KOMPARATIVNA AKTIVNOST CEKROPINA A I POLIMIKSINA B PROTIV BAKTERIJSKIH UZROČNIKA BOLESTI ŽABA

COMPARATIVE ACTIVITY OF CECROPIN A AND POLYMYXIN B AGAINST FROG BACTERIAL PATHOGENS

Schadic E., Cole A.L.J., Drusilla Mason

Abstract - The antimicrobial activity of two antimicrobial peptides, cecropin A and polymyxin B against different bacterial pathogens associated with bacterial dermatossepticemia, a fatal bacterial infectious disease of frogs was investigated. The peptides were tested in serial of concentrations (100-0.19 µg/ml) for growth inhibition of seven pathogens: Aeromonas hydrophila, Chryseobacterium meningosepticum, Citrobacter freundii, Klebsiella pneumoniae, Pseudomonas aeruginosa, Proteus mirabilis and Serratia liquefaciens. Their antimicrobial activity was compared with that of two antimicrobial peptides from frog skin, magainin 2 and aurein 2.1. Both cecropin A and polymyxin B, completely inhibited the growth of three pathogens: C. freundii, K. pneumoniae and P. aeruginosa at a concentration some sixteen times less than two skin peptides. Furthermore, cecropin A inhibited the growth of three pathogens resistant to the two skin peptides, A. hydrophila, C. meningosepticum and P. mirabilis. Polymyxin B also inhibited the growth of three pathogens resistant to the skin peptides, A. hydrophila, C. meningosepticum and S. liquefaciens. Cecropin A and polymyxin B have marked antibacterial activity against different frog bacterial pathogens indicating potential for therapeutic measures.

Keywords: frogs, antimicrobial, bacteria, cecropin, polymyxin, resistance

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Kratak sadržaj - Procijenjena je antimikrobna aktivnost dva antimikrobna peptida, cekropina A i polimiksina B protiv bakterijskih uzročnika fatalne bolesti žaba, bakterijske dermatoseptikemije. Peptidi su testirani u seriji koncentracija (100-0.19 µg/ml) za inhibiciju rasta sedam različitih uzročnika: Aeromonas hydrophila, Chryseobacterium meningosepticum, Citrobacter freundii, Klebsiella pneumoniae, Pseudomonas aeruginosa, Proteus mirabilis i Serratia liquefaciens, i bakterijskog saprofita, Lactococcus lactis. Njihova antibakterijska aktivnost je poređena sa aktivnošću dva peptida žablje kože, magaininom 2 i aureinom 2.1. Oba peptida su potpuno inhibirali rast tri uzročnika, C. freundii, K. pneumoniae i P. aeruginosa u koncentracijama koje su oko šesnaest puta manje od koncentracija dva kožna peptida. Što više, cekropin A je inhibirao rast tri uzročnika bolesti koji su rezistentni na kožne peptide, A. hydrophila, C. meningosepticum i P. mirabilis. Također, polimiks B je inhibirao rast tri uzročnika koji su rezistentni na kožne peptide, A. hydrophila, C. meningosepticum i S. liquefaciens. Cekropin A i polimiks B posjeduju značajnu antibakterijsku aktivnost protiv uzročnika bolesti, što pokazuje njihov potencijal za razvoj terapijskih tretmana, kako bi zamijenili konvencionalnu antibiotsku terapiju.

Ključne riječi: žabe, antimikrobici, bakterija, cekropin, polimiks, rezistencija

Introduction

Different opportunistic Gram-negative bacterial species are associated with bacterial dermatosepticemia, systemic bacterial infectious disease of frogs, including Aeromonas hydrophila, Chryseobacterium indolgenes, Chryseobacterium meningosepticum, Citrobacter freundii, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa and Serratia liquefaciens (4, 15, 19). Bacterial dermatosepticemia causes high mortalities in wild frog populations and major losses in captive frog populations (9, 19).

The multimicrobial nature of disease and association of multidrug resistance of bacterial pathogens to conventional antibiotics (10, 19) require novel approaches in the management of frog bacterial disease. One such approach could involve the use of the membrane acting cationic antimicrobial peptides with broad activity spectra and a mode of action less likely to induce multidrug resistance in bacterial cells.

Two different groups of cationic antimicrobial peptides that are known for antimicrobial activity against different Gram- negative bacterial species, the insect cecropins (A-D) of insects (5) and polymyxins (A-E) of the bacterium Paenibacillus polymyxa (18) could be potential candidates for effective therapeutic treatment. They bind to lipopolysaccharide complexes of Gram-negative bacteria and induce alterations in the hydrophobic-hydrophilic barrier of the cell membrane causing cell lysis (13). Their efficiency in inhibiting of growth of different Gram-negative bacterial pathogens of...
humans and different animals has led to their extensive use (3, 6, 17). Furthermore, cecropin genes have been successfully cloned in fish and used for the development of trans-genetic fish resistant to Gram-negative bacterial pathogens (12). Previous studies have shown that the natural mixtures of antimicrobial peptides of the skin innate immunity system of different frog species as well as purified single antimicrobial peptides that are effective against different frog bacterial pathogens do not have the antimicrobial activity against three notable bacterial pathogens including A. hydrophila, P. mirabilis and S. liquefaciens (14); however, the antimicrobial activity of other antimicrobial peptides including cecropins and polymyxins against any frog bacterial pathogen have not yet been studied.

The aim of this study was to characterize the antimicrobial activity of cecropin A and polymyxin B against different frog bacterial pathogens. The activity of cecropin A and polymyxin B was compared with two of two frog skin peptides, aurein 2.1 and magainin 2. Aurein 2.1 and magainin 2 are single peptides of two Litoria frog species, L. aurea and L. raniformis, and Xenopus laevis, respectively (11, 20).

Material and Methods

Bacterial Cultures

All bacterial isolates were obtained from the frog pathogen library, School of Biological Sciences, University of Canterbury (Christchurch, New Zealand). The collection includes different bacterial isolates collected from the hearts of septicemic animals during an extensive epidemiological survey between 2002 and 2006 (1, 14). Isolates of A. hydrophila, C. freundii, P. aeruginosa and S. liquefaciens were collected from captive X. laevis in 2003, and the isolate of C. meningosepticum was collected from captive L. aurea in 2002. A skin isolate of the saprophyte Lactococcus lactis was collected from captive L. raniformis in 2004. Isolates of A. hydrophila, K. pneumoniae and P. mirabilis were collected from L. ewingii taken from a site in Oxford Forest, New Zealand, in December 2005 at the time of a severe mortality outbreak. A standard reference strain, Escherichia coli (ATCC 25922) was purchased from the American Type Culture Collection (University Boulevard, Manassas, USA).

Peptides

Cecropin A, (KWKLFKKIEKVGQNIIRDGIKAGPAVAVGQATQIAK-NH2) and polymyxin B (cyclic peptide), were purchased from Sigma Chemical Co., (St Louis, USA.)

Antibacterial assays

Activities of cecropin A and polymyxin B were tested using the same growth inhibition assays for aquatic bacteria as described in previous studies (14, 16). Overnight
colonies of each isolate were selected from agar, transferred to soya tryptone broth and incubated for 8 h. Following incubation, the bacterial suspension was adjusted with phosphate buffered saline to achieve turbidity of 0.5 McFarland standard (corresponding to 1.5 x 10^8 colony forming units (cfu) per ml), and the adjusted suspension was used as inoculum. For the growth inhibition assay, 50 µl of Muller-Hinton broth with a bacterial concentration of 1 x10^6 cfu was plated into each well of a 96-well microtiter plate, and 50 µl of peptide was then added to each well, in serial dilutions (100, 50, 25, 12.5 6.25, 3.12, 1.56, 0.78, 039 and 0.19 µg/ml) corresponding to concentrations of effective antibiotic candidate. The exceptions were non-peptide wells, positive control wells, which received 50 µl of ddH_2O instead of peptides, and negative controls that received 0.4% paraformaldehyde, on separate plates. For growth inhibition assays with L. lactis, soya tryptone broth was used instead of Muller-Hinton broth. The samples were incubated aerobically for 24 h at 30 ºC and bacterial cell growth was measured as increased optical density at 490 nm with an ELISA plate reader. Five replicate reactions were tested for each peptide concentration and three independent assays were performed for all tested peptides. To determine minimum inhibitory concentration (MIC), the means of optical density values of the wells with peptide concentrations that completely inhibited growth were compared with the optical density values of the negative control reactions with no visible growth. The MIC was defined as the lowest concentration at which no significant growth was observed as in negative control reactions with 0.4% paraformaldehyde. They were compared with the published MICs of two frog skin peptides, aurein 2.1 and magainin 2 (14). Permission to reproduce the copies of MICs of two skin peptides was given by editorial board of Journal of Herpetology.

**Results**

Cecropin A inhibited the growth of two isolates of A. hydrophila and the single isolates of C. freundii, C. meningosepticum, K. pneumoniae, P. aeruginosa and P. mirabilis (Table 1) and standard reference strain E. coli (ATCC 25922). Polymyxin B inhibited the growth of the same isolates as cecropin except that it also inhibited the growth of S. liquefaciens while being inactive against the isolate of P. mirabilis (Table 1). Both of these two peptides were not active against L. lactis (Table 1). The MIC of cecropin A and polymyxin B for growth inhibition of the isolates of C. freundii, K. pneumoniae, P. aeruginosa, and standard reference strain E. coli (ATCC 25922) is at least sixteen times smaller than those of the two skin peptides, aurein 2.1 and magainin 2 (Table 1). The two skin peptides were inactive against the isolates of A. hydrophila and C. meningosepticum, P. mirabilis and S. liquefaciens (Table 1).
Table 1. Activities of cecropin A, polymyxin B, magainin 2, aurein 2.1

<table>
<thead>
<tr>
<th>Isolate</th>
<th>MIC (µg/ml)</th>
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<tbody>
<tr>
<td></td>
<td>Cecropin A</td>
</tr>
<tr>
<td>A. hydrophila Xl</td>
<td>3.12</td>
</tr>
<tr>
<td>A. hydrophila Le</td>
<td>3.12</td>
</tr>
<tr>
<td>C. freundii Xl</td>
<td>1.56</td>
</tr>
<tr>
<td>C. meningosepticum La</td>
<td>12.50</td>
</tr>
<tr>
<td>E. coli (ATCC 25922)*</td>
<td>0.78</td>
</tr>
<tr>
<td>K. pneumoniae Le</td>
<td>3.12</td>
</tr>
<tr>
<td>L. lactis Lr**</td>
<td>NA</td>
</tr>
<tr>
<td>P. aeruginosa Xl</td>
<td>1.56</td>
</tr>
<tr>
<td>P. mirabilis Le</td>
<td>50.00</td>
</tr>
<tr>
<td>S. liquefaciens Xl</td>
<td>NA</td>
</tr>
</tbody>
</table>

Peptides were tested for activity against bacterial isolates collected from four frog species: X. laevis (Xl), L. aurea (La), L. raniformis (Lr) and L. ewingii (Le). * denotes standard reference strain and ** denotes skin saprophyte. NA denotes no activity. \(^a\) Cited from Schadich (14).

Discussion

The marked activity of cecropin A and polymyxin B when compared with skin antimicrobial peptides suggests potential for the development of therapeutic measures for bacterial dermatosepticemia of frogs. This is also supported by observations that they are inactive against Gram-positive skin saprophyte L. lactis a component of skin and gut microbiota (Table 1), and is consistent with studies showing cecropin A and polymyxin B are not active against Gram-positive bacterial saprophytes of humans and different animals (2, 17).

Only two pathogens, S. liquefaciens and P. mirabilis were resistant to cecropin A and polymyxin B (Table 1) confirming the findings of previous studies on clinical and environmental isolates of these species, which showed they can develop resistance to either cecropin or polymyxin B (7, 8). The mechanism of resistance to cecropin A and polymyxin B is reported to involve the alteration of LPS components (7) although this has not been investigated for frog isolates of S. liquefaciens and P. mirabilis.

The overall importance of this study is that both cecropin A and polymyxin B were shown as potential candidates for therapeutic treatments of diseased frogs against frog bacterial pathogens. In order for these peptides to be effective they must gain access to infected tissues of diseased frogs. Further pharmacodynamic and pharmacotoxicological are indicated for this potential to be used.
Conclusions

Based on analysis of antimicrobial activity of cecropin A and polymyxin B against different frog bacterial pathogens, two conclusions are provided as follows.

1. Both cecropin A and polymyxin B have the activity against different bacterial pathogens including three pathogens susceptible to two skin peptides of frogs, aurein 2.1 and magainin 2, including *C. freundii*, *K. pneumoniae* and *P. aeruginosa* and two pathogens resistant to two skin peptides including *A. hydrophila* and *C. meningosepticum*, and cecropin A also has activity against *P. mirabilis* resistant to two skin peptides and polymyxin B has also activity against *S. liquefaciens* resistant to two skin peptides.

2. Their MICs required for complete inhibition of different bacterial pathogens have values within range of concentrations smaller than 100 µg/ml, which corresponds to concentrations of antibiotic candidates for development of effective antibacterial treatments of diseased frogs.

REFERENCES


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