 37 °C for 2 h. The reactions were then diluted ten-fold and spread onto plates. Colonies of bacteria from each reaction were counted and expressed as CFU/ml.

2.9. Determination of toxicity of antimicrobial peptide by hemolytic assay

Human red blood cells (HRBCs) were centrifuged and washed with phosphate buffered saline (PBS) pH 7.4. HRBCs were suspended with the same buffer (2%, w/v). One milliliter of 2% HRBCs was mixed with the peptides and incubated at 37 °C for 30 min. The reactions were centrifuged and the absorbance of the supernatant was measured at 525 nm. PBS and 1% Triton X-100 were used as negative and positive controls, respectively. The percentage of hemolysis was calculated and compared.

2.10. Analysis of antimicrobial substance mechanism of action by scanning electron microscopy

Scanning electron microscopy (SEM) was performed according to Lau et al. (2004) with slight modifications. S. epidermidis and S. typhi were grown in nutrient broth and harvested at the logarithmic phase of growth by centrifugation at 3000 × g for 5 min. The bacterial cells were then washed twice with PBS pH 7.0 and resuspended to a final concentration of 1 × 10⁶ CFU/ml. Aliquots of suspensions of S. epidermidis (100 μl) and S. typhi were individually incubated with Leucrocin I (10 μg/50 μl) or Leucrocin II (10 μg/50 μl) at 37 °C for 2 h. The 150 μl solutions 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 applied to a 0.2 μm cellulose acetate membrane filter (Sartorius AG, Germany) for 5 min and then washed twice with PBS. The fixed material was dehydrated by rinsing (for 15 min) repeatedly with a series of ethanol solutions containing 30%, 50%, 70%, 90% and finally 100% ethanol. The dehydrated material in absolute ethanol was dried in a critical point dryer (CPD7510; Thermo VG Scientific, England) with carbon dioxide as the drying agent. Dry materials were coated with a sputter coater (SC7620, Polaron, England) with gold palladium and examined by SEM (LEO1450VP, LEO Electron Microscopy Ltd., England) operating at 12–20kV. The negative control was performed in a similar manner except the bacterial cells were incubated with PBS buffer (pH 7.0) instead of the antibacterial compound.

2.11. Outer membrane permeabilization assay

1-N-phenylnaphthylamine (NPN) is a hydrophobic fluorescence probe (Loh et al., 1984; Falla et al., 1996) that is used to study the mode of action of the antimicrobial peptides. Gram-negative bacteria have two envelope membranes. Overnight cultures of S. typhi and V. cholerae were transferred to fresh nutrient broth medium and grown to OD₆₀₀ values of 0.5–0.6. Cells were harvested, washed with HEPES buffer pH 7.24 and resuspended in 5 mM KCN in HEPES buffer pH 7.24 (OD₆₀₀ around 0.5). To 3 ml of cells, 60 μl of 500 μM NPN was added, followed by the peptide samples after 30 seconds. Excitation and emission wavelengths were set at 315 nm and 400 nm, respectively. If peptides can bind and permeabilize the outer membrane, NPN will be taken up into the interior which is a hydrophobic environment. The increase in fluorescence as a result of partitioning of NPN into the interior outer membrane was measured 14 min after the addition of peptides using a fluorescence spectrophotometer. Streptomycin sulphate (100 μg) was used as a positive control.

2.12. Synthetic membrane permeabilization assay

2.12.1. Preparation of liposome encapsulated β-galactosidase

Lecithin from soy bean (1.2 g) was dissolved in 50 ml PBS pH 7.4. A solution of lecithin was placed in a 50 ml beaker and dispersed in β-galactosidase enzyme (0.001 g) in 50 ml PBS, pH 7.4. The solution was sonicated for 10 min to obtain vesicles. The mixture was incubated at 4 °C overnight. Liposomes were transferred into a new tube. The liposomes were then suspended in the same buffer and stored at room temperature prior to use.

2.12.2. Inner membrane permeabilization assay

The extent of cytoplasmic membrane permeabilization was determined by measurement of β-galactosidase activity in liposomes using o-nitrophenyl-β-D-galactopyranoside (ONPG), a non-membrane-permeable chromogenic substrate. Liposome
encapsulated β-galactosidase (50 μl) was added to a 96-well plate and then 1.5 mM ONPG (100 μl) was added. Peptides were mixed at the concentrations of Leucrocin I (0.015 μg/50 μl), Leucrocin II (0.25 μg/50 μl), Leucrocin III (0.8 μg/50 μl) and Leucrocin IV (0.6 μg/50 μl). The hydrolysis of ONPG to o-nitrophenol over time was monitored spectrophotometrically at 415 nm following the addition of peptide samples at five fold the MIC values.

2.13. Peptide mass analysis by LC/MS/MS mass spectrophotometer

Purified peptides Leucrocin I and II were frozen dry. Each fragment was digested with trypsin, and the tryptic peptides were resolved by reverse phase HPLC. After that, peptide fragments were analyzed by mass spectrometry.

2.14. Determination of N-terminal amino acid sequence

The N-terminal amino acid sequence of peptides was analyzed with tryptic peptide performed on an Applied Biosystem/MDS Sciex API QSTAR Pulsar Hybrid LC/MS/MS System equipped with o-MALDI ionization source (MDS Sciex, API-QSTAR, Applied Biosystems, Canada). The digested solution was purified on a RP-HPLC column using a Jasco 800 series HPLC instrument (Japan Spectroscopic Co., Japan). Automated Edman peptide sequencing was carried out on a glass fiber disk using a Procise 492 Protein Sequencer (Applied Biosystem, Japan), with pulsed liquid TFA delivery.

3. Results

3.1. Antimicrobial activity of crocodile white blood cell extracts measured by the disc diffusion technique

Antimicrobial activity of Siamese crocodile white blood cell extracts was screened using 14 species of Gram-positive bacteria, 12 species of Gram-negative bacteria, and 3 species of yeast. White blood cell extracts were tested against organisms with concentrations ranging from 30 to 60 μg/disc. The dose-dependent activity of the white blood cell extracts as measured by the size of the clear zones surrounding the sample discs was observed (Fig. 1). All 9 species of organisms: S. epidermidis, B. megaterium, B. subtilis TISTR 008, P. aeruginosa, P. aeruginosa ATCC 27853, S. typhi, S. typhi ATCC 5784, V. cholerae and C. albicans TISTR 5779 were sensitive to the crocodile white blood cell extracts. In this case, the zone of clearing was greater against V. cholerae than other species. The test was repeated with a 10 μg streptomycin disc as the positive control, demonstrating a clear zone. The negative control, a 0.01% sterile acetic acid disc resulted in no such clear zone. These activities are sensitive to pronase, are heat-stable and are unaffected by the presence of EDTA (data not shown).

3.2. Purification of antimicrobial agents in crocodile white blood cell extracts

White blood cell extract was first separated through an UNO Q1 (Bio-Rad, USA) anion-exchange column containing Q Sepharose fast flow resin. Four protein fractions were obtained and designated P1, P2, P3, and P4 (data not shown). All four protein fractions displayed broad spectrum antimicrobial activity against the test organisms. However, to confirm the antimicrobial activity of fractions P2, P3 and P4 which might contain some salt from the buffer used, fractions P1, P2, P3 and P4 were dialyzed against double distilled water, lyophilized, and dissolved in 0.01% sterile acetic acid, and assayed for antimicrobial activity by the disc diffusion technique. The results showed that the P1 and P3 fractions still displayed broad spectrum antimicrobial activity against the test organisms. Both peaks could inhibit the growth of S. epidermidis and V. cholerae. Peak B from the gel filtration step (MW<10 kDa) was further purified by C18 reverse phase HPLC. The RP-HPLC chromatogram is presented in Fig. 2A. Ten
Table 1
Minimal inhibitory concentration (MIC) for the peptides against three bacterial strains.

<table>
<thead>
<tr>
<th>Peptides</th>
<th>MIC (µg/ml)</th>
<th>S. epidermidis</th>
<th>S. typhi</th>
<th>V. cholerae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leucrocin I</td>
<td>25</td>
<td>25</td>
<td>0.156</td>
<td></td>
</tr>
<tr>
<td>Leucrocin II</td>
<td>0.66</td>
<td>0.66</td>
<td>2.88</td>
<td></td>
</tr>
<tr>
<td>Leucrocin III</td>
<td>&gt;156</td>
<td>&gt;156</td>
<td>16.56</td>
<td></td>
</tr>
<tr>
<td>Leucrocin IV</td>
<td>5.31</td>
<td>&gt;43.70</td>
<td>10.50</td>
<td></td>
</tr>
<tr>
<td>Streptomycin</td>
<td>0.73</td>
<td>5.82</td>
<td>11.64</td>
<td></td>
</tr>
</tbody>
</table>

peptide peaks were obtained, and designated according to their elution times as HP1 (6.29 min), HP2 (8.37 min), HP3 (9.41 min), HP4 (11.02), HP5 (11.59 min), HP6 (14.32 min), HP7 (18.39 min), HP8 (19.55 min), HP9 (21.41 min) and HP10 (20.31 min). All of the peptides were measured for antibacterial activity against S. epidermidis and V. cholerae. HP1 and HP9 showed antibacterial activity against S. epidermidis (Fig. 2B), and HP3 and HP4 inhibited the growth of V. cholerae (Fig. 2C). Peptides HP1, HP9, HP3 and HP4 were named Leucrocin I, Leucrocin II, Leucrocin III, and Leucrocin IV, respectively. Leucrocin I–IV were tested for antimicrobial activity towards S. typhi and C. albicans TISTR 5779 as well. The results showed that S. typhi was weakly inhibited by Leucrocin I and II, and that C. albicans TISTR 5779 was inhibited by Leucrocin III (data not shown).

3.3. Minimal inhibitory concentration

The minimal inhibitory concentrations of Leucrocin I–IV are shown in Table 1. Leucrocin II had the most potent activity against S. epidermidis (0.66 µg/ml), S. typhi (0.66 µg/ml) and V. cholerae (2.88 µg/ml). Leucrocin III (16.56 µg/ml) had good activity against V. cholerae and Leucrocin IV showed strong antimicrobial activity against S. epidermidis (5.31 µg/ml) which was greater than that of the other species tested. However, Leucrocin III was less active against S. epidermidis and S. typhi (>156 µg/ml). Therefore, these MICs values were chosen for further characterization of antimicrobial properties.

3.4. Microbicidal activity of Leucrocin I, II, III and IV

Killing properties of the Leucrocin I–IV peptides were determined by the total plate count method. Leucrocin I and II showed strong killing activity towards the S. epidermidis bacteria and weak killing activity towards the S. typhi (Fig. 3A), as seen by a decrease in the number of colonies of these bacteria. Leucrocin II decreased the amount of bacteria 10-fold when compared to the negative control. Leucrocin III and IV resulted in cell killing of V. cholerae (Fig. 3B). The number of colonies of V. cholerae decreased 5-fold (Leucrocin III) and 6-fold (Leucrocin IV) when compared to the negative control.

3.5. Toxicity to human red blood cells

Hemolytic assays have been used to assess the toxicity of peptides towards HRBCs in vitro. Incubation of 2% HRBCs (w/v) with Leucrocin I and II for 30 min at room temperature resulted in an increase in OD at 525 nm, reflecting disruption of the cells. The percentages of the hemolytic human red blood cells were calculated from the OD at 525 nm. Triton X-100 was used as positive control, and taken as the 100% hemolysis value. The results showed that Leucrocin I has hemolysis percentage values of around 12.97, 10.74 and 15.88 at concentrations of 12, 24 and 48 µg/ml, respectively. Leucrocin II showed percentages of hemolysis around 8.28, 11.63 and 91.73, at concentrations of 3.0, 6.0 and 10.0 µg/ml, respectively (Fig. 4A). When the toxicities of Leucrocin I and II were compared, the results showed that Leucrocin II has a higher toxicity with

Fig. 3. S. epidermidis, S. typhi and V. cholerae were killed by the peptide Leucrocin. (A) Colony of S. epidermidis and S. typhi after treatment with Leucrocin I (5 µg/ml) or Leucrocin II (5 µg/ml). (B) A colony of V. cholerae after mixing with Leucrocin III (19.7 µg/ml) and Leucrocin IV (12.34 µg/ml). The positive control is 70% ethanol and the negative control is PBS buffer pH 7.4 supplemented with 5 mM glucose.

Fig. 4. (A) Toxicity of Leucrocin I and Leucrocin II with HRBCs. The positive control is 1% Triton X-100, and the negative control is PBS. (B) Toxicity of Leucrocin III and Leucrocin IV with human red blood cells. The positive control is 1% Triton X-100, and the negative control is PBS.
HRBCs, greater than that of Leucrocin I. In addition, Leucrocin III and IV were assayed for their toxicity towards HRBCs. The results indicate that Leucrocin III and IV displayed no toxicity to HRBCs at the test concentrations (Leucrocin III: 4, 8, 16 μg/ml; Leucrocin IV: 87, 175 μg/ml). However, the concentration of Leucrocin IV of 346 μg/ml has been reported to be toxic to HRBCs (Fig. 4B).

3.6. Scanning electron microscopy

The effects of Leucrocin I and II on the cell membranes of S. epidermidis and S. typhi were examined by scanning electron microscopy (SEM) to gain insight into the direct effects of Leucrocin I and II on the morphology of S. epidermidis. The results showed that, bacteria left untreated for 1 or 2 h displayed a smooth bright surface with no apparent cellular debris (Fig. 5A and D). In contrast, peptide-exposed cells exhibited a wide range of significant abnormalities. The cell surfaces of S. epidermidis showed blebs upon Leucrocin I and II treatment (Fig. 5B and C) and the demonstrated a collapsed cell structure (Fig. 5E and F). In contrast, Leucrocin I and II treatment of S. typhi resulted in deep roughening of the cell surface and collapsed cell structure (data not shown).

3.7. Membrane permeabilization assay

3.7.1. Outer membrane permeabilization assay

The ability to permeabilize the outer membrane of S. typhi and V. cholerae was determined by using NPN. NPN is a hydrophobic fluorescent probe that fluoresces weakly in an aqueous environment and strongly when it enters a hydrophobic environment such as the interior of a membrane. Normally, NPN is excluded from Gram-negative bacterial cells. At this stage, the maximum excitation wavelength is 315 nm, and the maximum emission wavelength is 400 nm, similar to the maxima observed with NPN added to aqueous solution. From experiments performed in triplicate, we found that the polycationic antibiotic, streptomycin (20 μg/ml) and Leucrocin I, II, III and IV showed a rapid increase in fluorescence intensity, reaching a stable plateau after 7 min when compared to cells in buffer only and cells with NPN only. The increasing intensity of fluorescence was also seen in S. typhi cells which were treated with Leucrocin I and Leucrocin II (Fig. 6A). In addition, increasing intensity of fluorescence was also observed in intact V. cholerae cells. Leucrocin IV was able to disrupt the bacterial outer membrane better than Leucrocin III and able to permeabilize the outer membrane, albeit at a higher rate than Leucrocin III, as indicated by the slower increase in fluorescence intensity of Leucrocin III (Fig. 6B).

3.7.2. Inner membrane permeabilization

Liposome encapsulated β-galactosidase was used to measure inner membrane permeabilization. The results showed that Leucrocin II, III and IV have the ability to lyse the liposome (Fig. 7). After incubation of the peptides with the liposome for 10 min, the activity of the inner β-galactosidase was determined by measuring the absorbance of o-nitrophenol at 415 nm. Leucrocin II, III and IV treat-

![Fig. 5. SEM of Leucrocin I- and II-treated S. epidermidis. (A) Control bacteria after 1 h and (D) 2 h. (B and E) Bacteria after treatment with Leucrocin I at a sublethal concentration (40 μg/ml) for 1 h and 2 h. (C and F) Bacteria after treatment with Leucrocin II at a concentration of 80 μg/ml for 1 h and 2 h. See the results section for other experimental details and descriptions of the images. Each figure has been magnified 20,000 times.

![Fig. 6. Time course of Leucrocin effects on (A) S. typhi and (B) V. cholera.](image-url)
ment resulted in an increase in OD 415 nm, reflecting leakage of the enzyme into solution and digestion of ONPG to p-nitrophenol, while Leucrocin I treatment resulted in no detection of β-galactosidase in solution. Triton X-100 was used as the positive control. Liposome incubation with Triton X-100 showed more inner β-galactosidase activity than liposome treated with the peptides.

3.8. LC/MS/MS analysis

The amino acid sequences of Leucrocin I and II were analyzed by LC/MS/MS. The profile showed that Leucrocin I has a precursor mass of around 806.99 Da. Leucrocin II has a precursor mass of 956.37 Da. The deduced amino acid sequence of these peptides were shown in Table 2. The amino acid sequence of Leucrocin I was NGVQPKY with an approximate mass of around 804.90 Da and a pI of 8.59. The amino acid sequence of Leucrocin II was found to be NAGSLLSGWG with an approximate mass of around 847.88 Da, and pI of 5.52. These amino acid sequences were not homologous with any antimicrobial peptides in the database. However, amino acid sequences of Leucrocin III and IV could not be analyzed which might be due to the purity of the peptides.

4. Discussion

Antimicrobial peptides have been isolated from a broad variety of phylogenetically diverse organisms (Ganz and Lehrer, 2001). These peptides can be segregated into a variety of classes based on their structures (Roman, 1995). These classes exhibit different mechanisms of antimicrobial action (Yeaman and Yount, 2003). However, all antimicrobial peptides have several factors in common. They all tend to be cationic in nature so that they are able to interact with the negatively charged phospholipids in the membranes of microbes. Broad-spectrum antimicrobial activities have been reported for peptides isolated from amphibian skin (Lorin et al., 2005) and the white blood cells of humans (Garcia et al., 2001), sheep and goats (Shamova et al., 1999), rats (Eisenhaver et al., 1989), rabbits (Lehrer et al., 1975), guinea pigs (Selson and Harwig, 1987), bovine species (Tydell et al., 2006) and alligators (Merchant et al., 2006). Similar to these results, we report the discovery of the four Leucrokins from Siamese crocodile white blood cell extracts by the analysis of their amino acid sequences. The novel Leucrokins exhibited antimicrobial activities against a wide variety of microbial species. In addition, we ruled out the possibility that these observed activities are due to the serum complement system of proteins. To confirm the activity of Leucrokins, the peptides were synthesized and tested their activity. The MIC values of synthetic Leucrokins seem higher than native peptide (data not shown). This result might be due to they cannot fold to right functional structure because of microenvironmental conditions (temperature, pH, cationicity, anionicity etc.) and target cell growth phase which have influence on the microbicidal activity of the antimicrobial peptides.

The short amino acid sequence of Leucrocin I was analyzed by Gor IV secondary prediction method. Leucrocin I contains extended strand (28.57%) and random coil (71.43%) components. In the same manner as Leucrocin I, which showed a high content of random coil structure, the secondary structure of Leucrocin II was composed of extended strand (44.44%) and random coil (55.56%) components. From these results, Leucrocin I and Leucrocin II showed no core amino acid sequence, however both peptides might adopt a helical structure from a random coil which is important to their interaction with a membrane. From the reports of Brogden (2005), cationic antimicrobial peptides first bind to the lipopolysaccharide (LPS) or lipoteichoic acid (LTA) of bacterial cells through ionic interactions (ion binding), and after that they form a structure that disrupts and permeabilizes the bacterial cell membrane. However, amino acid sequences of Leucrocin II do not contain positively charged amino acids but high content of hydrophobic amino acid. Its mechanism to penetrate the bacterial membrane may be different from cationic antimicrobial peptides and its mode of killing bacteria might be due to inhibiting protein or DNA synthesis as reported for the antimicrobial peptide, PR-39 (Cabiaux et al., 1994). This result coincides with the purification process by anion exchanger column. The elution position of the active fraction P1 from anion exchange column (data not shown) indicates that the natures of active component were composed of cationic or no charge molecule because the fraction is not significantly retained in the Q Sepharose column in the condition used. Leucrocin II shows the most potent and wide antibacterial activity. This result might be due to the hydrophobicity property which is important to partitioning of the peptide into the membrane.

The target for the majority of amphipathic antimicrobial peptides is the cytoplasmic membrane. In this study, we demonstrated that the unique structure of Leucrokins with a low amount of positively charge amino acids causes a remarkable modification of the cellular shape, as shown by SEM. This activity of Leucrocin I and II towards Gram-negative bacteria is similar to the antimicrobial activity of Temporin-L on E. coli cells (Mangoni et al., 2004) and that

Table 2

Peptide sequences from LC/MS/MS of Leucrocin I and II.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Precursor Mass</th>
<th>Amino acid sequence</th>
<th>Charge</th>
<th>Mass analysis (Da)</th>
<th>% Hydrophobicity</th>
<th>pI Secondary structure prediction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leucrocin I</td>
<td>806.99</td>
<td>NGVQPKY</td>
<td>1</td>
<td>804.9</td>
<td>11</td>
<td>8.59</td>
</tr>
<tr>
<td>Leucrocin II</td>
<td>956.37</td>
<td>NAGSLLSGWG</td>
<td>0</td>
<td>847.88</td>
<td>40</td>
<td>5.52</td>
</tr>
</tbody>
</table>
of the serum of the Siamese crocodile on S. typhi cells (Preecharram et al., 2008). The shape of these bacterial cells is shriveled, and the structure collapsed as time went on. In contrast, Gram-positive bacteria formed blebs on the cell surface and then collapsed, similarly to that observed for the peptides Crocosin (Preecharram et al., 2010), Magainin 2 (Park et al., 2000), SMP29 and CAP18 (Kalfa et al., 2001). These results indicate that Leucrocin I have active target sites on the cell surface. Further Leucrocin I, II, and IV can reach the bacterial membrane, which might involve steps of interaction between membrane and peptide and lead to permeabilization of the outer and inner membranes. Firstly, Leucrocin I increase NPN fluorescence the same as streptomyacin which indicates that Leucrocin I, II, and IV have permeabilized the outer membrane of the intact cells. This result is similar to the cationic antimicrobial peptides SMP29 (Kalfa et al., 2001), Indolicidin (Falla et al., 1996) and Temporin-I (Mangoni et al., 2004). To determine the leakage of the inner membrane by these peptides, liposomes mimicking the cytoplasmic membrane with encapsulated β-galactosidase were used. The results indicated that Leucrocin I, II, and IV can disrupt the liposome membrane. However, for Leucrocin I, no β-galactosidase activity could be detected. This result may be due to the low concentration of Leucrocin I. Taking all the data into account, we believe that the Leucrocin peptides kill bacteria by perturbing the permeability of the bacterial cell membrane.

In conclusion, Siamese crocodile white blood cells containing antimicrobial proteins and peptides show broad spectrum antibacterial activity. These peptides act on the membrane target site of microorganisms. They have killing properties but are benign to HRBCs. Leucrocin I and II are not homologous with any antimicrobial peptides found in the data base. These results indicate that Leucrocin I and II are novel antimicrobial peptides.

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