Analysis and Prediction of Some Histone-derived Antimicrobial Peptides from Toads *Duttaphrynus melanostictus* and *Phyrinoidis asper*

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**ABSTRACT**

Antimicrobial peptides in skin secretions of toads are promising methods to combat a wide spectrum of bacteria. Histone H2A is a type of DNA-binding protein that acts as a precursor for several antimicrobial peptides. In toads (family Bufonidae) buforin I and buforin II are examples of antimicrobial peptides that derived from histone H2A. This study investigated the genetic diversity and phylogenetic analysis and in silico prediction of antimicrobial peptides derived from histone H2A of *Duttaphrynus melanostictus* and *Phyrinoidis asper*, which were collected from Bogor Agricultural University’s campus area. A new set of primers (Buf_fwd and Buf_rev) were designed by using PrimerBLAST, to amplify 393 nucleotides of the histone H2A gene that codes 131 amino acids. Haplotype diversity of both species are very low. Phylogenetic analysis shows the sample *D. melanostictus* and *P. asper* are separated to each other in two different clades. Several short predicted peptides from histone H2A show a potential as an antimicrobial peptides based on in silico prediction. Psychochemical characteristics and 3D structure of potent antimicrobial peptides are described.

Keywords: Antimicrobial Peptides, Histone H2A, Phylogenetic, In silico prediction, Toads

**INTRODUCTION**

Bacterial antibiotic resistance is a global problem today. Song [1] reported there is an increase of the resistance rate of various bacteria pathogens to conventional antibiotics, especially in the Asian Region. This is caused by misuses of antibiotics in the clinical and the animal husbandry sectors. Therefore, there is a need for a new generation of antibiotics that can combat resistant bacteria and which do not cause further resistance to become a critical issue. Antimicrobial peptides (AMPs) are short peptides that can act as an alternative for conventional antibiotics, because AMPs can combat most resistant bacteria [2] and natural resistance of bacteria to AMPs is very rare [3].

AMPs are natural compounds that are used as an innate immune system in many organisms to protect their body from pathogens. AMPs are found in many organisms, from microbes (bacteria and yeast) to higher organisms (animals and plants) [4]. One animal group...
that produces many AMPs is anuran, the amphibians. Anurans secrete a skin secretion that contains AMPs to protect themselves from microbial pathogens. Xu and Lai [5] reviewed all antimicrobial peptides of amphibians (1900 AMPs) that had been published up to 2013 and classified them into 100 families of peptides. However, in the family Bufonidae, few researchers have reported the presence of AMPs. Several antimicrobial peptides that have been reported from this genus are buforin I, buforin II, maximin and alyteserin-1a [6]. In addition, Garg et al [7] reported the potential of skin secretions of D. melanostictus from India as an antibacterial agent, and Utami et al [8] reported the antifungal activities from this species.

Buforin I and II are examples of AMPs that isolated from B. gargarizans. This buforin has an identical amino acid sequence with histone H2A, it is an AMPs that derived from histone H2A by endo-peptic cleavage. Kawasaki and Iwamuro [9] summarized the potential of histone as a precursor for antimicrobial peptides from various organisms. These facts, show that exploration and identification of AMPs derived from histones needs further study. On the other hand, exploration and identification AMPs by conventional methods are long and tedious. As a cutting edge solution, the screening and identification potential AMPs derived from histone using an in silico approach is a promising solution with many advantages. Several related studies on the exploration and identification of AMPs with the in silico approach have been undertaken for histones of microbes [10] and fish [11, 12]. In this study, we designed a pairs of primer that can amplify the histone H2A gene of D. melanostictus and P. asper. We report the nucleotide sequences of histone H2A from D. melanostictus and the phylogenetic relationship with previous reported histone H2A. In addition, we presented the potential AMPs that can be predicted from histone H2A based on the in silico approach.

**EXPERIMENT**

**Chemicals and instrumentation**

Chemicals used for this research were: Alcohol 96% (Novapharin), DNA Isolation Kit (Qiagen), Go Taq Green PCR Mix (Promega), Oligonucleotides Primers (IDT), TBE Buffer (Promega), Agarose (1st Base), Molecular Grade Water (GBioscience). Several consumable plastics that were used: micropipette tips (Axygen), Microtube 1.5 mL (Eppendorf), PCR Tube 0.2 mL (Corning).

Instrumentation applied in this research were: Micropipettes (Eppendorf), Microcentrifuge (Eppendorf Centrifuge 5424), PCR Thermal Cycler (MJ Mini Biorad PTC1148), Horizontal Electrophoresis tank (Thermoscientific Owl Easycast B1A), Microwave (Sanken), Electrophoresis Power Supplay (Enduro Labnet 300V), Gel doc (Biorad).

**Sample collection**

Live samples of D. melanostictus and P. asper were collected from Bogor Agricultural University campus area. Toe clips were collected and preserved in alcohol 96% as the DNA sources. The live samples were released to their habitat.

**DNA isolation and PCR amplification**

Total genomic DNA was isolated from toe-clip-tissue sample by using DNAeasy Blood and Tissue Kit (Qiagen). The histone H2A gene was amplified by PCR with primers Buf_fwd (5’-AAGAGAACGATGTCTGGACG-3’) and Buf_rev2 (5’-TTAGAAGAGCCTTTGGGTT CC GGG-3’) that were designed with PrimerBLAST NCBI [13] based on the sequence of histone H2A of B. gargarizans with accession code U70133.1 [14]. The PCR reaction was performed
with Go Taq green (Promega) in 50 µL reaction volume. The PCR conditions involved an initial denaturation at 95°C for 5 minutes, followed by 40 cycles of 94°C for 1 minute, 50°C for 1 minute, 72°C for 1 minutes and a final extension at 72°C for 10 minutes. The amplicons were analyzed by electrophoresis in 1.5% agarose gel in TBE buffer, stained with gel red, and visualized using Gel Doc. The PCR products were purified and sequenced at 1st Base, Malaysia.

Phylogenetic Analysis

Forward and reverse sequences were proof read, trimmed and assembled using software MEGA 5 [15]. The sequences were aligned by Clustall W and the amino acid sequences were deduced with standard genetic code provided by MEGA 5 [15]. The homology search of nucleotide was done by the Basic Local Search Alignment Tools (BLAST) algorithm of National Center for Biotechnology Information (NCBI) [16]. Phylogenetic tree was constructed with Maximum Likelihood and 1000 replication of bootstrap was performed during the tree construction. Best substitution model was chosen by model test.

Antimicrobial Peptides Prediction

In silico prediction of potent AMPs was conducted based on the methods used by Yoo et al [17] with a slight modification. Amino acid sequences of histone H2A were submitted to CAMP server [18] and each fragmented into 20 amino acid segments. All fragments were predicted with 4 algorithm provided by the server. Fragments, that were categorized as AMPs were further analyzed for their isoelectric point (8≤PI≤12), molecular weight and positive charge with EMBOSS PEPSTATs [19]. In vitro and in vivo aggregation levels of peptides were predicted with TANGO (AGG ≤ 500, 0 ≤ HELIX ≤ 25, 25 ≤ BETA ≤ 100) [20] and AGGRESCAN (-40 ≤ Na4vSS ≤ 60) [21], respectively. The possibility the candidate AMPs were degraded in the in vivo system was predicted based on the cleavage site of chymotrypsin using a peptide cutter [22] and PEST motif with EMBOSS EPESTFIND [19]. 3D structure of potent AMPs generated with PEPstr server [23] then visualized with VMD 1.9.1 [24].

RESULT AND DISCUSSION

Primer Design and Amplicon of histone H2A

A pairs of primer, forward Buf_Fwd (5’-AAGAGAACGATGTCTGGACG-3’) and reverse Buf_rev2 (5’-TTAGAAGAGCCTTTGGTTCGGG-3’) were designed and chosen from several alternatives primers that were suggested by PrimerBLAST NCBI (Figure 1). This primer successfully amplified gene that encodes histone H2A of D. melanostictus and P. Asper with PCR product reaching 400-500 bp. These primers have a good specificity to amplify gene targets in both species. This is showed by the single band with high concentration of amplicons in agarose-gel-visualization (Figure 1). However, the electropherograms of D. melanostictus sequences, the reverse sequences, it has a high noise peak that can be caused by unspecific annealing of the reverse primer to the DNA template of this species. For further research, we recommend the design of more general primers that can amplify histone H2A genes from all anuran species. The electropherograms of several sequences are provided in Figure 2.
Figure 1 (Right). (a) Pair of primers that were designed with the primerBLAST NCBI. The chosen primers are marked in black box. 1b. Electrophoresis gel visualization of amplicons of gene histone H2A from *D. melanostictus* (DM1-9) and *P. Asper* (PA1-9), M (marker), (-) (negative control). The length of these amplicons are around 400-500 bp.

Figure 2. (Right) Electropherograms of histone H2A sequence from both species. All forward sequences show the good quality of peak, however the reverse sequence from *D. melanostictus* has some noise at the bottom of the peak.

Nucleotide and Amino Acid Sequences of Histone H2A
A total of 393 base pairs of DNA, codes for 131 amino acid of histone H2A from both species. There were used for phylogenetic reconstruction. Nucleotide BLAST to NCBI database shows that the sequences from both species have an identity 97% in common to sequence of histone H2A of *B. gargarizans* with accession number AF255739.1 and have an
The sequences from *D. melanostictus* and *P. asper* have low genetic diversity. From the 393 bp nucleotides, there are only three haplotypes from all 18 individuals (Table 1). Compared to each group of samples, there is one polymorphic site in the *D. melanostictus* groups, which is in nucleotides position 124. This is caused by substitution from G to C, which is a transversion (Tv) mutation. On the other hand, there is no a polymorphic site in *P. asper* groups. This result shows, that histone H2A of *D. melanostictus* has a higher haplotype diversity compared to *P. asper*. This is in contrast with the Cytochrome oxidase 1 data [Unpublished data] that shows a higher haplotype diversity on *P. asper* groups than on *D. melanostictus* groups (from same samples).

**Table 1.** Haplotype groups of *D. melanostictus* and *P. asper*

<table>
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<th>No</th>
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<th>Sample</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>Haplotype 1</td>
<td><em>D. melanostictus</em> 1, 2, 3, 4, 6, 7, 9</td>
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<tr>
<td>2</td>
<td>Haplotype 2</td>
<td><em>D. melanostictus</em> 5, 8</td>
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<td>3</td>
<td>Haplotype 3</td>
<td><em>P. asper</em> 1, 2, 3, 4, 5, 6, 7, 8, 9</td>
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</table>

Figure 3. Nucleotide and amino acid sequences of histone H2A of *D. melanostictus* and *P. asper*. The nucleotides which differ among the 3 haplotype are shown with grey shading, and the amino acid change is in bold and underlined font.

Amino acid translation from histone H2A gene was conducted by using standard genetic code provided by MEGA 5 software [15] (Figure 3). Based on this translation, among the 3 haplotypes, there are 3 non-synonymous mutations. These amino acid sequence changes are
in amino acid positions 42, 126 and 127, that caused by substitution of the nucleotide in the first codon position. Based on redundancy of the genetic code, every mutation in the second position of a codon will give an amino acid change. Moreover, most of mutations in the third position of the codon will not change the amino acid, and some mutations in the first codon position also do not give amino acid changes.

**Figure 4.** Phylogenetic tree from nucleotide sequences of histone H2A of *D. melanostictus* and *P. asper* with several comparison sequences from NCBI.

**Phylogenetic Analysis**

A total 397 nucleotides that code for histone H2A were used for a phylogenetic tree reconstruction. As a comparison, 13 sequences of previous reported histone from other organisms were downloaded from the NCBI database. Substitution models of our dataset was
tested with a model test and chosen based on the Bayesian Invariance Criterion (BIC) value [25]. Tamura 3 parameter with gamma distribution (T93+G) was chosen as the best model with the lowest BIC value compared with other models. A maximum likelihood tree was constructed and tested using bootstrap methods with 1000 replications. The result was provided in Figure 4.

Our phylogenetic tree shows both groups of samples are separated into two different clades with support of bootstrap 80%. This clade is strongly separated from other anuran species which are B. gargarizans and Xenopus tropicaliss with 99% and 56% confident, respectively. In addition, inside of the D. melanostictus clade, samples 5 and 8 that have the same haplotype are separated from other samples that have a different haplotype. On the other hand, all sample of P. asper are together in one big clade. This because the P. asper sample have the same haplotype.

**Prediction of Potent Antimicrobial Peptides Derived from Histone H2A**

Prediction of fragment peptides that have potential as antimicrobial peptides (AMPs) was conducted by submitting the histone H2A amino acid sequence to collection of antimicrobial peptides release 2 (CAMP R2) server [18]. A total 130 amino acids were used as a template and resulted in 111 fragment peptides with 20 amino acids each. The four algorithms provided by the server are search vector machine (SVM), random forest (RF), Artificial Neural Network (ANN) and Discriminant analysis (DA), were used to calculate and give a prediction score for all peptides. A total 42 peptides considered as candidate AMPs based on its fourth algorithm. Peptides that are predicted Non-AMPs, based on one algorithm, will not be used for further analysis. From the 42 peptides, 13 peptides were chosen based on their positive charge and their isoelectric point (8-12). Peptides with positive charges will easily attach to the bacterial cell wall, which are negatively charged [26].

The aggregation of peptides in solution and in the outer membrane of bacteria, were predicted using software Tango (solution) [20] and Aggrescan (Outer membrane) [21]. Peptides with decrease aggregation in solution but which increase in the bacteria cell membrane is an ideal characteristic for AMPs. By this analysis, only 8 peptides (Table 2) were left for further analysis. In vivo stability based on the possibility that the peptides may be degraded by any peptidase enzymes is an important issue. An endopeptidase that can reduce the activities of AMPs is α-chymotrypsin. Also, PEST motif can be elevated the possibility of the peptides being degraded. This cleavage site in 8 potent peptides was searched by Peptide cutter [22] and EMBOSS EPESTFIND [19]. All 8 potent peptides did not have any of these peptidase cleavage sites. This shows that the peptides will be stable in vivo, because they will not be degraded by endopeptidase α-chymotrypsin. The predicted 3D structures of the 8 potent peptides are provided in Figure 5.
CONCLUSION

A pair of primers was designed and successfully amplified the histone H2A of both *D. melanostictus* and *P. asper*. The phylogenetic tree based on histone H2A sequence shows all samples of *D. melanostictus* and *P. asper* are separated onto 2 big clades. Three haplotype of these samples are separated in a phylogenetic tree. A total of 8 potent AMPs are successfully predicted based on in silico prediction. We recommend the synthesis these 8 AMPs and the bioassay of their antimicrobial activities in vitro.

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REFERENCES


Figure 5. 3D structure of potent AMPs derived from histone H2A of *D. melanostictus* and *P. asper*
Table 2. Potent peptides as AMPs and their molecular properties

<table>
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