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Cathelicidin-HR from *Hoplobatrachus rugulosus*: an antioxidant peptide that performs a protective effect against UV/H_2O_2-induced DNA damage

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ABSTRACT

Cathelicidins, a group of vertebrate multifunctional molecules, play a role in innate immunity. In this study, a cathelicidin was identified from the lungs of frogs, *Hoplobatrachus rugulosus*. A 474 base pairs complementary DNA sequence encoded a 157 amino acid residue prepropeptide of *H. rugulosus* cathelicidin (cathelicidin-HR), which consisting of a 20-residue signal peptide sequence, a 108-residue cathelin region, and a 29-residue cathelicidin-HR peptide. Amino acid sequence alignment and cladogram analysis illustrated that cathelicidin-HR have a high degree of similarity to other amphibian cathelicidins. The cathelicidin-HR peptide displays very low antimicrobial activity but exhibits dose-dependent antioxidant activity. Moreover, this peptide expresses DNA damage inhibition against UV/H_2O_2-induction. The molecular docking indicated that DNA damage protection of cathelicidin-HR might occur via DNA-peptide complex formation. This is the first amphibian cathelicidin peptide that possesses DNA damage inhibitory activity which might play a crucial role in oxidative stress.

1. INTRODUCTION

During cell metabolism, the reactive oxygen species (ROS) could be generated such as hydroxyl radical, superoxide anion radical, and hydrogen peroxide [1]. An imbalance between ROS level and the antioxidant capacity initiated oxidative stress [2], leads to many degenerative diseases such as diabetes, stroke, and cancer [3]. Bioactive compounds such as antioxidant peptides, found naturally in them, play a crucial role in preventing those diseases [4].

In amphibians, they can survive in a broad range of environmental systems due to the containing pharmacological substances to combat environmental factors such as microbes and ultraviolet (UV) radiation [5]. They have abundant peptides that play a role in defense mechanisms [6]. Amphibian bioactive peptides have been proven a variety of biological functions, including antimicrobial, antioxidant, and immunomodulatory activities [7]. Many studies have demonstrated that amphibian peptides have the potential for drug development [8,9]. Cathelicidin is a class of multi-functional peptides that has been determined and characterized from various amphibian tissues such as skin [10-18], lung [19,20], spleen [21], and ear-side gland [22]. Most of them play a vital role in antimicrobial activity such as cathelicidin-AL from *Amolops loloensis* [10], Lf-CATH1 and Lf-CATH2 from *Limnonectes fragilis* [21], cathelicidin-RC1 and cathelicidin-RC2 from *Rana catesbeiana* [19], cathelicidin-BG from *Bufo gargarizans* [22], AdCath from *Andrias davidianus* [14] and OL-CATH2 from *Odorrana livida* [23]. Moreover, some amphibian cathelicidin peptides perform wound healing activity such as cathelicidin-TV from *Tylototriton verrucosus* [12], cathelicidin-OA1 from *Odorrana andersonii* [15], cathelicidin-NV from *Nanorana ventripunctata* [16] and cathelicidin-DM from *Duttaphrynus melanostictus* [17]. Besides, cathelicidin-PY from *Paa yunnanensis* [11], FM-CATH1 and FM-CATH2 from *Fejervarya multistriata* [20], cathelicidin-PP from *Polypedates puerensis* [13], and PN-CATH1 and PN-CATH2 from *Pelophylax nigromaculata* [18] exhibit anti-inflammatory activity. Moreover, four cathelicidin peptides with antioxidant activity have been reported such as cathelicidin-OA1 [15], cathelicidin-NV [16], and PN-CATHs [18].

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The rice field frog, *Hoplobatrachus rugulosus* is an anuran amphibian in the Dicroglossidae family. This frog is widely spread throughout the Indochina countries and also in China (Amphibian Species of the World 6.1: https://amphibiansoftheworld.amnh.org/index). Recently, HR-CATH, cathelicidin peptide was identified from *H. rugulosus* skin play role in antimicrobial activity and immunomodulatory activity [24]. However, in general, multiple cathelicidins have been found in many species [25]. Therefore, it is possible to discover a new sequence of cathelicidin with distinct functions from different tissues. In this study, the cathelicidin gene was identified from the lung of *H. rugulosus* and the mature cathelicidin peptide function was as well characterized. Importantly, *H. rugulosus* cathelicidin (cathelicidin-HR) is the first amphibian cathelicidin to exhibit DNA damage protection activity.

2. MATERIALS AND METHODS

2.1. Sample Collection and Tissue Preparation

A captive adult *H. rugulosus* was purchased from the local market in Kalasin Province, Thailand. A frog was euthanized and the lung was immediately removed and stored in liquid nitrogen until extraction. The experimental procedure was approved by the Institutional Animal Care and Use Committee of Khon Kaen University (Record number IACUC-KKU-10162).

2.2. Rapid Amplification of Complementary DNA (cDNA) Ends (RACE) Reaction

Total RNA was extracted from frog lungs using GF-1 Total RNA extraction Kit (Vivantis, USA). RNA concentration was measured by spectrophotometer. The first-strand cDNA was produced by M-MuLV reverse transcriptase reaction (Vivantis, USA) with oligonucleotide d(T). The cDNA template was magnified with the forward primer rcCATH-F (5'-ATGAAGATCTGGCAGTGTGTG-3') and the reverse primer rcCATH-R (5'-GGTCAGGCTGACGCACTTC-3') with a design based on the conserved signal peptide sequence and C-terminal cathelin domain of *Rana catesbiena* cathelicidin (cathelicidin-RC) genes, respectively [19]. Furthermore, the 3'-RACE was synthesized using a 5'-specific forward primer (hrCATH34-F: 5'-GCAATCACATTGCAGTCAGC-3') and oligonucleotide d(T) primer. The polymerase chain reaction (PCR) was conducted by T100 Thermal Cycler (Bio-Rad, USA). The PCR product purification was carried out by gel electrophoresis with a GF-1 AmbiClean kit (Vivantis, USA). The purified PCR products were sequenced by Sanger sequencing (Macrogen, South Korea).

2.3. Bioinformatic Analysis

The amphibian cathelicidin amino acid sequences were obtained from NCBI database. The amphibian cathelicidin sequences were aligned using ClustalOmega (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Besides, phylogenetic analysis was determined using the neighbor-joining method (Mega version X; [26]. The prediction of the antimicrobial region was calculated through Antimicrobial Sequence Scanning System (AMPA) server (http://tcoffee.crg.cat/apps/ampa/) [27]. The peptide sequences were computed for the physicochemical properties analysis via APD2 database (http://aps.unmc.edu/AP/main.php) [28]. The secondary structure was performed by PEP-FOLD3 server [29]. The molecular docking structures were simulated and refined by PatchDock [30] and FireDock server [31], respectively. The PDB file from PEP-FOLD3 of cathelicidin-HR peptide secondary structure was used as a ligand molecule. The receptor molecules were conducted by using the structure of a double stand B-DNA containing dipyrimidine sites (5'-GCACGAATTAAG-3') (PDB: 1COC) [32]. The structure graphics were hence produced in *PyMol* (Schrödinger LLC).

2.4. Peptide Synthesis

The cathelicidin-HR peptide was produced by GenScript (NJ, USA). The amino acid sequencing was validated by electrospray ionization mass spectrometry while peptide purity was checked by reverse-phase high-performance liquid chromatography.

2.5. Antimicrobial Activity

The antimicrobial activity assay of peptides was determined using the broth assay. Briefly, bacterial cells were cultured in nutrient broth until the mid-log phase at 37°C. Then cultured cells were diluted to 10^4 CFU/ml. The 50 µl of diluted cells were aliquoted into microcentrifuge tubes then mixed with 50 µl of 4 mg/ml cathelicidin-HR peptide, followed by the incubation at 37°C for 16–18 h. the bacterial growth was observed by spectrophotometer. A decrease in optical density at 600 nm indicated the antimicrobial activity of the peptide. The melittin and double-distilled water (DW) were used as positive and negative controls, respectively. The percentage of bacterial growth inhibition was calculated the following formula: ([OD600 nm, control−OD600 nm, peptide]/OD600 nm, control) × 100.

2.6. Antioxidant Activity Assay

A 2, 2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) scavenging activity was determined according to previously described [15]. Briefly, the ABTS radical solution was prepared by mixing 2.8 mM potassium persulfate with 7 mM ABTS in water, followed by incubating for 6 h in the dark. The ABTS solution was diluted 50-fold with DW. Samples dissolved in water were added to the diluted stock solution, and the same volume of solvent was used as the negative control. The ascorbic acid was used as a positive control. The reactions were kept from light for 30 min. A decrease in absorbance at 415 nm indicated the antioxidant activity of the samples. The rate of free radical scavenging (%) was calculated by ([A415 nm, blank−A415 nm, sample]/A415 nm, blank) × 100.

A 2, 2-diphenyl-1-picrythydrazyl (DPPH) scavenging activity was assayed as earlier research [15]. The reaction contained 190 µl of 50 µM DPPH radical dissolved in ethanol and 10 µl of 2-fold dilution peptide. The mixture was incubated at room temperature for 30 min in the dark. Then, the absorbance was measured against a blank at 517 nm. The ascorbic acid was used as a positive control. The percentage of DPPH free radical scavenging activity was calculated the following formula: ([A517 nm, blank−A517 nm, sample]/A517 nm, blank) × 100.

2.7. DNA Damage Protection Assay

DNA damage inhibition activity was performed as a modification of previously described [33]. The DNA damage was induced using OH radicals produced from the UV/H_2O_2-radical system and evaluated on gel electrophoresis. The experiments were performed in a volume of 10 µl in a PCR tube containing 1 µl of 100 base pairs (bp) DNA ladder, 5 µl of different concentrations of peptide (50, 100, 200, and 400 µg/ml), and 4 µl of 30% H_2O_2. The reaction tubes were UV irradiated at a wavelength of 312 nm using a UV transilluminator (Major Science, Taiwan) for 5 min at room temperature. The control contained only untreated DNA as internal control while the negative control contained DNA and H_2O_2 without peptide treatment. The bovine serum albumin treated with UV/H_2O_2 was used as a positive control. All samples were visualized using 1% agarose gel electrophoresis.

2.8. Hemolytic Activity Assay

The hemolytic activity of the cathelicidin-HR peptide was investigated against the defibrinated sheep red blood cells (shRBCs). Briefly, the shRBCs were washed with phosphate-buffer saline (PBS), pH 7.4, and then diluted to 0.5% (v/v) in PBS. The 100 µl of shRBCs solution was divided into microcentrifuge tubes. The 10 µl of 2-fold serial dilution of cathelicidin-HR peptide was added, followed by incubation at 37°C for 1 h. The reactions were centrifuged at 1,000 g for 5 min. The 100 µl supernatants have measured absorbance at 415 nm with a spectrophotometer. The 1% (v/v) Triton X-100 and DW were used as positive and negative controls, respectively. The percentage of hemolysis was computed as ([A415 nm, peptide]/[A415 nm, 1% [v/v] Triton X-100]) × 100.

3. RESULTS AND DISCUSSION

3.1. Identification of Cathelicidin-HR

To identify the cathelicidin gene in *H. rugulosus*, the frog lung was collected then analyzed by RT-PCR with the conserved cathelicidin-RC gene primer. The 300 bp PCR product was obtained from the reaction with 80% sequence similarity to the cathelicidin-PY1 precursor (AFX61592) from *Nyssa yunnanensis* [Supplement Data Figure 1a]. This nucleotide sequence was further used as a template to obtain the complete 3´ ends of the cathelicidin-HR gene by RACE-PCR. The 800 bp 3' RACE-PCR product was amplified using specific cathelicidin-HR primers combined with oligonucleotide d(T) primer [Supplement Data Figure 1b]. The complete prepropeptide cathelicidin-HR cDNA sequence was presented in Figure 1. The 474 bp prepro-cathelicidin-HR nucleotide encoded 157 amino acid residues. The cathelicidin-HR was identified from the lung as the previous reports of cathelicidin-RC1-2 from *R. catesbeiana*, FM-CATH1-2 from *F. multistriata,* and OL-CATH1-2 from *O. livida* [19,20,23].

3.2. Analysis of Cathelicidin-HR Amino Acid Sequence

The amino acid sequence analysis with ExPASy showed that the molecular weight of prepro-cathelicidin-HR was 17.97 kDa with the 6.59 pI. Moreover, SignalP 4.0 sequence analysis indicated that the first 20 amino acid residues domain on the N-terminus site was the signal peptide region. The conserved cathelin region comprised 108 residues whereas the C-terminal end indicated the putative mature cathelicidin-HR peptide comprised 29 amino acid residues with a molecular weight of 3.18 kDa and 10.86 of pI [Supplement Data Table 1]. The amino acid sequence alignment illustrated that prepro-cathelicidin-HR showed 66% similarity with Lf-CATH1 (*L. fragilis*) and performed 64% and 60% with the cathelicidin-NV precursor (*N. ventripunctata*) and OL-CATH1 from *O. livida*, respectively [Figure 2a]. Among amphibian species, the N-terminus cathelin region performed a highly conserved sequence. Whereas, the C-terminus mature cathelicidin peptide presented a peptide sequences variation. The sequence alignment elucidated that among the group of amphibian cathelicidin has a highly similar degree of cathelin sequence region which not only four conserved cysteine residues but also two conserved cysteine residues have been found at mature peptide site [Figure 2a]. However, except for cathelicidin-AL and cathelicidin-OA1 which exhibit sequence similarities to reptilian cathelicidin [10,15]. The phylogenetic tree analysis of amphibian cathelicidins was divided into three clusters [Figure 2b]. Cluster I was the largest group of amphibian cathelicidin which can be split into two sub-groups (cluster I-a and cluster I-b). Cluster I-a comprised cathelicidin-HR, Lf-CATH1, cathelicidin-NV, and OL-CATH1. In addition, cluster I-b included cathelicidin-RC1-2, OL-CATH 2, PN-CATH1-2, cathelicidin-PP, cathelicidin-PY1, Lf-CATH2, and HR-CATH. Besides, cluster II had two cathelicidins from toads, cathelicidin-BG, and cathelicidin-DM. Cluster III presented the diversity of amphibian cathelicidin, consisting of cathelicidin-OA1 and cathelicidin-AL. The phylogenetic analysis illustrated that the cathelicidin-HR relate to other amphibian cathelicidins which are mostly located in cluster I [Figure 2b]. On the other hand, cathelicidin-AL and cathelicidin-OA1 presented that both sequences are distinct to amphibian sequence clusters that might be the connecting link between the amphibian and reptilia cathelicidin [10,15] [Figure 2b]. However, the sequence similarity result indicated that the cathelicidin-HR sequence showed the closest sequence related to Lf-CATH1 which was isolated from the spleen of *L. fragilis* [Figure 2b]. It was not

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atgaagatctggcagtgtgtgttctggattttcgcaatcacattgcagtcagctcactct      60
M  K  I  W  Q  C  V  F  W  I  F  A  I  T  L  Q  S  A  H  S       20
gagtctccagatcaggatgagtggatcagagaggccttagatctctacaaccagaaggat      120
E  S  P  D  Q  D  E  W  I  R  E  A  L  D  L  Y  N  Q  K  D       40
aatggaaagtgcttctataagcaactgtccgatctcccggctggcatcctggaggaagag      180
N  G  K  C  F  Y  K  Q  L  S  D  L  P  A  G  I  L  E  E  E       60
gaagactctcccacagtcaggttctttataaaggagacagaatgcctcaagtctgaagat      240
E  D  S  P  T  V  R  F  F  I  K  E  T  E  C  L  K  S  E  D       80
atagacttggcccagtgtgactacaagaaggatgggcaggtgaaggcctgtgcattgtac      300
I  D  L  A  Q  C  D  Y  K  K  D  G  Q  V  K  A  C  A  L  Y      100
ccggaggaggggagacctcaaagactctgaaatgcgtcagcttgaccaagacctcccgt      360
P  E  E  G  E  T  S  K  T  L  K  C  V  S  L  T  K  T  S  R      120
gttaaacgatcaaatagatcacaaccctgcaggggtatcttttgtagaactggatccaga      420
V  K  R  S  N  R  S  Q  P  C  R  G  I  F  C  R  T  G  S  R      140
agtccgattgccaagccaagcaaagacaatttagttcggatgtcactgtcttaa           474
S  P  I  A  K  P  S  K  D  N  L  V  R  M  S  L  S  *            157
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Figure 1: The cDNA sequence and the predicted prepropeptide sequence of cathelicidin-HR. The predicted mature peptide is displayed in grey box. The predicted signal peptides are underlined. An asterisk (*) indicates the stop codon.

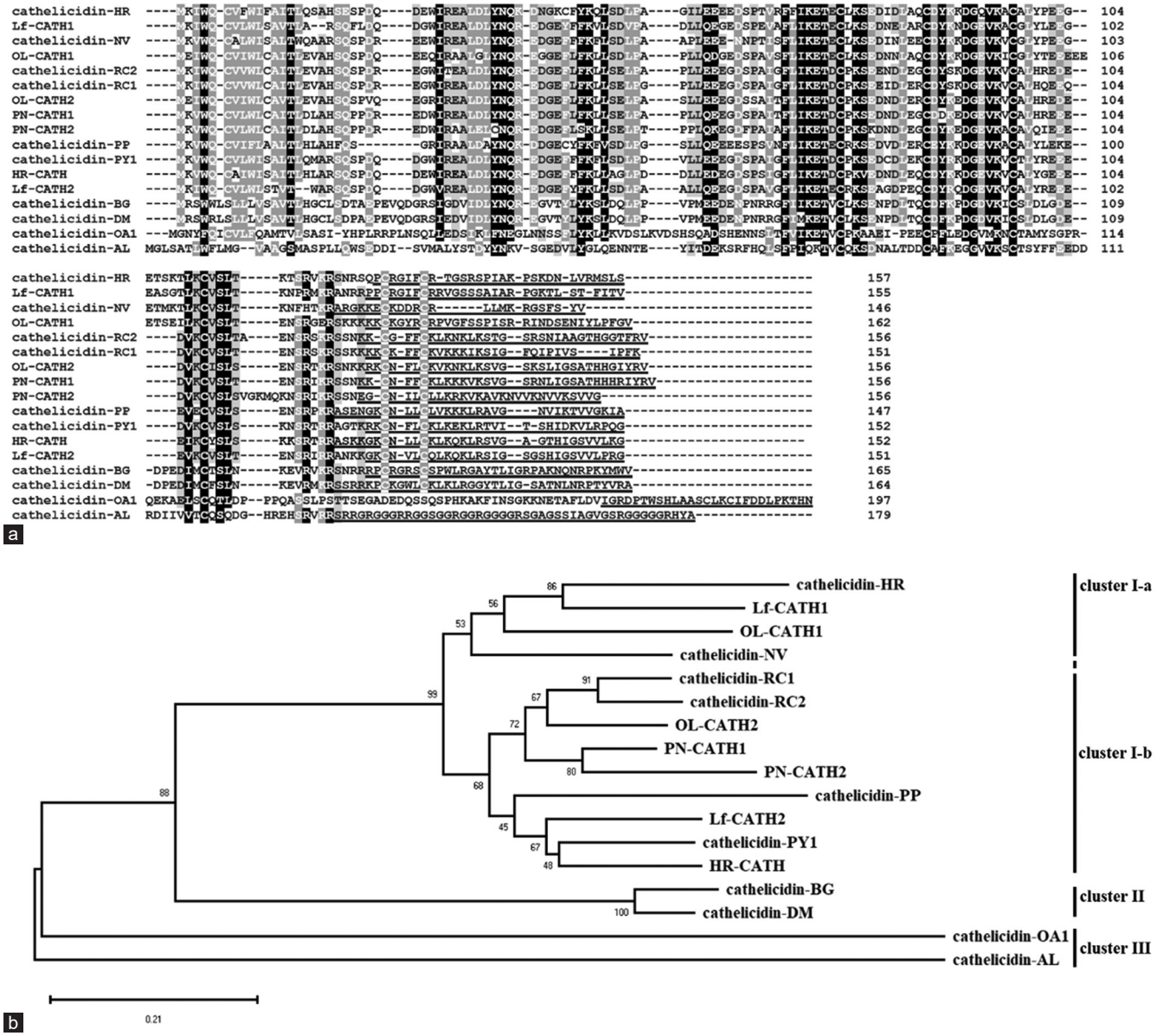

Figure 2: The cathelicidin-HR sequence analysis. The amino acid sequence alignment (a) and phylogenetic analysis (b) of cathelicidin-HR complete peptide compared with other amphibian cathelicidins. The highly conserved amino acid are shaded. The mature peptides are underlined. *Amolops loloensis* cathelicidin (cathelicidin-AL<AEI69698>), *Bufo gargarizans* cathelicidin (cathelicidin-BG<ANV28414>), *Duttaphrynus melanostictus* cathelicidin (cathelicidin-DM<AJQ20790>), *Hoplobatrachus rugulosus* cathelicidins (cathelicidin-HR<MW725232> and HR-CATH<QQG31491>), *Limnonectes fragilis* cathelicidins (Lf-CATH1 and Lf-CATH2<without accession No.>), *Nanorana ventripunctata* cathelicidin (cathelicidin-NV<AZW10343>), *Odorrana andersonii* cathelicidin (cathelicidin-OA1<AWO14611>), *Odorrana livida* cathelicidins (OL-CATH1<AXR75913> and OL-CATH2<AXR75914>), *Pelophylax nigromaculata* cathelicidins (PN-CATH1<QPL18198> and PN-CATH2<QPL18199>), *Polypedates puerensis* cathelicidin (cathelicidin-PP<ASU44943>), *Paa yunnanensis* cathelicidin (cathelicidin-PY1<AFX61592>), and *Rana catesbeiana* cathelicidins (cathelicidin-RC1< AHW58220> and cathelicidin-RC2<AHW58221>).

surprising that cathelicidin-HR and HR-CATH showed distinct clade in phylogram analysis since cathelicidins were the protein family which generally found more than one sequence in the same species. In addition, *H. rugulosus* was widely spread in southern China and Thailand might be a cryptic species complex [34].

3.3. Analysis of Candidate Cathelicidin-HR Peptide

In general, cathelicidin was essential enzymatically cleaved for proteolytic maturation. Enzymatic processing in most of the cathelicidins was mediated by elastase. This enzyme was typically sensitive to valine or alanine residue. However, the proteolytic processing for amphibian cathelicidins is remaining unclear. This research was studied based on antimicrobial peptide domain prediction and amino acid sequence alignment results. The AMPA server analysis indicated that the region from Pro129 to Arg140 represented the bactericidal stretch with 13% probability (data not shown) which is located on the mature cathelicidin peptide domain. Therefore, the candidate putative mature peptide denoted as cathelicidin-HR peptide; NH2-PCRGIFCRTGSRSPIAKPSKDNLVRMSLS-COOH derived from the C-terminus site. The cathelicidin-HR peptide comprised 29

amino acid residues and performed the cationic peptide property +5 net charge. However, cathelicidin-HR peptide expressed a positive total net charge which presented a 34% of hydrophobic ratio with lower than at the level of a major group of AMPs. The predicted cathelicidin-HR peptide secondary structure was showed in Supplement Data Figure 2. The secondary structure modeling illustrated that the cathelicidin-HR peptide exhibited the α-helix model.

3.4. Antimicrobial Activity of the Cathelicidin-HR Peptide

Most amphibian cathelicidins exert direct antimicrobial activity against a broad range of bacteria, fungi, and drug-resistant pathogens [10,11,19,21,23]. To evaluate the antimicrobial property of the cathelicidin-HR peptide, the cathelicidin-HR peptide was chemically synthesized had a 3177.80 observed molecular weight and 98.18% purity [Supplement Data Figure 3]. The antimicrobial activity results performed that a high concentration of cathelicidin-HR peptide could inhibit the growth of only *Bacillus subtilis* TISTR124 and *Enterococcus faecalis* TISTR927 [Supplement Data Table 2]. However, the minimum inhibition concentration of this peptide was performed at 1 mg/ml for both bacterial strains (data not shown). These results indicated that cathelicidin-HR peptide exhibited low antimicrobial activity. Although the cationic α-helix character of the cathelicidin-HR peptide was a common AMPs character, the hydrophobic ratio percentage was at 34% which is lower than the range of a main group of AMPs which commonly hydrophobic ratio percentage between range 40% and 50% (APD). The optimum hydrophobicity of peptides may lead to the efficient action of the peptide on membranes especially penetration and disruption [35]. In cluster I, only Lf-CATH1 (40% hydrophobic ratio) exhibits antimicrobial activity but OL-CATH1 and cathelicidin-NV with hydrophobic ratio percentages between range 30% and 33% were lacking antimicrobial activity although all peptides presented +5 net charge [Supplement Data Table 1].

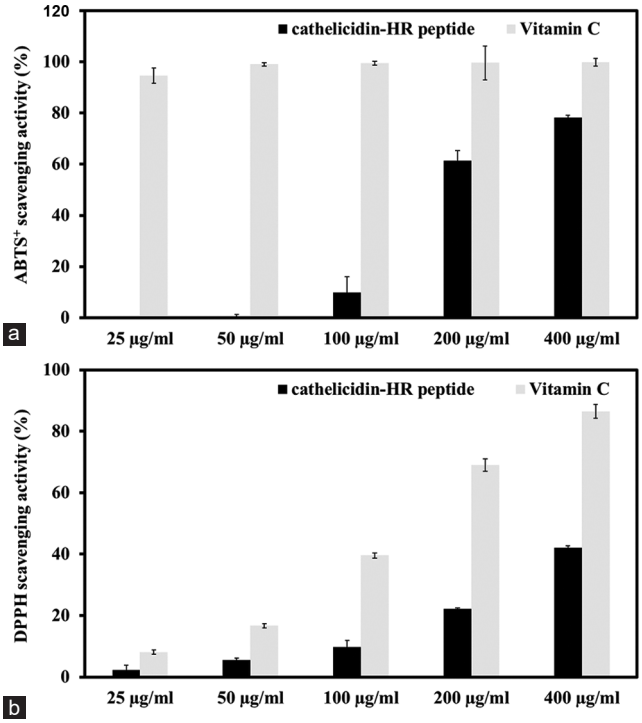

Figure 3: The antioxidant activity of cathelicidin-HR peptide. The $ABTS^+$ (a) and DPPH (b) scavenging activity of cathelicidin-HR peptide.

3.5. Antioxidant Activity of Cathelicidin-HR Peptide

The antioxidant activity of the cathelicidin-HR peptide was investigated by the ABTS and DPPH scavenging activity. The results clearly showed that the cathelicidin-HR peptide performs antioxidant capacity both ABTS [Figure 3a] and DPPH [Figure 3b] scavenging activity. Wherewith, the cathelicidin-HR peptide exerts the free radical scavenging activity of both ABTS and DPPH with the dose-dependent characteristic. These results illustrated that the cathelicidin-HR peptide presented antioxidant properties. The two cysteine residues of cathelicidin-HR peptide were estimated that might play a role in the free radicals scavenging activity. However, the antioxidant activity of cathelicidin peptide was disrupted by the formation of an intramolecular disulfide bridge [15]. Thus, it is assumed that two cysteine residues probably were free cysteine and performed the scavenging activity. Furthermore, the predicted cathelicidin-HR peptide secondary structure also established that no disulfide bond formation [Supplement Data Figure 2]. According to the results it can be concluded that the cathelicidin-HR peptide expressed the bi-functional peptide with antimicrobial and antioxidant activities. Recently, PN-CATHs from *P. nigromaculata* also showed both antimicrobial and antioxidant activities [18].

3.6. DNA Damage Inhibition Activity of Cathelicidin-HR Peptide

The DNA damage protection activity of the cathelicidin-HR peptide was investigated by the UV/H_2O_2 induced DNA damage system [Figure 4]. The results presented that the cathelicidin-HR peptide exhibit DNA damage protection especially at a concentration of 100 µg/ml. Whereas, at high concentrations (400 and 200 µg/ml), the inhibition activity was declined as the appearance of fainter DNA bands. This result might assume that this peptide act as pro-oxidants at high concentration for this activity. Not only cathelicidin-HR performed DNA damage protection but peptide from hoki frame protein hydrolysate (APHPH) also illustrated the ability of DNA damage inhibition at various concentrations [36]. Recently, sour meat peptides also exhibited the efficiency to protect DNA from hydroxyl radicals [37].

To understand the action of cathelicidin-HR peptide against DNA damage inhibition. The model of cathelicidin-HR-DNA interaction was investigated. The peptide-DNA docking indicated that the loop region of cathelicidin-HR peptide employed the peptide-DNA interaction [Figure 5a]. The hydrophobic interaction was the major

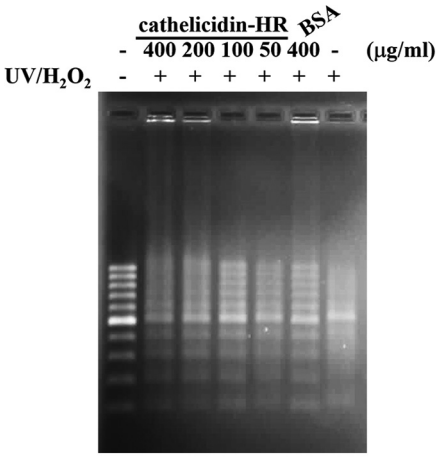

Figure 4: The DNA damage protection activity of cathelicidin-HR peptide.

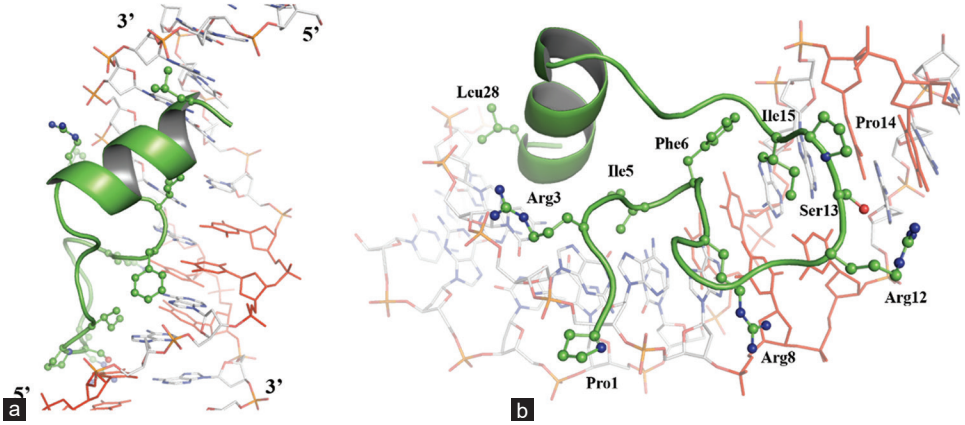

Figure 5: The molecular modeling of cathelicidin-HR peptide-DNA complex structure. The overall structure of cathelicidin-HR-DNA complex (a). Cathelicidin-HR is represented in green cartoon and DNA molecule is showed in stick. Close-up view of cathelicidin-HR loop domain interacting with DNA (b). Amino acid residues are represented in green balls and sticks. Thymine bases are showed in red sticks

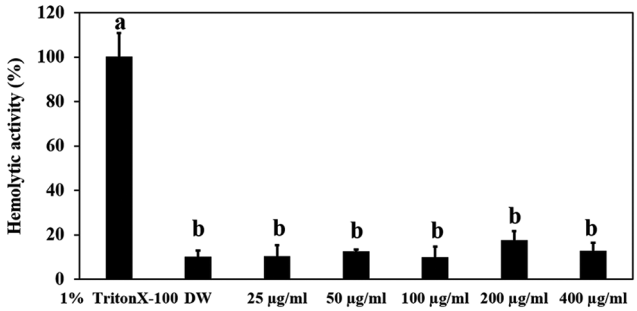

Figure 6: The hemolytic activity of cathelicidin-HR peptide against shRBCs. The same letters above the error bars indicate no statistically significant differences at $P<0.05$ (Duncan's Multiple Range Test).

force between cathelicidin-HR peptide and DNA which Ile5, Phe6, Pro14, Ile15, and Leu28 play a role in this interaction [Figure 5b]. Besides, the electrostatic interaction was found between three residues (Pro1, Arg3, and Arg8) on the loop domain of peptide and phosphate groups of DNA molecule whereas Arg12 presented this interaction with nucleotide base [Figure 5b]. A recent report also revealed that Lys and Arg play an important role in camel Lactoferrin chimera-DNA complex with electrostatic interactions and particularly salt bridges [38]. In addition, hydrogen bonding was found between Ser13 residue and pyrimidine ring of thymine base [Figure 5b]. These results indicated that the cathelicidin-HR peptide might protect DNA damage through the peptide-DNA interacting complex. Although, the DNA binding ability of cationic antimicrobial peptides was not always present. The cationic human cathelicidin (LL-37) exhibited the DNA binding activity whereas cationic Feline cathelicidin and magainin could not perform this activity [39,40]. However, here is the first report of amphibian cathelicidin showed DNA damage protection which might process via the DNA-peptide complex formation.

3.7. Hemolytic effect of Cathelicidin-HR Peptide against Red Blood Cells

The hemolytic activity of cathelicidin-HR peptide on sheep erythrocyte presented that cathelicidin-HR peptide expressed insignificant hemolysis activity between range 10% and 18% even at a concentration of 400 µg/ml [Figure 6]. Whereas, the negative control (DW) also showed hemolysis against red blood cells about 10%. Besides, the positive control (1% Triton-X 100) destroyed red blood cells about 100%. These results concluded that the cathelicidin-HR peptide presented a low toxicity activity. As mentioned above, cathelicidin-HR exhibits very low hydrophobicity that could affect the low hemolytic activity because hydrophobicity level was correlated with hemolysis pattern [41].

4. CONCLUSIONS

In summary, a cathelicidin peptide was identified from *H. rugulosus* that offers the diverse role of cathelicidin in an amphibian. The amino acid sequence analysis revealed cathelicidin-HR shares the common conserved sequence among the amphibian cathelicidins. The cathelicidin-HR peptide was predicted as a mature peptide released from cathelicidin-HR that exhibits low antimicrobial activity and performs the antioxidant activity in a dose-dependent manner. This peptide presents the protection of UV/H_2O_2-induced DNA. The molecular modeling illustrated that DNA-peptide interaction might occur during the DNA damage induction. The results provide a new role of amphibian cathelicidin peptide in oxidative stress through DNA damage protecting activity.

5. ACKNOWLEDGMENTS

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6. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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8. CONFLICT OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS

This experiment was approved by the Institutional Animal Care and Use Committee of Khon Kaen University (Record number IACUC-KKU-10162). This article does not contain any studies involving human participants performed by any of the authors.

10. DATA AVAILABILITY

The authors confirm that the data supporting the findings of this study are available within the article and its Supplement Data.

11. PUBLISHER'S NOTE

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SUPPLEMENT DATA

Supplement Data Table 1: Physicochemical properties of cluster I-a peptides.

Peptide	Sequence	Length	Net charge	Theoretical pI	Mw	Hydrophobic ratio (%)
cathelicidin-HR	PCRGIFCRTGSRSPIAKPSKDNLVRMSLS	29	+5	10.86	3177.77	34
Lf-CATH1 [1]	PPCRGIFCRRVGSSSAIARPGKTLSTFITV	30	+5	11.54	3178.76	40
OL-CATH1 [2]	KKCKGYRCRPVGFSSPISRRINDSENIYLPFGV	33	+5	9.89	3788.41	30
cathelicidin-NV [3]	ARGKKECKDDRCRLLMKRGSFSYV	24	+5	9.90	2847.38	33

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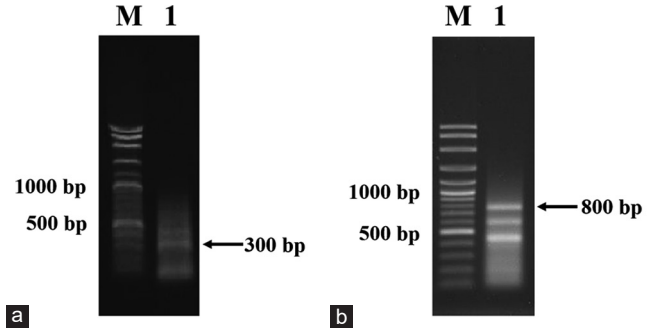
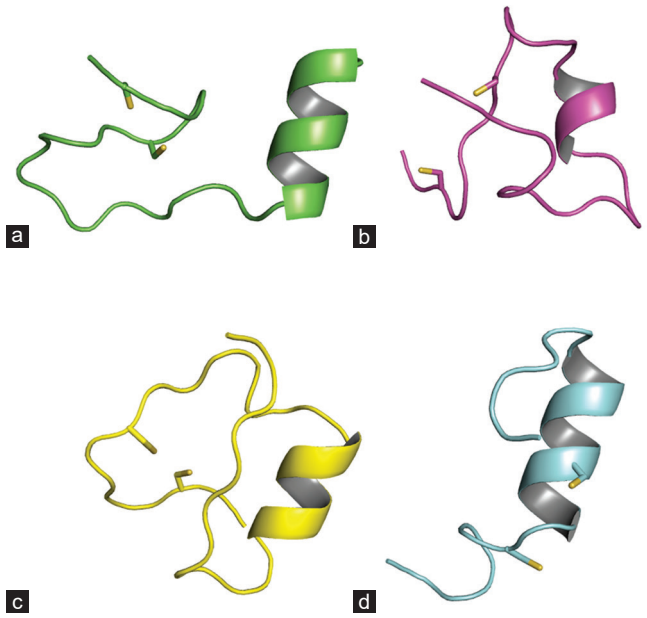
Supplement Data Table 2: Antimicrobial activity of cathelicidin-HR peptide.

Microorganism	cathelicidin-HR peptide	Melittin
Gram-positive bacteria		
Bacillus megaterium TISTR067	−	−
Bacillus subtilis TISTR1248	+	+
Bacillus cereus TISTR1449	−	−
Staphylococcus aureus ATCC25923	−	+
Enterococcus faecalis TISTR927	+	−
Gram-negative bacteria		
Escherichia coli ATCC25922	−	+
Pseudomonas aeruginosa TISTR1287	−	+
Salmonella thyphimurium TISTR1472	−	−
Fungi		
Candida albicans TISTR5554	−	−
Aquatic pathogenic bacteria		
Aeromonas hydrophila TISTR1321	−	+
Salmonella derby DMST16881	−	+
Edwardsiella tarda DMST38217	−	−

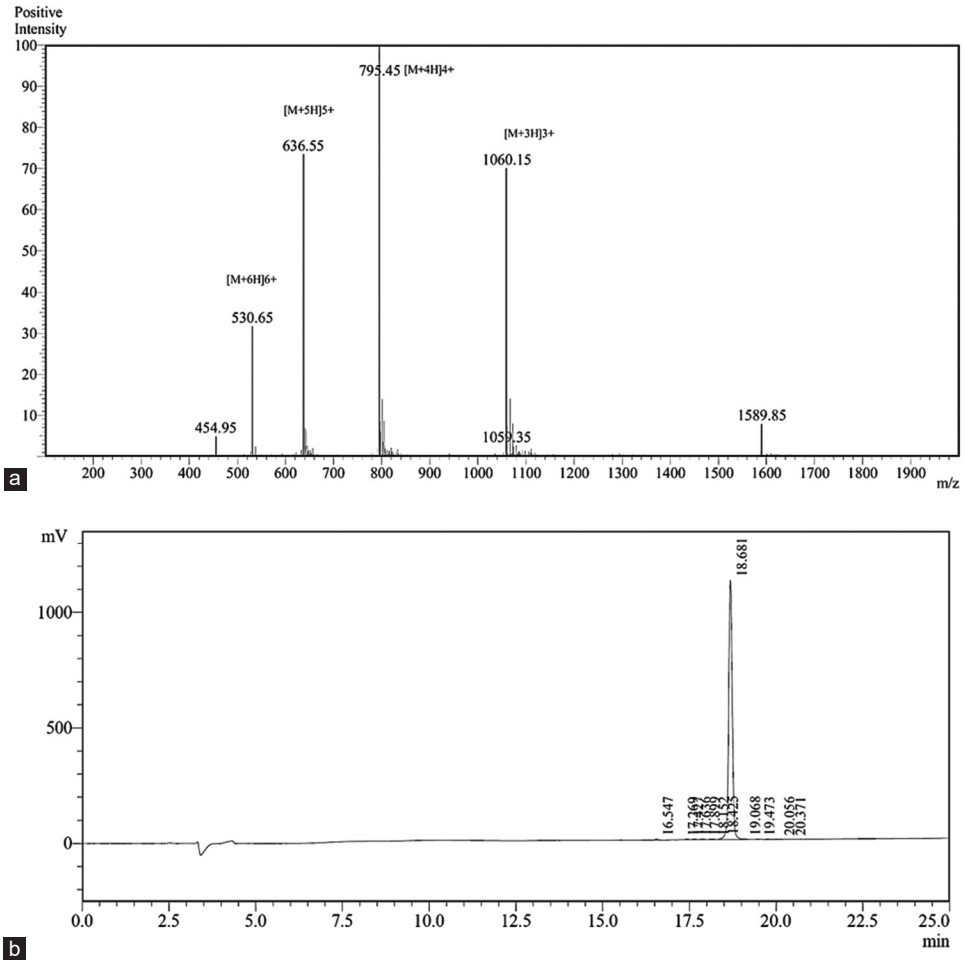
(+) Growth inhibition≥50% and (−) Growth inhibition<50%.



Supplement Data Figure 1: The RACE-PCR product of *Hoplobatrachus rugulosus* cDNA. The arrows are indicated the 300 bp product of PCR reaction with a conserved domain of cathelicidin-RC genes forward primer rcCATH-F and reverse primer rcCATH-R (a) and the 3'-RACE-PCR product (800 bp) using 5'-specific forward primer hrCATH34-F and oligonucleotide d(T) primer (b).



Supplement Data Figure 2: Predicted secondary structure of cathelicidin-HR peptide using PEP-FOLD3 server. The free cysteine residues are represented in stick. The cathelicidin-HR (a), Lf-CATH1(b), OL-CATH1 (c), and cathelicidin-NV (d) were presented in cartoon.



Supplement Data Figure 3: The mass spectrum (a) and HPLC analysis (b) of synthetic cathelicidin-HR peptide.