Identification and characterization of novel reptile cathelicidins from elapid snakes

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1. Introduction

Cathelicidins are cationic host defense peptides that play an important role in innate immune system. Members of the cathelicidin family all include N-terminal signal peptides and proregions highly homologous to cathelin, a cathepsin L inhibitor, then followed by highly divergent C-terminal antimicrobial domains [2,14,20,26]. Only one cathelicidin termed LL-37 has been characterized in humans and LL-37 represents one of the most extensively studied members of

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cathelicidin family so far. LL-37 is released by proteolysis from the C-terminal end of the human CAP18 protein and is expressed mainly in leukocytes and epithelial cells. Besides its direct antimicrobial activity against both Gram-positive and Gram-negative bacteria, LL-37 also participates in adaptive immune system and is considered as an important immunoregulator [23,24]. LL-37 binds and neutralizes lipopolysaccharide (LPS) and protects host against endotoxic shock in rodent animal models of septicemia [5]. Furthermore, it is chemotactic for neutrophils, monocytes, mast cells and T cells, induces degranulation of mast cells and stimulates wound vascularization and re-epithelialization of healing skin [4]. Meanwhile, LL-37 also has antitumor and anti-HIV activity [9]. Recently, a cathelin domain derived peptide with antimicrobial activity isolated from ovine blood was reported, indicating that the cathelin domain might be digested by protease and generate antimicrobial activity [1].

Up to now, most of the identified cathelicidins were from mammalian species [25–27]. Five cathelicidins termed fowlucidin-1, -2, -3, B1 and myeloid antimicrobial peptide 27 were characterized from chicken [8,22]. Cathelicidins were also found in fish species of Atlantic hagfish Myxine glutinosa, rainbow trout Oncorhynchus mykiss and Atlantic salmon Salmo salar. Hagfish cathelicidins were considered as ancient members of the cathelicidin family [6,7,21]. Recently, a phylogenetic tree was constructed using cathelin protein sequences of mammalian, chicken and fish cathelicidins. Analysis of the constructed phylogenetic tree reveals that chicken fowlidicins fall between the two major clusters of mammalian cathelin regions of classical cathelicidins and neutrophilic granule proteins (NGPs) [8]. However, the evolutionary relationship among chicken fowlidicins, classical cathelicidins and NGPs cannot be solved without additional cathelicidins information. The molecular evolutionary analyses of vertebrate cathelicidins will be greatly improved by obtaining cathelicidin sequences from amphibian or reptile species.

Here, we reported the molecular cloning of cathelicidins from elapid snake species. Two cathelicidin-encoding full cDNA sequences and one partial cDNA sequence were cloned from constructed venom gland cDNA libraries of Naja atra, Bungarus fasciatus and Ophiophagus hannah, respectively. The partial cathelicidin-encoding cDNA sequence from king cobra covers the open reading frame. The putative king cobra mature cathelicidin was synthesized and its antibacterial and haemolytic activities were reported. We provided for the first time the close relationship among snake cathelicidins, rodent NGPs, avian fowlidicins (1–3) and chicken myeloid antimicrobial peptide 27.

2. Materials and methods

2.1. Materials

Living N. atra snakes were collected in Hunan province, China. Living O. hannah and B. fasciatus snakes were collected in Yunnan province, China. Total RNA isolation kit and first-strand cDNA synthesis kit were from Invitrogen. High-fidelity Pyrobest DNA polymerase, DNA fragment extraction kit and pMD18-T vector were from TaKaRa Biotechnology Co. Ltd. (Dalian, China). Microorganisms used were as follows: Escherichia coli ATCC 25922, E. coli ML-35 (ampicillin-resistant), E. coli clinically isolated strain (producing extended-spectrum β-lactamase and resistant to I, II, III and IV generation cephalosporins), Pseudomonas aeruginosa ATCC 27853, P. aeruginosa PA 01, multi-drug resistant clinic strains of P. aeruginosa, Enterobacter aerogenes and E. cloacae. All the microorganisms were obtained from The First Affiliated Hospital of Kunming Medical College, China. Human red blood cells were prepared from whole blood provided by Yunnan Blood Center (China) by conventional centrifugation. All other reagents used were analytic grade from commercial sources.

2.2. Molecular cloning of elapid cathelicidins

Elapid venom gland cDNA libraries were constructed with some modifications as described previously [30]. Briefly, mRNAs were prepared from the total RNA of one venom gland from N. atra, B. fasciatus or O. hannah snake species by oligo(dT) cellulose chromatography, respectively. A directional cDNA library was then constructed for each of the above mentioned three elapid species with a plasmid cloning kit (SuperScript Plasmid System, GIBCO/BRL). Linkers containing a NotI restriction site and a SalI site were added to size-selected cDNAs. These cDNAs were cloned into the pSPORT1 vector (GIBCO/BRL) by insertion at the NotI and SalI site of pSPORT1 vector arms and used to transform E. coli HB101 competent cells. Each of the constructed elapid cDNA libraries has approximately 5 × 10⁶ independent colonies.

In attempt to analyze the expression profile of the N. atra, a strategy of random sequencing of the cloned cDNAs was used. At first, the bacterium library was tittered and bacteria were plated out in LB plates containing 100 μg/ml ampicillin. Single colony was picked out and analyzed by PCR to confirm the presence of an insert sequence and its size, under the following conditions: 2 min at 94 °C, followed by 30 cycles of 10 s at 92 °C, 30 s at 50 °C, 90 s at 72 °C with a Peltier Thermal Cycler (model PTC-200, M. J. Research, USA). The last cycle was followed by an extension step at 72 °C for 10 min. A vector T₇ primer (5′-GTTACAGCCTACACTATAGGGCGA-3′, in the sense direction) and a vector SP₆ promoter primer (5′-CATAGGATT-TAGGTGACACTATAG-3′, in the anti-sense direction) located in 5′ and 3′ of the cloned insert, respectively, were used in PCR analysis. Totally more than 2000 independent clones with insert >300 bp were sequenced for the constructed N. atra venom gland cDNA library. Fortunately, a full cDNA sequence coding for cathelicidin-related protein precursor (NA-CATH) was identified by Blast search of the GenBank database. A pair of oligonucleotide primer was designed according to the obtained NA-CATH cDNA sequence, the two primers used were C5 (5′-ATGGAAGGGTTTCTCGG-3′, in the sense direction) and C3 (5′-AGCCCTCTCTTGAAGG-3′, in the anti-sense direction). C5 and C3 are located in the 5′-coding and 3′-stop codon regions of NA-CATH cDNA sequence, respectively. A PCR-based method for high stringency screening of cDNA libraries was used for screening and isolating the clones as previously described [12]. C5 and C3 primers were used in PCR reactions under the following conditions: 2 min at 94 °C, followed by 35 cycles of 10 s at 92 °C, 30 s at 50 °C and 40 s at 72 °C.
72 °C. The last cycle was followed by an extension step at 72 °C for 10 min. Finally, a full cDNA sequence coding for B. fasciatus cathelicidin-related protein precursor (BF-CATH) was successfully cloned from a constructed B. fasciatus venom gland cDNA library. However, this method failed to clone cathelicidin-related protein precursor (OH-CATH) from the constructed king cobra venom gland cDNA library. Then, a new strategy was carried out to screen OH-CATH from the constructed king cobra venom gland cDNA library. A new pair of oligonucleotide primer was designed according to the obtained N. atra and B. fasciatus cathelicidin-encoding cDNA sequences, the two primers used were CSU (5’-GCTCTGTAGCAGAAGAACC-3’, in the sense direction) and C3D (5’-CCTGGGATTGGAGAGCCGA-3’, in the anti-sense direction). CSU and C3D are located in the 5’- and 3’-noncoding regions of N. atra cathelicidin-encoding cDNA, respectively. Using the constructed king cobra venom gland cDNA library as template and high-fidelity DNA polymerase (Pyrobest), the PCR reaction was carried out by using CSU and C3D as primer under the following conditions: 2 min at 94 °C, followed by 35 cycles of 10 s at 92 °C, 30 s at 55 °C and 60 s at 72 °C. The last cycle was followed by an extension step at 72 °C for 10 min. The PCR products were separated by agarose gel electrophoresis, the target fragment was cut and recovered by DNA fragment extraction kit. The extracted PCR products were subcloned into pMD18-T vector after an A-tailing reaction. The ligation products were transformed into E. coli strain JM109 competent cells. A PCR-based method using C5 and C3 as primers for high stringency screening of positive clones was used for screening and isolating the clones as mentioned above. Finally, five positive clones coding for king cobra cathelicidin-related protein precursor were sequenced and all the cDNAs were the same.

All the oligonucleotide primers for PCR were prepared with a DNA synthesizer (Model 381A, Applied Biosystems). For obtaining cathelicidin-related full-length cDNAs, both strands of the clones were sequenced on an Applied Biosystems DNA sequencer, model ABI PRISM 377.

The nucleotide sequence data reported in this paper are available from GenBank database with accession number of EU622892, EU622893 and EU622894 for cathelicidins from N. atra, B. fasciatus and O. hannah, respectively.

2.4. Antimicrobial assays

Antimicrobial activity was assayed according to the standard procedures as described previously [13]. Briefly, a serial 1:1 dilution of the test peptides was added to the same number of log-phase bacteria in a 96-well microtiter plate. Minimal inhibitory concentration (MIC) was determined in liquid LB medium at pH 7.0 by incubating the bacteria in LB broth with variable amounts of the sample tested for 16–18 h. The MIC at which no growth occurred, as measured at absorbance of 600 nm, was recorded. Under our assay conditions, the sample concentrations were the same for each of the tested bacterial strains in three independent experiments and the MIC values were obtained without inter-experiment variations and expressed as μg/ml.

2.5. Haemolytic assays

Haemolytic assay was tested with human red blood cells in liquid medium as previously reported [13]. Serial dilutions of the peptides were used, and after incubation at 37 °C for 30 min, the cells were centrifuged and the absorbance in the supernatant was measured at 595 nm. Maximum haemolysis was determined by adding 1% Triton X-100 to the cell samples.

2.6. Sequence analysis and phylogenetic tree construction

Multi-sequence alignment was performed with CLUST W [19] and phylogenetic tree was constructed with MEGA 3.1 using the neighbor-joining method [11]. The reliability of each branch was tested by 2000 bootstrap replications. Neutrophilic granule proteins from mouse and rat have no detectable cleavage site and the full amino acid sequences are used in the phylogenetic analysis. The rest of all sequences used only contain signal peptide and cathelin domain.

3. Results and discussion

3.1. Molecular cloning of elapid cathelicidins

A full-length cDNA sequence coding for NA-CATH was identified by large random sequencing (~2000 independent clones) of a constructed N. atra venom gland cDNA library. A highly similar full-length cDNA clone (BF-CATH) was isolated and sequenced from the constructed B. fasciatus venom gland cDNA library by PCR method using the designed primers (C5 and C3). However, molecular cloning of OH-CATH from the constructed king cobra venom gland cDNA library failed under the same experimental conditions. Our results suggested that the clone(s) bearing the king cobra cathelicidin might grow slowly and disappear after serial dilution of the original library. Thus, we could only get the open reading frame of OH-CATH by direct PCR method. The method used the constructed venom gland cDNA library as template and high-fidelity DNA polymerase together with a new pair of primer located in the 5’- and 3’-noncoding regions of NA-CATH. Finally, the open reading frame of OH-CATH was successfully cloned and sequenced. Recently, an expressed sequence tags (ESTs)...
database from viperid snake *Lachesis muta* venom gland was reported [10]. A partial cDNA sequence coding for cathelicidin-related protein precursor from *L. muta* was deposited to GenBank EST database with accession number of DY403262. The nucleotide and deduced amino acid sequences coding for cathelicidin-related protein precursor from three elapid species and one viperid species are shown in Fig. 1. cDNA sequences of NA-CATH, BF-CATH and OH-CATH are composed of 777 bp, 764 bp and 645 bp, respectively. All of the three cloned elapid cathelicidin cDNA sequences code for a 191 amino acid residue preprotein. The deduced protein precursors all have a typical signal peptide sequence, a highly conserved cathelin domain and a catolic C-terminal mature peptide sequence.

### 3.2. Prediction of elapid cathelicidin mature peptides

The three cloned elapid cathelicidin cDNAs code for precursors of *N. atra*, *B. fasciatus* and *O. hannah* snakes containing 191 amino acid residues. A signal peptide composed of 22 amino acid residues was predicted for NA-CATH, BF-CATH and OH-CATH by SignalP 3.0 software package [3]. Elastase is generally regarded to be responsible for processing of cathelicidin precursors to generate mature antimicrobial peptides in most of the cathelicidins characterized so far, from fish, bird and mammals. Valine and alanine represent the most common elastase-sensitive residues in the elastase-processed cathelicidins [18]. Thus, three mature elapid cathelicidins were predicted to be cleaved at Val157-Lys158.
and generate 34 amino acid residue mature peptides. The deduced amino acid sequences of the mature NA-CATH, BF-CATH and OH-CATH are shown in Fig. 2. Unlike the highly divergent cathelicidins in mammals, the snake cathelicidins represented by the present work are conserved in three elapid species of N. atra, B. fasciatus and O. hannah. Interestingly, the deduced mature elapid cathelicidins all contained a repeated amino acid motif of KRA(K/E)FF(K/R)K at the N-terminal and KRAK(K/E)FFKK at the middle of the mature peptides, see Fig. 2.

3.3. Antimicrobial and haemolytic activities of OH-CATH

The deduced three elapid snake cathelicidins were very similar in their amino acid sequences. Thus, a deduced king cobra cathelicidin termed OH-CATH was chemically synthesized and used to evaluate its antimicrobial and hemolytic activities. The effects of the synthetic peptide against various tested bacteria in the presence of 1% NaCl are shown in Table 1. Synthetic OH-CATH exhibited broad-spectrum antimicrobial activity against all tested bacteria, especially clinical samples of multi-drug resistant bacterial strains. OH-CATH showed the highest antimicrobial activity against E. cloacae clinical isolates of multi-drug resistant strain with a MIC value of 1 μg/ml. The MIC values of OH-CATH against P. aeruginosa PA 01 and clinical isolates of multi-drug resistant strain of P. aeruginosa and E. aerogenes were all determined to be 2 μg/ml. Comparatively, OH-CATH showed a relatively lower antimicrobial activity against standard laboratory E. coli strains. OH-CATH showed the lowest antimicrobial activity against E. coli clinical isolates of multi-drug resistant bacterial strain with MIC of 20 μg/ml. The MIC values of OH-CATH against standard laboratory strains of E. coli ATCC 25922 and ML-35P were 8 and 6 μg/ml, respectively. It has been observed that NaCl has a strong effect on the antimicrobial activity caused by cathelicidins. LL-37 lost its antibacterial activity in the presence of 100 mM NaCl [28]. The MIC value of OH-CATH against E. coli ATCC 25922 without NaCl was determined to be 2 μg/ml (data not added in Table 1), which is four times lower than that in the presence of NaCl for the same bacterial strain. OH-CATH showed no haemolytic activity on human red blood cells at concentrations up to 200 μg/ml (results not shown). It was reported that human LL-37 has antibacterial activities against E. coli ML-35 and P. aeruginosa ATCC 27853 in the absence of NaCl with MIC values of 2 μM (=9 μg/ml) and 4 μM (=18 μg/ml), respectively. The same research group also confirmed that human LL-37 has dose-dependent haemolytic activity on human red blood cells, the haemolytic activity are 45% and 85% under LL-37 concentrations of 10 μM (=45 μg/ml) and 100 μM (=450 μg/ml), respectively [29]. Comparatively, the antimicrobial activities against E. coli ML-35 and P. aeruginosa ATCC 27853 by OH-CATH are stronger than those by human LL-37 even though the data obtained were not obtained under the same experiment conditions. Taken together, the strong antimicrobial activity, salt insensitivity and low haemolytic activity indicate that elapid cathelicidins, represented by OH-CATH, could be a good model for the development of novel therapeutic drugs.

Table 1 – Antimicrobial activity of OH-CATH in the presence of 1% NaCl

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>MIC (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OH-CATH</td>
</tr>
<tr>
<td>Escherichia coli ATCC 25922</td>
<td>8</td>
</tr>
<tr>
<td>E. coli ML-35P</td>
<td>6</td>
</tr>
<tr>
<td>E. coli clinic strain</td>
<td>20</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa ATCC 27853</td>
<td>4</td>
</tr>
<tr>
<td>P. aeruginosa PA 01</td>
<td>2</td>
</tr>
<tr>
<td>P. aeruginosa clinic strain</td>
<td>2</td>
</tr>
<tr>
<td>Enterobacter aerogenes clinic strain</td>
<td>2</td>
</tr>
<tr>
<td>E. cloacae clinic strain</td>
<td>1</td>
</tr>
</tbody>
</table>

The data are obtained from three independent experiments.
cathelicidins and rodent NGPs showed a close relationship with the subgroup of fowlicidins-1, -2, -3 and chicken myeloid antimicrobial peptide 27. The phylogenetic tree constructed in this paper provided new information on the evolutionary relationship among snake cathelicidins, rodent NGPs, avian fowlicidins and chicken myeloid antimicrobial peptide 27. The third and the most divergent group is represented by mammalian cathelicidins. Our results suggest that snake cathelicidins are evolutionary very close to mammalian NGPs, avian fowlicidins and chicken myeloid antimicrobial peptide 27. Our results also support that hagfish cathelicidins might be considered as ancient members of the cathelicidin family as hagfish cathelicidin seems independently evolved in the constructed phylogenetic tree.

In summary, cathelicidins existed in reptile were first confirmed in the present work. Three cathelicidins from elapid species of N. atra, B. fasciatus and O. hannah were identified by molecular cloning procedures. The nucleotide and deduced amino acid sequences of elapid cathelicidins were comparatively conserved among different snake species. Predicted mature cathelicidin from king cobra was chemically synthesized and it showed strong antimicrobial activity against various tested bacteria (MIC = 1–20 μg/ml) in the presence of 1% NaCl but no haemolytic activity was observed even at high concentrations (200 μg/ml). Phylogenetic analysis suggests that snake cathelicidins are closely related to rodent NGPs, avian fowlicidins and chicken myeloid antimicrobial peptide 27. Elapid cathelicidins might serve as model molecules for the development of antimicrobial drugs.

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Fig. 3 – Phylogenetic analysis of vertebrate cathelicidins. The tree was constructed by the neighbor-joining method based on the proportion difference of aligned amino acid sites of the signal peptide and cathelin domain of the preprotein sequences. A total of 2000 replicates were used to test the reliability of each branch. The numbers on the branches indicate the percentage of 2000 bootstrap samples supporting the branch. Bootstrap values are set to be ≥50%. The bar indicates the branch length. GenBank database accession number of the sequences and their common names are indicated in the tree.


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