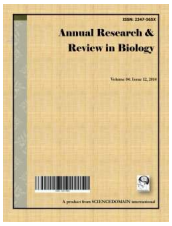

Annual Research & Review in Biology

14(6): 1-10, 2017; Article no.ARRB.34211
ISSN: 2347-565X, NLM ID: 101632869

Ecology and Protein Composition of *Polypedates leucomystax* (Gravenhorst, 1829) (Anura: Rhacophoridae) Foam Nests from Peninsular Malaysia

Shahriza Shahrudin[1*], Mohd Nazri Ismail[2], Soon Hong Kwan[2] and Nazalan Najimudin[3]

[1]School of Pharmaceutical Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia.
[2]Analytical Biochemistry Research Centre, Universiti Sains Malaysia, 11800 Penang, Malaysia.
[3]School of Biological Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia.

Authors' contributions

This work was carried out in collaboration between all authors. Author SS designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors MNI and SHK managed the analyses of the study. Author NN managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/ARRB/2017/34211
Editor(s):
(1) Reinhold J. Hutz, Department of Biological Sciences, University of Wisconsin-Milwaukee, USA.
(2) George Perry, University of Texas at San Antonio, USA.
Reviewers:
(1) Exbrayat Jean-Marie, Lyon Catholic University, France.
(2) F. A. Egwumah, University of Agriculture, Nigeria.
Complete Peer review History: http://www.sciencedomain.org/review-history/20096

Original Research Article

Received 18th May 2017
Accepted 12th July 2017
Published 18th July 2017

ABSTRACT

The four-lined tree frog, *Polypedates leucomystax*, spawns its eggs in a moist structure called a foam nest. Four foam nests constructed by this species were collected from the Sungai Sedim Recreational Forest, Kedah, Peninsular Malaysia. Two foam nests were found deposited on the leaves of low vegetation hanging over a rock pool. One was attached inside a water tank, and one was found on grass near an ephemeral puddle. In the laboratory, the foam nests were freeze-dried and the protein concentrations quantified, fractionated, and analyzed using LC-MS/MS. Twenty-two

**Corresponding author: E-mail: shahriza20@yahoo.com;*

proteins, including seven enzymes, six structural proteins, five regulatory proteins, three receptors, and one antimicrobial peptide (AMP) were found in the foam nests. The function of the AMP (brevinin-2 type) is believed to protect the frog eggs from pathogenic microorganisms.

Keywords: Amphibian; foam nest; protein and peptide; pharmacological effect; Polypedates leucomystax; Peninsular Malaysia.

1. INTRODUCTION

Polypedates leucomystax is a medium to large-sized frog. The snout-vent length (SVL) of males and females reaches 37–50 and 57–75 mm, respectively [1]. This commensal species is widely distributed throughout Bangladesh, Brunei, Cambodia, China, India, Indonesia, Laos, Malaysia, Myanmar, Nepal, the Philippines, Singapore, Thailand, and Vietnam [2]. They occupy various habitats and are usually found around human habitations in urban and rural areas [3,4,2]. During the breeding period, the females produce eggs in a white moist structure called a foam nest. The foam nest is created from skin secretions, produced by both the male and female frogs, and wiped by hind limbs onto the posterior dorsum. Usually, this foam nest is deposited on tree branches, twigs, or leaves and hangs over stagnant water bodies such as ephemeral pools and puddles [3,4,1].

Many studies have focused on the bioactive compounds contained in the skin secretions of frogs [5,6,7]. These secretions are believed to contain various chemical substances, which produce a variety of pharmacological effects including antimicrobial activities [8,9]. Two frog species from Peninsular Malaysia, namely *Odorrana hosii* and *Hylarana picturata*, produce a variety of antimicrobial peptides (AMPs) in their skin secretions [10]. Eight AMPs, belonging to the esculentin-1 (1 peptide), esculentin-2 (1 peptide), brevinin-1 (2 peptides), brevinin-2 (2 peptides), and nigrocin-2 (2 peptides) families, were detected in the skin secretions of *O. hosii.* Eight peptides, belonging to the brevinin-1 (2 peptides), brevinin-2 (5 peptides), and temporin (I peptide) families, were detected in the skin secretions of *H. picturata* [10]. Skin secretions of *Hylarana erythraea*, from Vietnam, contain several AMPs belonging to the brevinin-1 (3 peptides), brevinin-2 (2 peptides), esculentin-2 (4 peptide), and temporin (I peptide) families [11].

Amphibian foam nests perform various functions including: protection against predators and pathogens [12]; prevention of egg-mass dehydration [13,14]; provision of respiratory advantages to embryos [15]; gliding substrates for larvae [14]; and provision of nutrients for the development of embryos [16]. The composition of anuran foam nests has been studied by several researchers. Kabisch et al. [14] analyzed the foam nests of *P. leucomystax*, which contained more than 93% protein. The proteins consisted of 17 amino acids; the main proteins were asparagines, lysine, and glutamate. Cooper et al. [17] discovered unusual primary structures and surfactant activity in ranaspumins proteins in the foam nests of *Physalaemus pustulosus*. McMahon et al. [18] described the crystal structure of a 13 kDa surfactant protein, ranasmurfin, which was isolated from the foam nests of *P. leucomystax*. Hissa et al. [16] detected a strong surfactant protein, *Lv*-ranaspumin, in *Leptodactylus vastus*. These findings indicate the variety of chemical substances, especially proteins, contained in amphibian foam nests. Therefore, this study was conducted to analyze the ecology and protein composition of *P. leucomystax* foam nests.

2. MATERIALS AND METHODS

2.1 Foam Nest Collection

Four *P. leucomystax* foam nests were collected from the Sungai Sedim Recreational Forest, Kedah, Peninsular Malaysia (5°25'N, 100°46'E; elevation <150 m above sea level). The foam nests were collected by searching around stagnant water bodies such as rock pools and temporary puddles. The foam nests were collected by hand and placed into moist plastic bags. To confirm that the foam nests belonged to the *P. leucomystax* species, each mating pair was collected. Visual examination indicated that the morphology of each mating pair was in accordance with *P. leucomystax* [3].

2.2 Freeze Drying

Eggs were separated from the foam nests using forceps. The foam nests were then placed into 50 mL Falcon tubes and frozen at -35℃. After 24 hours, the frozen foam nests were transferred into a freeze-dry machine (Labconco) and

processed for 24 hours. The optimal temperature and vacuum conditions were -47℃ and 0.025 mbar, respectively. Fifteen milligrams of each freeze-dried foam nest was added to 1 mL of 40 mM Tris-HCl (pH 8.8) extraction buffer. The mixtures were left for 20 minutes and occasionally stirred. The mixtures were then centrifuged at 12,000 x g for 30 minutes. The supernatants were collected and stored at -35℃.

2.3 Bradford Assay

The total protein concentration of the foam nests was quantified using a Bradford assay [19]. Five micro liters of each supernatant was mixed with 250 µL of Bradford reagent in a 96-well plate and incubated at ambient temperature for 15 minutes. A standard curve ranging from 0–2.0 mg/mL was constructed with absorbance set at 595 nm. The total protein concentration of each sample was determined and averaged by comparing the absorbance value against the standard curve.

2.4 Protein Fractionation

Protein fractionation was conducted using a Gel free 8100 fractionation system (Expedeon, CA, USA). The procedure was carried out according to the protocol of Witkowski and Harkins [20]. Two hundred micrograms of each sample was loaded into a 10% Tris-Acetate cartridge. Twelve fractions were collected and concentrated using a vacuum concentrator.

2.5 Protein Digestion

Protein digestion was carried out according to Kinter and Sherman [21]. The samples were re-suspended in 100 µL of 6 M urea and 10 mg/mL of 100 mM Tris buffer. DTT (200 mM) was added to each sample and the mixture held at room temperature for one hour. Iodoacetamide (200 mM) was then added and incubated at room temperature for a further hour followed by addition of excess DTT to consume unreacted iodoacetamide. The concentration of urea in the samples was then reduced by adding 775 µL of water. For digestion purpose, 20 µg of trypsin solution (Promega, WI, USA) was added to each sample and incubated overnight at 37℃. The digestion was halted the next day by adjusting the pH of the buffer to pH < 6.

2.6 LC-MS/MS Analysis

Each sample was mixed with 100 µL of 0.1% formic acid in de-ionized water and filtered using a 0.45 µm regenerated cellulose (RC) membrane syringe filter (Sartorius AG, Goettingen, Germany). The analysis was conducted using a LTQ-Orbitrap Velos Pro mass spectrometer, coupled with an Easy-nLC II nano liquid chromatography system. A C18 Easy column (10 cm, 0.75 mm i.d., 3 µm) (Thermo Scientific, San Jose, CA, USA) was used as the analytical column and a C18 Easy column (2 cm, 0.1 mm i.d., 5 µm) (Thermo Scientific, San Jose, CA, USA) was used as the pre-column. The analytical column was equilibrated at a flow rate of 0.3 µL/min for 4 µL; the pre-column was equilibrated at 3 µL/min for 15 µL; and 5 µL of each sample was injected and chromatographically separated at a flow rate of 0.3 µL/min. Running buffers (A) 0.1% formic acid in de-ionized water and (B) 0.1% formic acid in acetonitrile were used. The samples were eluted using a gradient of 5% to 100% of buffer B for 80 minutes. The eluent was sprayed into the mass spectrometer at 2.1 kV (voltage source), and the capillary temperature was set at 220℃. Protein and peptides were detected using full-scan mass analysis from m/z 300–2,000 at a resolving power of 60,000 (at m/z 400, FWHM; 1-s acquisition). Data-dependent MS/MS analyses (ITMS) were triggered by the eight most abundant ions from a parent mass list of predicted peptides, with rejection or unassigned charge states. Collision-induced dissociation (CID) was used as a fragmentation technique with a collision energy of 35. Each sample was analyzed twice.

2.7 Protein and Peptide Identification (*De Novo* Sequencing)

PEAKS Studio Version 7 (Bioinformatics Solution, Waterloo Canada) was used to perform *de novo* sequencing and database matching. The National Centre for Biotechnology Information (NCBI) amphibian database (as of October 2014) was used for database matching. Parent mass and precursor mass tolerance were set at 0.1 Da. A false detection rate (FDR) of less than 0.1% and a significant score of -10lgP for proteins greater than 30 were used for protein acceptance. A minimum unique peptide was set at 1 and a maximum variable post-translational modification was set at 4.

3. RESULTS AND DISCUSSION

Mating pairs (Fig. 1) and foam nests (Fig. 2) of *P. leucomystax* were collected from the Sungai

Sedim Recreational Forest. In Fig. 1, the small-sized frog is male (top) and the larger frog is female (bottom). Two foam nests were found deposited on the leaves of low vegetation approximately 1 m above a rock pool. The rock pool was intermediate-sized, being approximately 4 m in length, 2 m wide, and 5–35 cm deep; contained clear water; had a sandy-gravel bed; and was covered with leaf litter and twigs. It was bordered by low vegetation and was directly exposed to sunlight. Tadpoles of other frog species, *Microhyla heymonsi* and *Fejervarya limnocharis*, were also found living in the pool. A single foam nest was collected from the inner surface of a water tank, which was located near a toilet. The water tank was about 1.5 m long and 1 m wide and was filled with clean tap water. This foam nest was located approximately 22 cm above the water level. The fourth foam nest was found deposited on grass at the edge of an ephemeral puddle. This intermediate-sized puddle was approximately 2 m long, 1 m wide, 2–15 cm deep, and located 6–7 m from a main river. It contained cloudy water, had a silty bottom, and was covered with sediments. The puddle was bordered by small sized grass and exposed to sunlight.

Anuran species lives in different types of environment and reproduce by a variety of modes. To date, 39 types of reproduction modes have been recognized and described in anurans, including the formation of foam nests [22,23]. Various frog species from Malaysia, especially tree frogs such as *Polypedates macrotis, P. otilophus, P. leucomystax, Rhacophorus nigropalmatus,* and *R. pardalis* deposit their eggs in foam nests during their breeding seasons [24]. Usually, foam nests are located adjacent to stagnant water bodies. Inger [25] found the foam nests of *P. leucomystax* attached to vegetation and rocks at the edges of standing or slowly flowing water. Berry [3] discovered foam nests in tubs around houses, tanks, rainwater butts, or on leaves overhanging small pools of water. Sheridan [26] found foam nests belonging to *P. leucomystax* above water in emergent vegetation or other suitable substrates. It breeds in standing water bodies such as natural ponds, cattle tanks, cisterns, and flowerpots. In this study, the foam nests of *P. leucomystax* were found deposited on leaves hanging over a rock pool, among grass, and attached inside a water tank.

Fig. 1. Male (top) and female (bottom) *P. leucomystax* in amplexus

Fig. 2. The foam nest of *P. leucomystax*

The average total protein concentration of the foam nests in the current study was 1.10 mg/mL. The seven-point calibration curve, ranged from 0–2.0 mg/mL ($y=0.3652x0.006$; $R^2=0.9914$) (Fig. 3). Twenty-two proteins, including seven enzymes (32%), six structural proteins (27%), five regulatory proteins (23%), three receptors (13%), and one AMP (5%) were detected in the *P. leucomystax* foam nests (Table 1). The enzymes detected in the foam nests were prohormone convertase 1, duodenase-1, $Ca2^{+}$-ATPase, membrane protease subunit 2, exosome complex exonuclease RRP43, and NADH-ubiquinone oxidoreductase chain 2. The structural proteins were collagen alpha-2(1) chain, alpha 1 type 1 collagen, adult keratin RAK, larval type 1 keratin, and larval specific keratin 1. The regulatory proteins were pumilio-related protein, protein 90B, C10orf27, and tropomyosin-1 alpha chain. The receptors were ryanodine receptor alpha isoform, ryanodine receptor beta isoform, and progesterone receptor. The single AMP belongs to the brevenin-2 family. The

proteins, based on their biological functions, are shown in Fig. 4.

Various chemical substances including proteins, peptides, steroids, and biogenic amines have been detected in the skin secretions of amphibians [27,28]. Previous reports have documented AMPs in the skin secretions of amphibian species belonging to the families of Pipidae, Hylidae, Hyperoliidae, and Ranidae [29,30,31]. These AMP molecules are 10–50 amino acid residues in length, with remarkably diverse structures [31,32]. From our results, a single AMP belonging to the brevinin-2 family was detected in the foam nest of *P. leucomystax*.

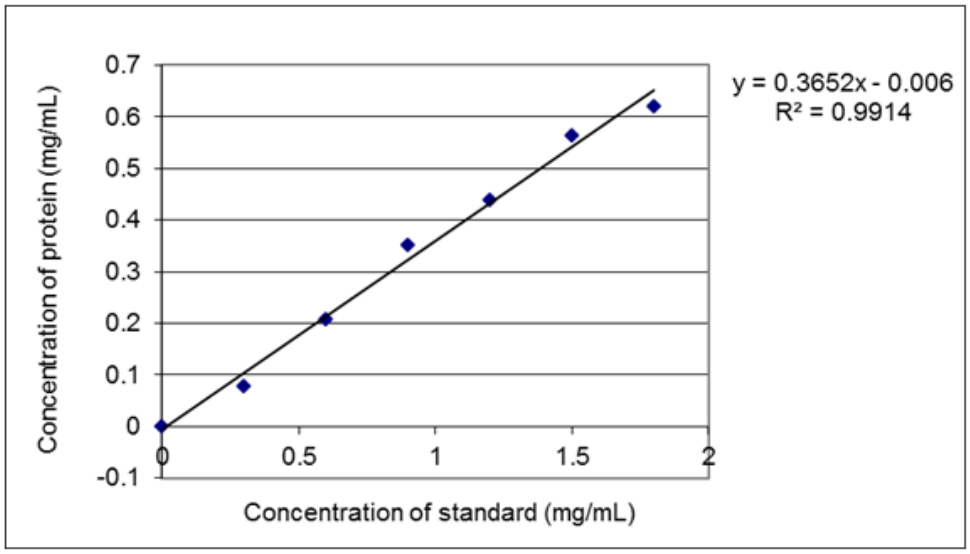

Fig. 3. The seven-point calibration curve (protein concentration of foam nests = 1.10 mg/mL)

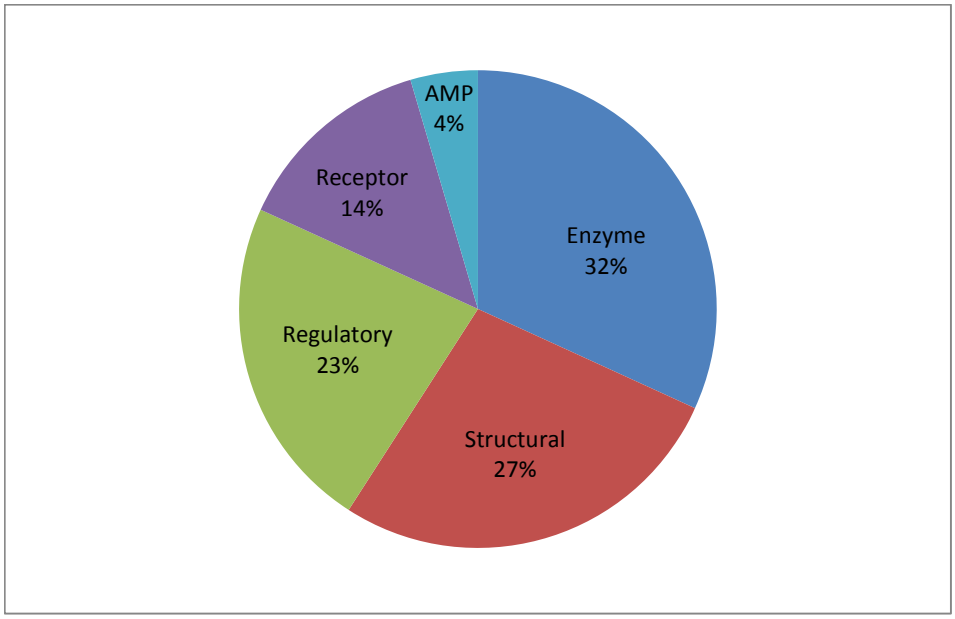

Fig. 4. Types of protein detected in *P. leucomystax* foam nests

Table 1. List of proteins detected in *P. leucomystax* foam nests

Accession	Score (%)	-10l gP	Coverage (%)	#Peptides	#Unique	Avg. Mass	Types of protein	Description
sp|O42350|CO1A2_LITCT	14.1	74.74	19	11	11	127644	Structural	Collagen alpha-2(I) chain OS=Lithobates catesbeiana GN=COL1A2 PE=2 SV=1
tr|O93251|O93251_LITCT	13.2	72.95	26	16	16	137251	Structural	Alpha 1 type I collagen OS=Lithobates catesbeiana GN=alpha 1 type I collagen PE=2 SV=1
tr|D8L948|D8L948_RANLE	12.3	40.71	6	3	3	129202	Regulatory	Pumilio-related protein OS=Rana lessonae GN=pum1 PE=2 SV=1
tr|Q76FG1|Q76FG1_LITCT	11.4	36.06	10	2	2	82881	Enzyme	Prohormone convertase 1 OS=Lithobates catesbeiana GN=pc1 PE=2 SV=1
tr|O93583|O93583_PELRI	10.2	36.06	10	2	2	82966	Enzyme	Prohormone convertase 1 OS=Pelophylax ridibundus PE=2 SV=1
tr|C1C3U9|C1C3U9_LITCT	20.6	31.31	7	1	1	28870	Regulatory	Coiled-coil domain-containing protein 90B, mitochondrial OS=Lithobates catesbeiana GN=CC90B PE=2 SV=1
tr|C1C450|C1C450_LITCT	5.1	29.97	13	2	2	27687	Enzyme	Duodenase-1 OS=Lithobates catesbeiana GN=DDN1 PE=2 SV=1
sp|P0C5X5|B2DYE_RANDY	5.3	27.07	35	1	1	3675	AMP	Brevinin-2DYe OS=Rana dybowskii PE=1 SV=1
tr|Q91313|Q91313_LITCT	12.2	25.59	0	1	1	571299	Receptor	Ryanodine receptor alpha isoform OS=Lithobates catesbeiana GN=FROG-ARR PE=2 SV=1
tr|Q91319|Q91319_LITCT	12.0	25.59	0	1	1	553055	Receptor	Ryanodine receptor beta isoform OS=Lithobates catesbeiana GN=FROG-BRR PE=2 SV=2
tr|Q9DDB8|Q9DDB8_RANSY	12.1	24.62	2	1	1	109266	Enzyme	Ca2+-ATPase OS=Rana sylvatica GN=atp2A1 PE=2 SV=1

Accession	Score (%)	-10l gP	Coverage (%)	#Peptides	#Unique	Avg. Mass	Types of protein	Description
tr|Q910C2|Q910C2_LITCT	10.9	24.44	3	1	1	46595	Structural	Adult keratin RAK (Fragment) OS=Lithobates catesbeiana GN=rak PE=2 SV=1
tr|A7TUG6|A7TUG6_LITCT	6.2	24.44	2	1	1	51694	Structural	Larval type I keratin OS=Lithobates catesbeiana GN=RLKI PE=2 SV=1
tr|F5BBF0|F5BBF0_RANLU	5.2	24.44	10	1	1	12783	Structural	Larval type I keratin (Fragment) OS=Rana luteiventris GN=RLKI PE=2 SV=1
tr|K9M0N1|K9M0N1_RANCL	5.2	24.44	3	1	1	36407	Structural	Larval-specific keratin 1 (Fragment) OS=Rana clamitans GN=rlk1 PE=2 SV=1
tr|C1C4Y0|C1C4Y0_LITCT	5.2	22.88	9	1	1	19605	Enzyme	Mitochondrial inner membrane protease subunit 2 OS=Lithobates catesbeiana GN=IMP2L PE=2 SV=1
tr|C1C4G7|C1C4G7_LITCT	5.1	22.71	4	1	1	40431	Regulatory	C10orf27 OS=Lithobates catesbeiana GN=CJ027 PE=2 SV=1
tr|C1C521|C1C521_LITCT	9.8	22.21	5	1	1	29713	Enzyme	Exosome complex exonuclease RRP43 OS=Lithobates catesbeiana GN=EXOS8 PE=2 SV=1
tr|C1C502|C1C502_LITCT	5.1	22.04	5	1	1	32739	Regulatory	Tropomyosin-1 alpha chain OS=Lithobates catesbeiana GN=TPM1 PE=2 SV=1
sp|P13105|TPM1_RANTE	5.1	22.04	5	1	1	32665	Regulatory	Tropomyosin alpha-1 chain OS=Rana temporaria GN=tpm1 PE=2 SV=1
tr|G3XF03|G3XF03_9NEOB	10.5	21.51	7	1	1	37529	Enzyme	NADH-ubiquinone oxidoreductase chain 2 OS=Odorrana ishikawae GN=ND2 PE=3 SV=1
sp|Q8AYI2|PRGR_RANDY	6.1	21.37	1	1	1	80126	Receptor	Progesterone receptor OS=Rana dybowskii GN=pgr PE=1 SV=1

Brevinin peptides were first identified in the skin secretions of *Rana brevipoda porosa* (reclassified as *Pelophylax porosa*) [33]. Further studies demonstrated that AMPs belonging to the brevinin-1 family are widely distributed in both Eurasian and North American anuran, whereas brevinin-2 peptides have only been found in Eurasian ranids [34]. Currently, brevinin peptides have been found in various amphibian species, including *H. picturata, O. hosii, H. erythraea,* and *Hylarana spinulosa*. Brevinin-1 and -2 peptides isolated from *H. picturata* and *O. hosii* showed antimicrobial activities against Gram-negative bacteria (*Escherichia coli*) and Gram-positive bacteria (*Staphylococcus aureus*) [10]. Brevinin-2 peptides from *H. erythraea* inhibits the growth activities of *E. coli* and *S. aureus* and a fungal, *Candida albicans* [11]. Antimicrobial peptides isolated from different anuran species exhibit various effects against different types of microorganisms. For example, brevinin-1 peptide isolated from *Hylarana spinulosa* (Ranidae) inhibits the growth of a variety of Gram-positive bacteria (*S. aureus, Enterococcus faecium, E. faecalis,* and *Nocardia asteroides*), Gram-negative bacteria (*Pseudomonas aeruginosa, Klebsiella pneumonia, Enterobactercloacae, E. coli,* and *Psychrobacter faecalis*), and fungi (*Candida glabrata* and *C. albicans*) at differing concentrations [7].

The current study's findings notably include the first time that brevinin peptides coming from the foam nests of anuran species have been reported. Almost all peptides that have been extracted, isolated, and documented have come from amphibian skin secretions. In amphibians, these peptides have specific functions including protection from pathogenic microbes, defense from potential predators, wound healing, and oxidant scavenging [27,31,35]. In addition, peptides can also exert multiple functions such as chemotactic and immunomodulating activities, endotoxin neutralization, induction of angiogenesis, and wound repair [36].

4. CONCLUSION

Polypedates leucomystax deposits its eggs in a moist structure called a foam nest. Twenty-two proteins including seven enzymes, six structural proteins, five regulatory proteins, three receptors, and a single AMP (brevinin-2 type) were detected in the foam nests of this species in this study. The importance and functions of these proteins needs to be further investigated. The AMP (brevinin-2 type) is believed to play a vital role in protecting *P. leucomystax* eggs from pathogenic microorganisms, which also requires further study. The peptide should be isolated and its activities against several bacterial and fungal species tested.

ACKNOWLEDGEMENTS

We wish to express our heartfelt gratitude to Universiti Sains Malaysia, Penang for the facilities and amenities provided. This research project was funded by Universiti Sains Malaysia, Research University Grant (1001/PFARMASI/8011004) and Fundamental Research Grant Scheme (203/PDOPING/6711389).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Grismer LL. Amphibians and reptiles of the Seribuat Archipelago. Edition Chimaira, Frankfurt; 2011.
2. IUCN. The IUCN red list of threatened species. Version 2015;2. Available:www.iucnredlist.org (Accessed 04 March 2015)
3. Berry PY. The amphibians fauna of Peninsular Malaysia. Tropical Press, Kuala Lumpur; 1975.
4. Ibrahim HJ, Shahrul Anuar MS, Norhayati A, Chan KO, Mohd Abdul Muin MA. The common amphibians and reptiles of Penang Island. The State Forestry Department of Penang; 2008.
5. Yang X, Lee WH, Zhang Y. Extremely abundant antimicrobial peptides existed in the skins of nine kinds of Chinese odorous frogs. Journal of Proteome Research. 2011;11:306-319.
6. Wu J, Liu H, Yang H, Yu H, You D, Ma Y, Ye H, Lai R. Proteomic analysis of skin defensive factors of tree frog *Hyla simplex.* Journal of Proteome Research. 2011;10: 4230-4240.
7. Yang X, Hu Y, Xu S, Hu Y, Meng H, Guo C, Liu Y, Liu J, Yu Z, Wang H. Identification of multiple antimicrobial peptides from the skin of fine-spined frog, *Hylarana spinulosa* (Ranidae). Biochimie. 2013;95:2429-2436.

8. Ma Y, Liu C, Liu X, Wu J, Yang H, Wang Y, Li J, Yu H, Lai R. Peptidomics and genomics analysis of novel antimicrobial peptides from the frog, *Rana nigrovittata.* Genomics. 2010;95:66-71.
9. Wang G, Wang Y, Ma D, Liu H, Li J, Zhang K, Yang X, Lai R, Liu J. Five novel antimicrobial peptides from the Kuhl's Wart frog skin secretions, *Limnonectes kuhlii.* Mol Biol Rep. 2013;40:1097-1102.
10. Conlon JM, Kolodziejek J, Nowotny N, Leprince J, Vaudry H, Coquet L, Jouenne T, King JD. Characterization of antimicrobial peptides from the skin secretions of the Malaysian frogs, *Odorrana hosii* and *Hylarana picturata* (Anura: Ranidae). Toxicon. 2008;52:465-473.
11. Nadia A, Kolodziejek J, Nowotny N, Coquet L, Jouenne T, Leprince J, Vaudry H, King JD, Conlon JM. Antimicrobial peptides from the skin secretions of the South-East Asian frog *Hylarana erythraea* (Ranidae). Peptides. 2009;31:548-554.
12. Downie JR. Functions of the foam in the foam nesting leptodactylid: The nest as a post-hatching refuge in *Physalaemus pustulosus.* Herpetological Journal. 1993;3: 35-42.
13. Downie JR. Function of the foam in the foam nesting leptodactylid *Physalaemus pustulosus.* Herpetological Journal. 1988; 1:302-307.
14. Kabisch K, Herrmann HJ, Klossek P, Luppa H, Brauer K. Foam gland and chemical analysis of the foam of *Polypedates leucomystax* (Gravenhorst, 1829) (Anura: Rhacophoridae). Russian Journal of Herpetology. 1998;5(1):10-14.
15. Seymour RS, Loveridge JP. Embryonic and larval respiration in the arboreal foam nests of the African Frog *Chiromantis xerampelina.* Journal of Experimental Biology. 1994;197:31-46.
16. Hissa DC, Vasconcelos IK, Carvalho AFU, Nogueira VLR, Cascon P, Antunes ASL, Macedo GR, Melo VMM. Novel surfactant proteins are involved in the structure and stability of foam nests from the frog *Leptodactylus vastus.* The Journal of Experimental Biology. 2008;211:2707-2711.
17. Cooper A, Kennedy MW, Fleming RI, Wilson EH, Videler H, Wokosin DL, Su T, Green RJ, Lu JR. Absorption of frog foam nest proteins at the air-water interface. Biophysic Journal. 2005;88:2114-2125.
18. McMahon SA, Walsh MA, Ching RTY, Carter LG, Dorward M, Johnson KA, Liu H, Oke M, Bloch C, Jr, Kennedy MW. Crystallization of ranasmurfin, a blue-coloured protein from *Polypedates leucomystax*. Acta Crystallographica. 2006;F62:1124-1126.
19. Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry. 1976;72(1):248-254.
20. Witkowski C, Harkins J. Using the Gelfree 8100 Fractionation system for molecular weight-based fractionation with liquid phase recovery. Journal Visualized Experimental. 2009;E1842.
21. Kinter M, Sherman NE. Protein sequencing and identification using tandem mass spectrometry. John Wiley and Son, New York. 2005;9.
22. Duellman WE, Trueb L. Biology of amphibians. McGraw-Hill Book Company. New York; 1986.
23. Haddad CFB, Prado CPA. Reproductive modes in frogs and their unexpected diversity in the Atlantic Forest of Brazil. Bioscience. 2005;55(3):207-217.
24. Inger RF, Stuebing RB. Frogs of Sabah. Kota Kinabalu: Sabah Parks Trustess; 1989.
25. Inger RF. The systematics and zoogeography of the amphibians of Borneo. Fieldiana Zoology. 1966;52:1-402.
26. Sheridan JA. Ecology and behaviour of *Polypedates leucomystax* (Anura: Rhacophoridae) in Northeast Thailand. Herpetological Review. 2008;39(2):165-169.
27. Clarke BT. The natural history of amphibian skin secretions, their normal functioning and potential medical applications. Biol Rev Camb Philos Soc. 1997;72:365-379.
28. Conlon JM, Leprince J. Identification and analysis of bioactive peptides in amphibians skin secretions. Peptidomics. 2010;1:145-157.
29. Barra D, Simmaco M. Amphibian skin: A promising resource for antimicrobial peptides. Trends Biotechnology. 1995;13: 205-209.
30. Duda TF, Jr., Vanhoye D, Nicholas P. Roles of diversifying selection and

coordinated evolution in the evolution of amphibian antimicrobial peptides. Molecular Biology Evolution. 2002;19:858-864.
31. Conlon JM, Kolodziejek J, Nowotny N. Antimicrobial peptides from ranid frogs: Taxonomic and phylogenetic markers and a potential source of new therapeutic agents. Biochim Biophys Acta. 2004;1696: 1-14.
32. Li J, Xu X, Xu C, Zhou W, Zhang K, Yu H, Zhang Y, Zheng Y, Rees HH, Lai R, Yang D, Wu J. Anti-infection peptidomics of amphibian skin. Molecular Cell Proteomics. 2007;6:882-894.
33. Morikawa N, Hagiwara K, Nakajima T. Brevinin-1 and -2, unique antimicrobial peptide from the skin of the frog, *Rana brevipoda porsa*. Biochemical and Biophysical Research Communications. 1992;189:184-190.
34. Suzuki H, Iwamuro S, Ohnuma A, Coquet L, Leprince J, Jouenne T, Vaudry H, Taylor CK, Abel PW, Conlon JM. Expression of genes encoding antimicrobial and bradykinin-related peptides in skin of the stream brown frog *Rana sakuraii.* Peptides. 2007;28:505-514.
35. Yang H, Wang X, Liu X, Wu J, Liu C, Gong W, Zhao Z, Hong J, Lin D, Wang Y, Lai R. Antioxidant peptidomics reveals novel skin antioxidant system. Molecular Cell Proteomics. 2009;8:571-583.
36. Guani-Guerra E, Santos-Mendoza T, Lugo-Reyes SO, Teran LM. Antimicrobial peptides: General overview and clinical implication in human health and disease. Clinical Immunology. 2010;135:1-11.

Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/20096