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RESEARCH ARTICLE

Identification and Characterization of a Novel Gene-encoded Antioxidant Peptide from Odorous Frog Skin

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Abstract: ***Background***: Amphibian skin plays an essential role in protecting organisms from harmful external factors such as UV radiation. How amphibians protect themselves from reactive oxygen species following long-term sun exposure is an important and interesting question. Amphibian skins possess a novel antioxidant system composed of various antioxidant peptides (AOPs), which maintain redox homeostasis. However, only a few AOPs have been identified so far.

Method: Using combinational methods of peptidomics and genomics, we characterized a novel gene-encoded antioxidant peptide (herein named OA-VI12) from *Odorrana andersonii* skin secretions, which was produced by the post-translational processing of a 59-residue prepropeptide. The amino acid sequence of the OA-V112 was 'VIPFLACRPLGL', with a molecular mass of 1298.6 Da and no observed post-transcriptional modifications. Functional analysis demonstrated that OA-VI12 was capable of scavenging $ABTS^{+}$, DPPH, NO and decreasing the Fe^{3+} production.

Results: We determined that the C7 amino acid was responsible for $ABTS^{+}$ and Fe^{3+} scavenging, activities, the F4, C7, and P9 amino acids were crucial for DPPH scavenging activity, and the P9 amino acid was responsible for NO scavenging activity. Unlike several other amphibian peptides, OA-VI12 did not accelerate wound healing in a full-thickness skin-wound mouse model and did not demonstrate direct microbial killing. Here, we identified and named a novel gene-encoded antioxidant peptide from the skin secretions of an odorous frog species, which may assist in the development of potential antioxidant candidates.

Conclusion: This study may help improve our understanding of the molecular basis of amphibians' adaptation to environments experiencing long-term UV radiation.

Keywords: *Odorrana andersonii,* OA-VI12*,* antioxidant peptide, molecular basis, amphibian adaptation, UV radiation.

1. INTRODUCTION

Free radicals, which contain unpaired electrons in their atomic or molecular orbitals or simply contain reactive oxygen species, are generated by cell respiration and cell-mediated immune functions [1]. They are produced naturally in the body and play important roles in cellular activity [1], with a complex system of endogenous and exogenous antioxidants employed to mitigate damage from these species [2]. Excess free radicals participate in the pathogenesis of many human diseases, including cancer, atherosclerosis, hyperpigmentation, and neurodegeneration [3-6]. In biological systems, antioxidants maintain free radical balance through scavenging [6], thereby protecting the organism from oxidative stress-induced diseases [1].

Amphibians form a link between reptilian and aquatic animals and show global distribution and the ability to survive in complex environments [6-8]. Their bare skin is highly susceptible to physical harm due to UV radiation and pathogenic microorganism infection. Thus, due to the combination of vulnerable skin and harsh habitats, amphibians have evolved an excellent defensive system to minimize

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these threats [6, 9]. Amphibian bioactive peptides, such as antimicrobial peptides, tachykinins, dermorphins, cholecystokinin, and bradykinins, have been found to influence biological injuries; however, few peptides showing non-biological injury effects have been reported [10-15]. Injury from UV radiation is an important non-biological stress for amphibians, with long-term radiation resulting in the development of a unique and effective antioxidant peptide skin defense system [7]. Amphibian antioxidant peptides, which are used to scavenge free radicals, are rapidly produced after long-term exposure to sunlight radiation to prevent skin injury [9, 16-19].

To date, three different antioxidant systems have been identified in amphibian skin [20-22]; namely, gene-encoded enzymes with high molecular weight, including superoxide dismutase, catalase, ascorbate peroxidase, and glutathione reductase [7]; non-gene-encoded antioxidants of low molecular weight (LMWA) secreted from the epidermis and separated into dietary (*e.g*., vitamin C and E) and endogenous antioxidants (*e.g*., reduced glutathione) [7, 21, 23]; and, gene-encoded small molecular weight peptides (*e.g*., from amphibian skin secretions) [20]. Currently, however, only a few novel gene-encoded antioxidant peptides have been reported [9], such as fragilin-A1 and -B1 from *Limnonectes fragilis* [9] and antioxidin-I from tropical frog species [6], *et al*.

Odorous frogs are among the most abundant natural sources of antimicrobial peptides, though many such peptides await discovery and identification [8]. In the present study, we investigated antioxidant peptides from skin secretions of *Odorrana andersonii*, an odorous frog species from the Yun-Gui Plateau of southwest China. This species resides at very high altitudes (surpassing 2,300 m) and experiences extreme UV radiation exposure, which has led to the development of a robust antioxidant system to protect their skin [7]. Here, we isolated a novel antioxidant peptide (OA-VI12) that demonstrated considerable scavenging ability, including the scavenging of $ABTS^+$, DPPH, Fe^{3+}, and NO. Our results further indicated that the C7 amino acid was responsible for $ABTS^+$ and Fe^{3+} scavenging activities, whereas F4, C7, and P9 were responsible for DPPH scavenging activity and P9 was responsible for NO scavenging activity. Unlike other peptides, however, OA-VI12 did not increase wound healing in a full-thickness skin-wound mouse model or show direct microbial killing activity. In this study, we discovered a new gene-encoded antioxidant peptide from the skin secretions of a frog species, which may be a potential candidate for the advance of novel antioxidant agents. Furthermore, this study adds to our knowledge of the molecular basis of amphibian adaptation to strong UV radiation.

2. MATERIALS AND METHODS

2.1. Sample Collection and Animal Care

Adult *O. andersonii* (n = 25) individuals were collected from Lijiang in Yunnan Province, China, and safely transferred to our laboratory. Frogs were housed collectively in a 50 cm × 60 cm container with mealworms provided *ad libitum*. After one week of adjustment to their environment, *O. andersonii* skin was stimulated using an electronic massager with a 6-V alternating current. The resulting secretions were rinsed off the frogs using 25 mM Tis-HCl buffer (pH 7.8). The procured secretions were centrifuged at 4000 g for 15 min at 4°C, with supernatants then collected and lyophilized. All samples were stored until further analysis at -80°C.

The Ethics Committee of Kunming Medical University approved all animal-based experiments and welfare protocols, which were conducted in accordance with the university's Guidelines for Animal Care and Use.

2.2. Purification Procedure

The peptide purification procedure was performed as per earlier research [8]. Briefly, the lyophilized skin secretion samples (500 µl, OD_{280} = 50) were dissolved in deionized water and applied to a Sephadex G-75 gel filtration column (1.5 × 31 cm, superfine, GE Healthcare, Sweden) equilibrated with 25 mM Tris-HCl (pH 7.8) plus 0.1 M NaCl as the elution buffer at a 0.1 ml/min flow rate. Fractions were collected every 10 min using a BSA-30A automatic fraction collector (Huxi Company, Shanghai, China), with absorbance monitored at 280 nm. Fractions were then subjected to an $ABTS^+$ antioxidant activity assay, and those that demonstrated activity were collected and purified using a Hypersil BDS C18 RP-HPLC column (4.0 × 300 mm, Elite, China) pre-equilibrated with 0.1% (v/v) trifluoroacetic acid (TFA) in water. Elution was conducted using a linear gradient (0–60% acetonitrile in 60 min) of 0.1% (v/v) TFA in acetonitrile at a 1 ml/min flow rate and monitored at 220 nm (Figure **1B**). Again, fractions that demonstrated $ABTS^+$ antioxidant activity were gathered and lyophilized for a second round of HPLC purification (as above).

2.3. Peptide Primary Structure Determination

An AXIMA-CFRTM-MALDI-TOF mass spectrometer (Shimadzu/Kratos, Manchester, UK) in linear mode with α-cyano-4-hydroxycinnamic acid was used to determine the observed molecular weight and purity of native OA-VI12. All protocols were carried out and all data were analyzed according to the manufacturer's standard procedures and software. Edman degradation on a Shimadzu PPSQ-31A protein sequencer (Japan) was applied to clarify the complete amino acid sequence using the manufacturer's standard GFD protocols. Lastly, the peptide's cysteine residues were determined based on the procedures provided by the manufacturer.

2.4. Cloning of cDNA Encoding OA-VI12

We screened the cDNA encoding the mature peptide based on our previously established *O. andersonii* skin cDNA library [8]. The primers used, namely, 5' PCR primer (5'-CCAAA(G/C)ATGTTCACC(T/A)TGAAGAAA-3') and 3' PCR primer (5'-ATTCTAGAGGCCGAGGCGGCCG ACATG-3'), were produced by the BGI Company (China). Polymerase chain reaction (PCR) was performed (2 min at 94°C, 25 cycles of 10 s at 92°C, 30 s at 50°C, and 40 s at 72°C), with the resulting products retrieved using a Bioteke DNA gel extraction kit (China) and ligated into a pMD19-T vector (TaKaRa Biotechnology Co., Ltd., Dalian, China). The PCR products were then cloned into *Escherichia coli*

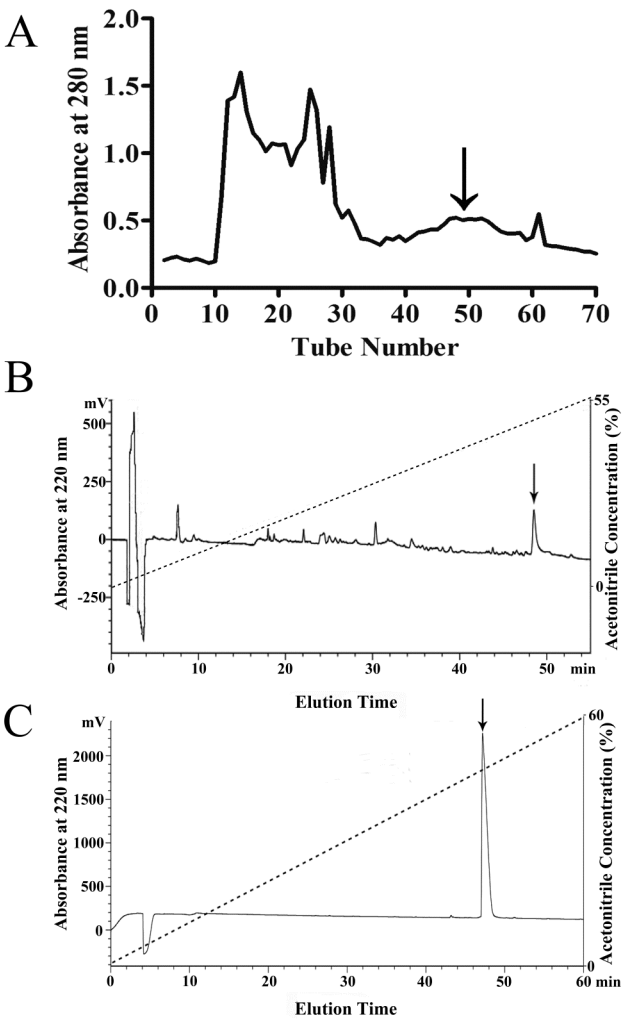

Figure 1. Peptide purification procedure from *O. andersonii* skin secretions.
(A) *O. andersonii* skin secretions were separated by a Sephadex G-75 gel column (arrow indicates peak exhibiting $ABTS^+$ free radical scavenging activity). (B) Eluted peak in (A) showing activity underwent further RP-HPLC column purification. Absorbance was monitored at 220 nm. (C) Sample exhibiting $ABTS^+$ scavenging activity (arrow in (B)) underwent further RP-HPLC purification. A final peptide showing $ABTS^+$ scavenging activity was then obtained (arrow in C).

DH5α, with the independent clones used for DNA sequencing on an ABI 3730XL DNA sequencer (Applied Biosystems, Foster City, CA, USA).

2.5. Peptide Synthesis

The mature OA-VI12 peptide ('VIPFLACRPLGL') was synthesized by Bioyeargene Biotechnology Co., Ltd. (Wuhan, China). The commercially produced OA-VI12 co-eluted with natural OA-VI12 was analyzed using mass spectrometry and was confirmed to be identical with native OA-VI12. Five mutated peptides (OA-VI12 (P3/A): VIAFLACRPLGL; OA-VI12 (F4/A): VIPALACRPLGL; OA-VI12 (C7/A): VIPFLAARPLGL; OA-VI12 (P9/A): VIPFLACRALGL; OA-VI12 (P3, F4, C7, and P9): VIAALAARALGL) were also produced by Bioyeargene Biotechnology Co., Ltd. (Wuhan, China).

The synthesized samples were stored in powder at -20°C. Before the experiment, samples were dissolved in deionized water, with mass spectrometry applied to determine that there was no dimer, after which the samples were used without delay.

2.6. Antioxidant Activity

Free radical scavenging activity was determined by 2, 2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as described previously [8], with some modification. In short, the ABTS stock solution was prepared by incubating 2.8 mM potassium persulfate (Sigma-Aldrich, St Louis, MO, USA) with 7 mM ABTS in water for 6 h in the dark. The stock solution was diluted with deionized water and used immediately. Samples dissolved in water (50 μL) were combined with 50 μL of diluted stock solution and maintained in the dark at room temperature for 30 min, with the sample solvent (same volume) used as a negative control. A decrease in absorbance at 415 nm indicated antioxidant activity. The $ABTS^+$ scavenging activity (%) was calculated by $(A_{blank} - A_{sample}) \times 100 / A_{blank}$.

The 2, 2-diphenyl-1-picrythydrazyl (DPPH) (Sigma-Aldrich, St Louis, MO, USA) free radical scavenging test was applied following our previous study [8], with some modification. In short, the assay mixture, which consisted of 190 μl of 5×10^{-5} M DPPH dissolved in ethanol and 10 μl of sample solution, was added to 96-well microtiter plates for 30 min at room temperature, with the sample solvent used as a negative control. Absorbance was determined at 517 nm and DPPH scavenging activity (%) was calculated by $(A_{blank} - A_{sample}) \times 100 / A_{blank}$.

Fe^{3+} scavenging was tested following earlier research [15], with some amendments. Various concentrations of samples combined with 2.5 ml of 1% $K_3Fe(CN)_6$ and 2.5 ml of 0.2 M phosphate buffer (pH 6.6) were incubated for 20 min at 50°C, followed by the addition of 2.5 ml of 10% TCA. The solution was then centrifuged at 3000 g for 10 min at room temperature, with the top layer (2.5 ml) then mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml; 0.1%). Absorbance was read against a blank at 700 nm. The reducing power was determined according to the increase in reaction mixture absorbance, with vitamin C used as a positive control.

NO scavenging activity was tested as per prior methods [15], with some modification. Samples at various concentrations were incubated with 5 mM sodium nitroprusside dihydrate (SNP; Sigma-Aldrich, St Louis, MO, USA) in distilled water. After 120 min of incubation at 25°C, 0.5 ml of solution was removed and mixed with 0.5 ml of Griess reagent (Promega). Absorbance was then measured at 550 nm using a SpectraMax 190 microplate reader (Molecular Devices, USA). The NO scavenging activity (%) was calculated by $(A_{blank} - A_{sample}) \times 100 / A_{blank}$.

2.7. Antimicrobial Activity Assay

The antimicrobial activity of OA-VI12 was assayed according to our previous research [8]. We obtained gram-

positive bacterial strains *Staphylococcus epidermidis* (ATCC 12228), *Staphylococcus haemolyticus* (ATCC 29970), and *Enterococcus faecalis* (ATCC 29212), gram-negative bacterial strains *Escherichia coli* (ATCC 25922), *Salmonella paratyphi A* (ATCC 9150), *Pseudomonas aeruginosa* (ATCC 27853), and *Acinetobacter junii* (ATCC 17908), fungal strains *Candida glabrata* (ATCC 66032) and *Candida albicans* (ATCC 14053), and marine bacterial strains *Aeromonas hydrophilia* (ATCC 49140), *Vibrio splendidus* (ATCC 33869), and *Streptococcus iniae* (ATCC 29177) from the Kunming Medical University. Except for the marine bacterial strains, which were grown in marine culture medium, all microbes were grown in Luria Bertani (LB) broth to an OD_{600} of 0.8. A 10-μl aliquot of each microbe was collected and added to 10 ml of fresh LB broth or marine culture medium (marine bacterial strains) with 1% Type I agar (Sigma-Aldrich, St Louis, MO, USA), then placed in 90-mm Petri dishes. A small hole was created in the agar after it hardened, with a 7-μl aliquot of OA-VI12 (1 mM) then added to the hole. Lastly, the microbes were incubated at 37°C for 16-18 h, with antimicrobial activity determined by the formation of a clear zone on the agar surface, which represented bacterial growth inhibition.

2.8. Animal Wound-healing Assay

Adult male mice (18-22 g, same generation) were acquired from the Kunming Medical University Experimental Animal Central and used to construct a skin wound-healing model. The mice were caged individually at room temperature with free access to water and laboratory food. After 7 d of acclimation, 10 mice were randomly selected, with all experiments carried out in triplicate. The mice were anesthetized with an intraperitoneal injection of 100 ml of 1% pentobarbital sodium solution (0.1 ml/20 g body weight). Dorsal back hair was removed by electric clipper, after which the shaved area was sterilized with 75% alcohol to prevent infection. Dual full-thickness wounds (8 × 8 mm) were surgically generated on the backs the mice. The mice were then rested and allowed to recover from the anesthesia near a heating device. All right-sided wounds were treated twice daily with 20 μl of OA-VI12 (5 μM) and all left-sided wounds were treated twice daily with the same volume of saline control. Wounds were imaged at certain time intervals every 2 d.

2.9. Wound Healing Measurement

Wound closure was recorded using a digital camera (D3000, Nikon, Japan) and wound area (percentage of residual to original wound area) was calculated using Image J software (NIH, USA). Residual Wound Area (%) = [R (0, 4, 8, 12) / R(0)] × 100%, where R(0) and R(4, 8, 12) denote the residual wound area on the operation day and postoperative days 4, 8, and 12, respectively. GraphPad Prism software (v.5) was used to construct the wound-repair curves.

3. RESULTS

3.1. Peptide Purification

As illustrated in Figure **1A**, *O. andersonii* skin secretions were divided into about 10 fractions after Sephadex G-75 gel filtration. The fractions were collected at 10 min intervals and their $ABTS^+$ scavenging activities were tested. Samples with scavenging $ABTS^+$ free radical activity (arrow in Figure **1A**) were pooled and subjected to C18 column RP-HPLC for further purification. Samples again demonstrating $ABTS^+$ scavenging activity (arrow in Figure **1B**) were collected for a second round of C18 RP-HPLC purification, with one peak obtained showing an ideal shape and identical elution time (arrow in Figure **1C**) to that of the former HPLC procedure. This purified sample exhibited $ABTS^+$ free radical scavenging activity and its primary structure was established.

3.2. Primary Structure of OA-VI12

The primary structure of the purified sample exhibiting antioxidant activity (arrow in Figure **1C**) was determined. Using an Edman sequencer, the amino acid sequence was 'VIPFLACRPLGL' from the N-terminus to C-terminus (Figure **2A**) and thus the peptide was named OA-VI12 (OA: species abbreviation VI: two initial amino acids 12: peptide length). As shown in Figure **2A**, the peptide precursor was composed of 59 amino acid residues. To confirm this sequence, we screened the cDNA clone encoding the peptide precursor from the skin cDNA library, which was well-matched with the Edman-determined sequence. The observed molecular mass of OA-VI12 (1298.6 Da, Figure **2B**) also well-matched the theoretical molecular mass (1297.76 Da), as calculated at http://web.expasy.org/compute_pi/. We Blast searched the NCBI database, and the peptide showed an 83% sequence similarity to brevinin-1 DY1 from *Amolops daiyunensis* (Figure **2C**). Unlike brevinin, however, the current peptide contained no intramolecular disulfide bridge but did contain a free C in the sequence. These results suggested that the peptide belonged to a novel family.

4. ANTIOXIDANT ACTIVITY OF OA-VI12

4.1. $ABTS^+$ Scavenging Activity

As OA-VI12 had a single C residue, we presumed it could be readily dimerized. Using mass spectrometry, we confirmed that no dimer existed (data not shown), after which the peptide was used immediately. As shown in Figure **3A**, at the concentration of 0.3 μM, OA-VI12 scavenged 7.9% of $ABTS^+$, but scavenged 66.6% at 5 μM. Thus, OA-VI12 demonstrated dose-dependent $ABTS^+$ free radical scavenging activity. In addition, OA-VI12 (5 μM) reached its maximum scavenging rate within 10 s (Figure **3B**).

We applied a point-mutant strategy to determine the amino acid residues essential for the $ABTS^+$ scavenging activity of OA-VI12. Earlier studies have shown that peptides act as free radical scavengers due to the presence of C, P, and F amino acid residues [6]. Thus, we substituted these residues with A and synthesized five OA-VI12 mutants. As seen in Figure **3A**, at concentrations of 5 μM, the OA-VI12 (P3/A), (F4/A), and (P9/A) mutants showed similar activities as naturally-occurring OA-VI12; however, when the above-mentioned residues were substituted by A, the OA-VI12 (P3F4C7P9/A) mutant lost this activity, strongly suggesting that the C7 amino acid was crucial for $ABTS^+$ scavenging activity.

A

```
atgttcacctagaagaaatccctgttactccttttcttccttgggaccatcaacttatct 60
 M  F  T  M  K  K  S  L  L  L  L  F  F  L  G  T  I  N  L  S  20
ctctgtcacaaagagagagatgctgatgaggaagaaagaagagatgatccagatgaaagg 120
 L  C  H  K  E  R  D  A  D  E  E  E  R  R  D  D  P  D  E  R  40
gatgttgaagtggaaaaacgagttataccatttcttgcatgtcggccgctcggcctctag 180
 D  V  E  V  E  K  R  V  I  P  F  L  A  C  R  P  L  G  L  *  60
tagtaacgggctggctaaagcttgaatcggaaatcatctgatgtggaatatcatttagct 240
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aaaaa                                                        305
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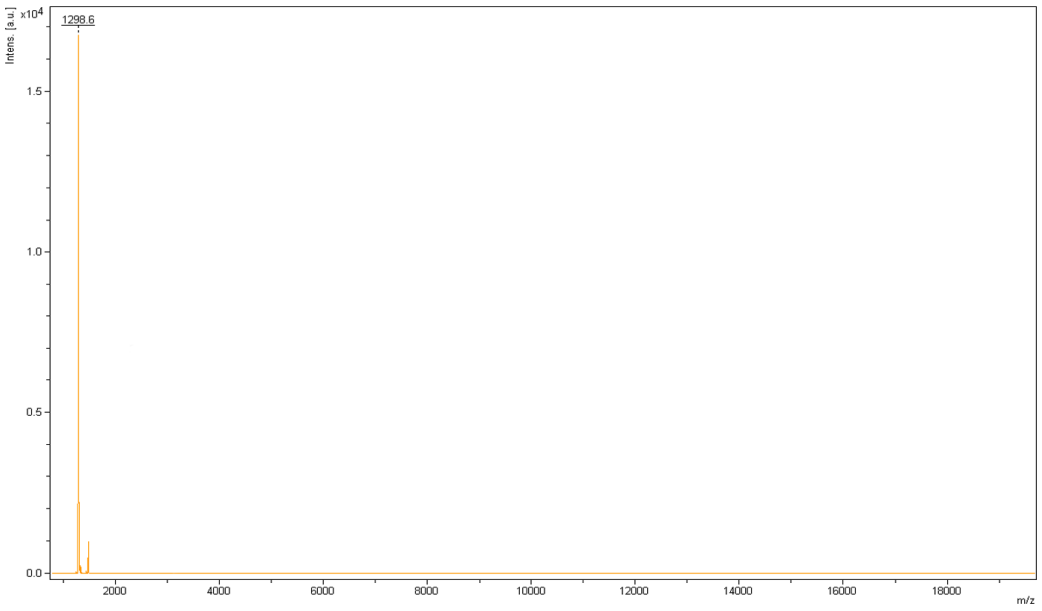
B



C

Enzyme cleavage cite

Name	Species	Signal peptide	Acidic peptide	Mature peptide	Accession number
OA-VI12	*O. andersonii*	MFTMKKSLLLLFFLGTINLSLCHKERDADEEERRDDPDERDVEVEKRVIPFLACRPLGL			
Brevinin-1DY1	*A. daiyunensis*	MFTLKKSMLLLFFLGTINLSLCEQERDADEEERRDDPDETNVEVEKRFLPLLAGLAANFLPTIICKIARKC			AIU99873.1
Brevinin-1RTa antimicrobial peptide precursor	*A. ricketti*	MFTFKKSMLLLFFLGTINLSLCEQERNADEEERRDDPDETNVEVEKRFLPLLAGVVANFLPQIICKIARKC			ACZ52156.1
Temporin-1GY	*P. nigromaculatus*	MFTTKKSILLLFFLGTINLSLCEQERDADEEERRDDPYETDTEVQKRVIPIVSGLLSSLLGK			AGE10574.1
Brivinin-1 precursor	*A. wuyiensis*	MFTLKKSMLLLFFLGTINLSLCEQERNADEEERRDNPDEMDVVVEKRFLPLLAGLAANFLPQIICKIARKC			CCV01178.1
Temporin-HB3	*P. hubeiensis*	MFTLKKSLLLIFFLGTITSSLCEQERNADEEQRRDEPEERDVEVQKRFLPFLAGLFSKIFGK			AIU99906.1
Temporin-HB2 antimicrobial peptide precursor	*P.hubeiensis*	MFTSKKSLLLIFFLGTITSSLCEQERNADEEQRRDEPEERDVEVQKRFLPFLAGLFGKIFGK			AIU99904.1

Figure 2. Primary structure of OA-VI12.
(A) cDNA sequence of OA-VI12. Mature peptide ('VIPFLACRPLGL') was 12 amino acid residues in length (red) and was produced by the post-translational processing of a 59-residue prepropeptide. (B) Observed molecular mass of OA-VI12 (arrow in Figure 1C). (C) Multiple sequence alignments of OA-VI12 compared with several similar peptides. Figure shows: peptide name, species, signal peptide, acidic peptide, mature peptide, and accession number. (*Color figure is available online*)

We also determined if substitution of these residues influenced the efficiency of $ABTS^+$ scavenging. OA-VI12 reached its maximum scavenging rate within 10 s (Figure **3B**); however, when P3, F4, and P9 were substituted, the scavenging rate declined to ~180 s (Figure **3D**), whereas replacement of C7 resulted in a decrease to ~16 m (Figure **3C**). These findings indicated that C, P, and F influenced $ABTS^+$ scavenging efficiency, but C7 was most responsible for the scavenging efficiency of OA-VI12.

4.2. DPPH Scavenging Activity

At the maximum concentration of 5 μM, OA-VI12 scavenged about 10.1% of DPPH, but activity was abolished when the concentration declined to 2.5 μM (Figure **4**). These results indicated that OA-VI12 showed weaker DPPH scavenging activity than $ABTS^+$ scavenging activity.

We further ascertained the DPPH scavenging activity of the five OA-VI12 mutants and determined the amino acid residues crucial for DPPH scavenging activity. As shown in Figure **4**, at concentrations of 5 μM, the OA-VI12 (P3/A) mutant showed similar activity as native OA-VI12; however, when A was used to replace the F4, C7, and P9 residues, the activity was eliminated for the OA-VI12 (F4/A), (C7/A), and (P9/A) mutants. Furthermore, the OA-VI12 (P3F4C7P9/A) mutant also lost this activity when A was used to replace the above residues. These findings demonstrated that F4, C7, and P9 were jointly responsible for the DPPH scavenging activity of OA-VI12.

4.3. Fe^{3+} Scavenging Activity

We applied Fe^{3+}–Fe^{2+} transformation to determine Fe^{3+} reducing antioxidant capacity [15]. As shown in Table **1**, OA-VI12 exhibited Fe^{3+} scavenging activity at the maximum concentration of 5 μM, but this activity was eliminated when the concentration decreased to 2.5 μM. These results indicated that OA-VI12 exhibited weak Fe^{3+} scavenging activity.

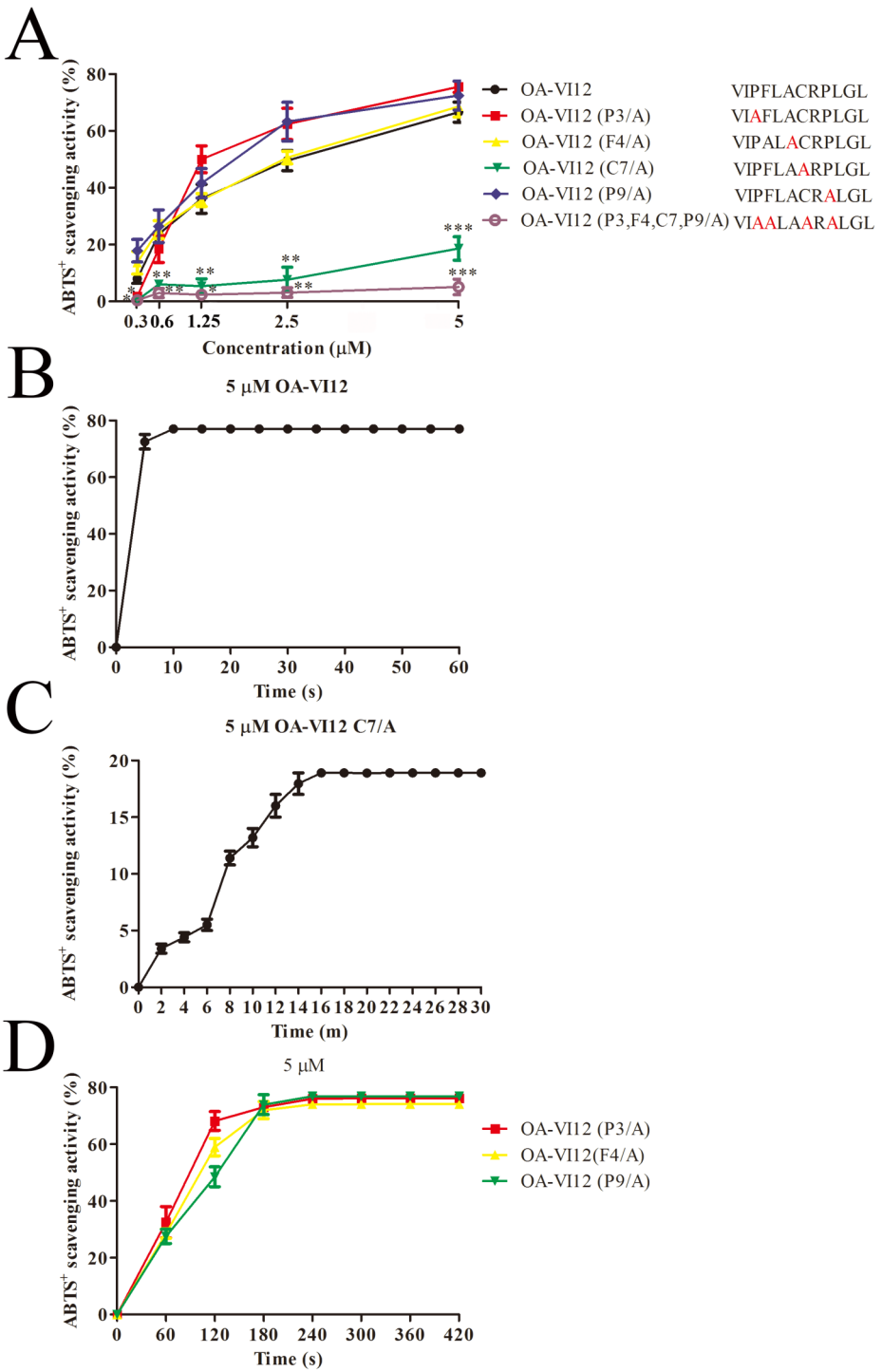


Figure 3. $ABTS^+$ scavenging activities of native OA-VI12 and mutants.
(A) Five OA-VI12 mutants were designed. OA-VI12 and mutants exhibited dose-dependent $ABTS^+$ scavenging activity. (B) OA-VI12 scavenging efficiency against $ABTS^+$ free radicals. (C, D) Scavenging efficiency of mutants against $ABTS^+$ free radicals. *P < 0.05, **P < 0.01, and ***P < 0.0001 indicate significant differences from natural OA-VI12 (Students *t*-test). Data are means of three independent experiments performed in triplicate.

We also determined the Fe^{3+} scavenging activity of the five OA-VI12 mutants and identified the amino acid residues responsible for the scavenging activity. As shown in Table **1**, at concentrations of 5 μM, the OA-VI12 (P3/A), (F4/A), and (P9/A) mutants exhibited comparable activities as native OA-VI12. However, this activity was eliminated for the OA-VI12 (P3F4C7P9/A) mutant after A was used to replace the above residues. These results suggested that the free C might determine OA-VI12 Fe^{3+} scavenging activity.

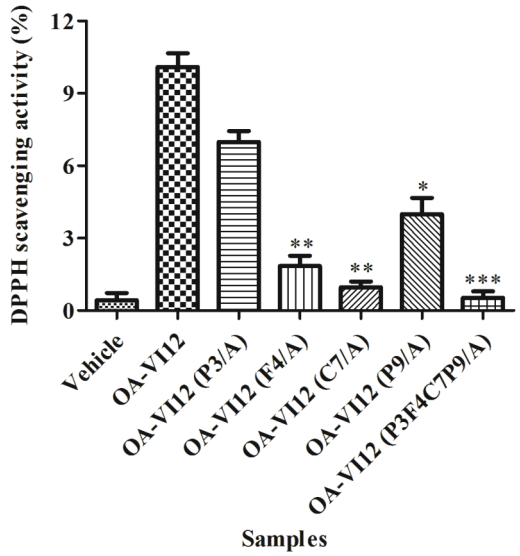


Figure 4. DPPH scavenging activities of native OA-VI12 and mutants.

DPPH scavenging activity of OA-VI12 and mutants at a concentration of 5 μM. *P < 0.05 and **P < 0.01 indicate significant differences from natural OA-VI12 (Students *t*-test). Data are means of three independent experiments performed in triplicate.

Table 1. Fe^{3+} reducing power of native OA-VI12 and mutants.

Sample	Fe^{3+} Reducing Power (Absorbance at 700 nm)	P Value
H_2O	0.03 ± 0.009	
Vitamin C	0.127 ± 0.021	
OA-VI12	0.113 ± 0.003	
OA-VI12 (P3/A)	0.088 ± 0.003	NO
OA-VI12 (F4/A)	0.106 ± 0.01	NO
OA-VI12 (C7/A)	0.036 ± 0.01	*
OA-VI12 (P9/A)	0.101 ± 0.015	NO
OA-VI12 (P3,F4,C7,P9/A)	0.045 ± 0.002	*

'NO' indicates no differences from natural OA-VI12; *P < 0.05 indicates differences from natural OA-VI12 (Students *t*-test)

4.4. NO Scavenging Activity

As shown in Figure **5**, the peptide also quenched NO release by SNP. Incubation of SNP solution in distilled water at 25°C for 120 min resulted in the release of NO. In this mixture, OA-VI12 quenched NO release by an NO donor of SNP. At the concentration of 1.25 μM, OA-VI12 did not demonstrate NO scavenging activity, but the scavenging rate reached 51.25% at 5 μM. These results demonstrate that OA-VI12 showed dose-dependent NO free radical scavenging activity.

Furthermore, the OA-VI12 (P3/A), (F4/A), and (C7/A) mutants showed similar activities as that of naturally-occurring OA-VI12; however, the OA-VI12 (P9/A) and OA-VI12 (P3F4C7P9/A) mutants lost this activity (Figure **5**). These results suggest that P9 plays a crucial role in the NO scavenging activity of OA-VI12.

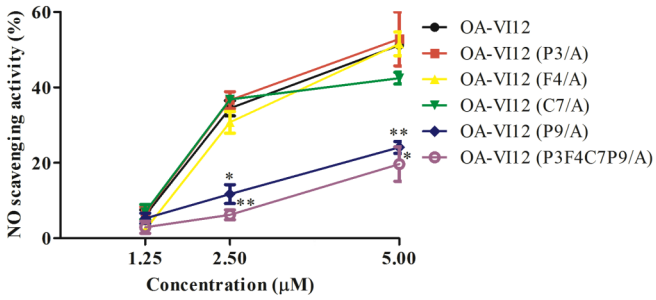


Figure 5. NO scavenging activities of native OA-VI12 and mutants.

At concentrations of 1.25, 2.5, and 5 μM, OA-VI12 and its mutants showed dose-dependent NO scavenging activity. *P < 0.05 and **P < 0.01 indicate significant differences from natural OA-VI12 (Students *t*-test). Data are means of three independent experiments performed in triplicate.

4.5. OA-VI12 Showed no Antimicrobial Activity

According to sequence analysis, OA-VI12 showed similarity to the identified antimicrobial peptide brevinin. As other amphibian antioxidant peptides have shown antimicrobial activity, including antioxidant-I [6], taipehensin-1 TP1, and taipehensin-2 TP2 [16], we next investigated the effectiveness of the OA-VI12 peptide against various pathogens. Interestingly, distinct from previous amphibian antioxidant peptides, OA-VI12 showed no direct killing effect on gram-positive bacterial strains *S. epidermidis*, *S. haemolyticus*, and *E. faecalis*, gram-negative bacterial strains *E. coli*, *S. paratyphi A*, *P. aeruginosa*, and *A. junii*, fungal strains *C. glabrata* and *C. albicans*, and marine bacterial strains *A. hydrophilia*, *V. splendidus*, and *S. iniae*, even at the maximum concentration of 1 mM (Table **2**).

4.6. OA-VI12 Showed No Accelerated Wound Healing in a Mouse Skin Wound Model

As oxidative stress and inflammation can influence the wound healing process, antioxidant activity may therefore accelerate wound healing [8, 24]. Because OA-VI12 illustrated antioxidant activity, we tested its wound-healing activity using a full-thickness skin excisional wound mouse model. Following twice daily application of OA-VI12 (5 μM, 20 μl) or saline (negative control, 20 μl), OA-VI12 did not demonstrate any differences in wound healing activity compared with the vehicle control (Figure **6**).

5. DISCUSSION

Free radicals are known to be involved in the pathogenesis of many human diseases [2, 17]. However, scavengers can reduce the levels of free radicals and provide disease protection [15, 25], and thus the discovery of novel antioxidants from natural sources has attracted increasing attention [16]. As a link between terrestrial and aquatic organisms, amphibians can survive in hostile environments, despite the susceptibly of their bare skin to physical harm [11, 26]. In recent years, amphibian skin studies have demonstrated the

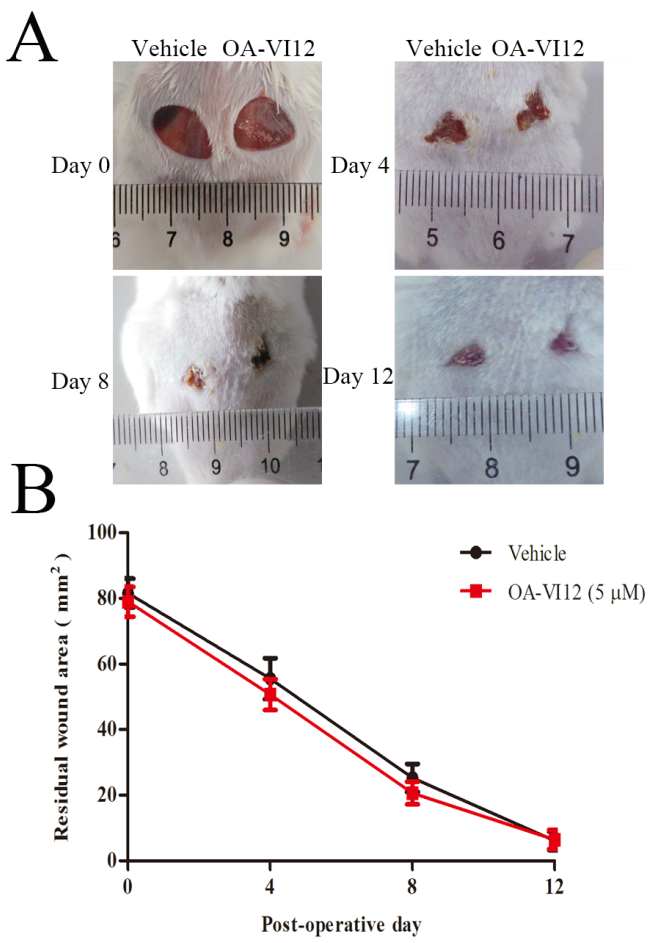


Figure 6. Effects of OA-VI12 on wound healing.
(A) Macroscopic view of wound healing on day 0 and postoperative days 4, 8, and 12. Two 8 × 8 mm surgical wounds were made on the back of each mouse, followed by treatment with 20 μl of saline (vehicle) or 5 μM OA-VI12 twice daily, respectively. (B) Images of wounds were taken on day 0 and postoperative days 4, 8, and 12.

existence of an effective defense system, with skin secretions containing multiple peptides with various biological activities, including antimicrobial, antioxidant, and immunomodulatory activities [12, 15, 27]. Thus, amphibian skin secretions could be a potent source of novel antioxidant peptides. To date, three antioxidant defense systems have been recognized in amphibian skin: namely, gene-encoded antioxidant enzymes and proteins of large molecular weight; non-gene-encoded LMWAs [15, 22, 28]; and gene-encoded LMWAs that scavenge free radicals either directly or indirectly [15]. These gene-encoded antioxidant peptides form a novel antioxidant system, with only a few gene-encoded amphibian antioxidant peptides identified thus far [9].

O. andersonii frogs reside on the Yun-Gui Plateau in southwest China at considerable elevations, and thus endure intense UV radiation exposure. This harsh environment has resulted in the evolution of the effective antioxidant system in their skin. In the present study, we identified a novel gene-encoded antioxidant peptide (OA-VI12) from *O. andersonii* skin secretions. We tested the scavenging activity of the peptide on $ABTS^{+}$, DPPH, $Fe^{3+,}$ and NO. Results showed that OA-VI12 exhibited obvious antioxidant activity, albeit weaker than that of previously reported odorous frog antioxidant peptides such as andersonin-AOP1 [7]. Earlier studies have indicated that peptide antioxidant activity can be attributed to the presence of critical amino acid residues F, C, M, W, and P [6, 15]. In the current study, OA-VI12 contained three of the amino acids listed above (F, C, and P), suggesting that these amino acids may be related to the antioxidant activities of OA-VI12. Thus, we tested the antioxidant activities of several OA-VI12 mutants. Results showed that C7 was the determinant residue for $ABTS^{+}$ and Fe^{3+} scavenging activity. For activity against the DPPH free radical, the determinant residues were F4, C7, and P9, and for NO scavenging activity, the responsible residue was P9. Prior studies have demonstrated that free C is crucial for the scavenging efficiency of $ABTS^{+}$ by antioxidant peptides [7]. Here, we established that P3, F4, C7, and P9 were crucial for the peptide's $ABTS^{+}$ scavenging efficiency, though C7 played the most critical role (Figure **3**). Considering these results, the antioxidant activity of amphibian peptides appears to be related to the amino acid sequence, composition, and structure; moreover, the amino acids in the sequence may play different roles in $ABTS^{+}$, DPPH, Fe^{3+}, and NO scavenging activities. In this study, OA-VI12 exhibited the third antioxidant system characterized by 'gene-encoded peptides secreted from cells'. Specifically, OA-VI12 was of gene-encoded origin and was secreted, but had no enzyme activity. Furthermore, OA-VI12 directly scavenged free radicals, like LMWAs, but had a higher molecular weight. Thus, identification of the OA-VI12 peptide enhances our understanding of odorous frog skin secretions and their activities and compositions.

In recent years, gene-encoded antioxidant peptides from amphibian skin secretions have been shown to possess both antioxidant and antimicrobial activities (*e.g.*, TP1, TP2, and antioxidin-I) [6, 16]. The evolution of amphibian peptides containing both antioxidant and antimicrobial activities may help to save energy in the production of abundant peptides. Because OA-VI12 showed high similarity with the antimicrobial peptide brevinin, we tested the antimicrobial activity of OA-VI12. However, as shown in Table **2**, OA-VI12 did not exhibit any antimicrobial activity against gram-positive or gram-negative bacterial strains nor against fungal or marine bacterial strains.

Natural wound repair is impacted by many factors, including inflammation and oxidative stress [8]. As our previously reported amphibian antioxidant peptide (cathelicidin-OA1) accelerated wound healing activity, we used a mouse skin-wound model to assess the wound healing activity of OA-VI12. As seen in Figure **6**, however, OA-VI12 did not accelerate wound healing. These results indicate that OA-VI12 may possess other functions, such as anti-inflammatory activity, enabling *O. andersonii* to adapt to its complex and harsh habitats.

CONCLUSIONS

We isolated and identified a novel gene-encoded peptide from purified secretions obtained from *O. andersonii* skin. This peptide (named OA-VI12) exhibited an amino acid sequence of 'VIPFLACRPLGL'. Assessment showed that OA-

Table 2. Antimicrobial activity of OA-VI12.

Microorganism	Antimicrobial Activity	
	AMP	OA-VI12
Gram-Positive Bacteria		
Staphylococcus epidermidis (ATCC 12228)	+	-
Staphylococcus haemolyticus (ATCC 29970)	+	-
Enterococcus faecalis (ATCC 29212)	+	-
Gram-Negative Bacteria		
Escherichia coli (ATCC 25922)	+	-
Salmonella paratyphi A (ATCC 9150)	+	-
Pseudomonas aeruginosa (ATCC 27853)	+	-
Acinetobacter junii (ATCC 17908)	+	-
Fungal Strains		
Candida glabrata (ATCC 66032)	+	-
Candida albicans (ATCC 14053)	+	-
Marine Bacterial Strains		
Aeromonas hydrophilia (ATCC 49140)	-	-
Vibrio splendidus (ATCC 33869)	+	-
Streptococcus iniae (ATCC 29177)	+	-

'+': indicates antimicrobial activity; '-': indicates no antimicrobial activity

VI12 successfully scavenged $ABTS^{+}$, DPPH and NO, as well as decreased the Fe^{3+} production, with the C7 amino acid residue found to be responsible for $ABTS^{+}$ and Fe^{3+} scavenging activities, the F4, C7, and P9 amino acid residues responsible for DPPH scavenging activity, and the P9 amino acid residue responsible for NO scavenging activity. Compared with other amphibian peptides, OA-VI12 did not accelerate wound healing or demonstrate direct microbial killing activity. This newly identified gene-encoded antioxidant peptide may help in the development of novel antioxidant agents. Furthermore, this research improves our understanding of the unique molecular basis for amphibian adaptation to strongly UV-radiated habitats.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the local ethics committee of Kunming Medical University.

HUMAN AND ANIMAL RIGHTS

No humans were used in the study. The reported experiments on animals, were in accordance with the guidelines of the Kunming Medical University, Kunming, China.

CONFLICT OF INTERESTS

The authors declare no competing commercial interests in relation to this work.

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AUTHOR CONTRIBUTIONS

X.W.Y. and Y.W. designed the study; X.Q.C. and J.T. performed most of the research, including the collection of animals, purification procedures, primary structures of peptides, determination of activity, and preparation of the manuscript; Z.F. and S.Y.W. participated in the purification procedures; M.F.Y. participated in the antioxidant activity determination of peptides. C.Y.W. participated in the collection of animals. Z.F. participated in the construction of the wound healing model. All authors contributed substantially to this research and reviewed this manuscript.

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