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## Free Radical Biology &amp; Medicine

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## Original Contribution

## Frog skins keep redox homeostasis by antioxidant peptides with rapid radical scavenging ability

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## ARTICLE INFO

## Article history:

Received 18 December 2009

Revised 14 January 2010

Accepted 26 January 2010

Available online xxxx

## Keywords:

Radical scavenging peptide

Amphibian skin

Redox homeostasis

Free radicals

## ABSTRACT

The question of how amphibians can protect themselves from reactive oxygen species when exposed to the sun in an oxygen-rich atmosphere is important and interesting, not only from an evolutionary viewpoint, but also as a primer for researchers interested in mammalian skin biology, in which such peptide systems for antioxidant defense are not well studied. The identification of an antioxidant peptide named antioxidin-RL from frog (*Odorrana livida*) skin in this report supports the idea that a peptide antioxidant system may be a widespread antioxidant strategy among amphibian skins. Its ability to eliminate most of the 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) radical tested within 2 s, which is much faster than the commercial antioxidant factor butylated hydroxytoluene, suggests that it has a potentially large impact on redox homeostasis in amphibian skins. Cys10 is proven to be responsible for its rapid radical scavenging function and tyrosines take part in the binding of antioxidin-RL to radicals according to our nuclear magnetic resonance assay.

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The ability to eliminate toxic chemicals either from the environment or by a metabolic procedure is essential for the survival of all organisms. Formation of ROS is an unavoidable consequence of aerobic metabolism during respiration. Evidence suggests that mitochondria convert 1–2% of the oxygen consumed into superoxide [1]. These active radicals may cause damage to cells or tissues easily by lipid peroxidation, denaturation of proteins, or nucleic acid damage with severe consequences to overall metabolism [2]. For amphibians, the life transition from the aquatic environment to the terrestrial environment, which is characterized by the presence of higher oxygen concentration, means more endogenous production of ROS, which may also be accelerated by the intensive ultraviolet rays. For instance,

frog skin plays an important role in oxygen uptake both in water and in air [3]. When the oxygen concentration is higher, the skin will consume more of the absorbed oxygen instead of satisfying the oxygen requirements of other tissues [4]. In addition, the loss of water from the body may increase susceptibility to oxidative damage [5]. In any case, amphibian skin is destined to be the front line in the battle against radicals.

To cope with the increasing oxidative stress, skin has developed two groups of antioxidant systems. The first group is composed of several enzymes including superoxide dismutase, catalase, peroxidase, and glutathione reductase. The second group is composed of many low-molecular-weight antioxidants (LMWA) such as GSH, NADH, carnosine, uric acid, carotene, polyphenols, and lipoic acid [6–9]. They can scavenge ROS by donating electrons. No gene-coded LMWA has been reported [10,11].

In our previous work, we have identified a novel antioxidant system composed of various antioxidant peptides from skin secretions of *Rana pleuraden*, which are different from antioxidant enzymes and LMWA [12]. Further work is needed to confirm that amphibian skins have a common peptide antioxidant system and to identify the key amino acid residues responsible for the rapid radical scavenging function. In this work, we continue the effort to confirm the peptide antioxidant system in amphibians; in addition we tried to investigate the possible rapid ROS scavenging mechanism.

**Abbreviations:** ABTS, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); ABTS<sup>+</sup>, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) radical; BHT, butylated hydroxytoluene; GSH, reduced glutathione hormone; IAA, iodoacetamide; LMWA, low-molecular-weight antioxidant; LMG, lipocalin  $\alpha$ -microglobulin; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; NADH, reduced form of nicotinamide adenine dinucleotide; NMR, nuclear magnetic resonance; NOESY, nuclear overhauser enhancement spectroscopy; ROS, reactive oxygen species; RP-HPLC, reverse-phase high-performance liquid chromatography; TOCSY, total correlation spectroscopy.

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<sup>1</sup> These authors have contributed equally to this work.

**A**

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atgttcaccatgaagaatccctgttactcctcttttgttcttggaccatcaacttctct 60
M F T M K K S L L L L F V L G T I N L S 20
ctctgtgagcaagagagaggtgctgatgaagaatggagggaagctaaactggaagac 120
L C E Q E R G A D E E D G G G E A K L E D 40
ataaaaaagagccatgcgcttgacatataaccgcccctgtatataatgccacaaaagaaca 180
I K R A M R L T Y N R P C I Y A T K R T 60
aaagaaatgtaactactggaatctcttctgatgtggaatatcatttagctaaatgcaaaa 240
K E M * 63
cagatacagtatctagtaaaaaataaaatttcacatataaaaaaaaaaaaaaaaaaaaaa 300
aaaaaa 306

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**B**

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Antioxidin-RL  AMRLTYNRPCIYAT
Antioxidin-RP1 AMRLTYNKPLYGT
Antioxidin-RP2 SMRLTYNKPLYGT
                *****  *****  *

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**Fig. 1.** (A) The cDNA sequence encoding antioxidant-RL and the deduced amino acid sequence. The mature antioxidant-RL is boxed. The stop codon is indicated by an asterisk. (B) Sequence comparison of antioxidant-RL with the antioxidantins-RP [12]. Identical amino acid residues are indicated by asterisks.

## Materials and methods

### Collection of frog skin secretions

Adult specimens of *Rana (Odorrana) livida* of both sexes ( $n=30$ ; weight range 30–40 g) were collected in Yunnan Province of China. Skin secretions were collected as follows: frogs were put into a cylinder container containing a piece of absorbent cotton saturated

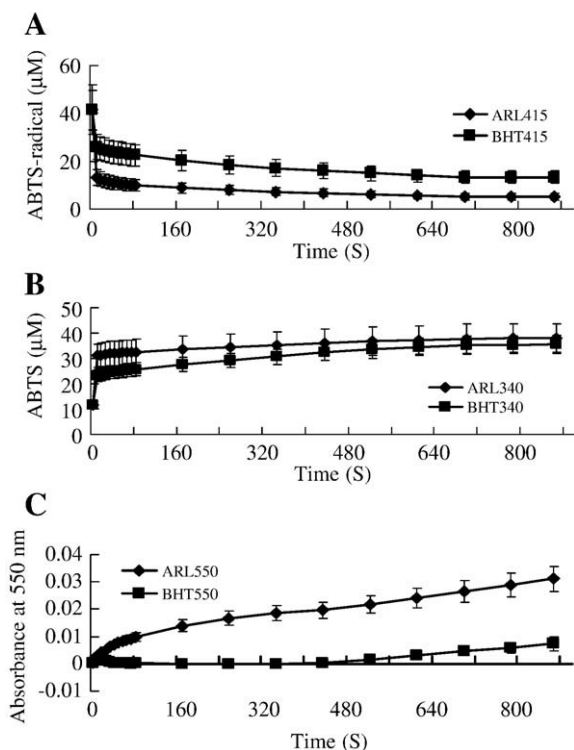
with anhydrous ether. On exposure to anhydrous ether for 1–2 min, the frog skin surface was seen to exude copious secretions. Skin secretions were collected by washing the dorsal region of each frog with 0.1 M NaCl solution (containing protease inhibitor cocktail; Sigma). The experimental animals were washed with clean water and awake after 30 min recovery. The collected solutions (100 ml total volume) were quickly centrifuged and the supernatants were lyophilized. All the experiments were approved by the Kunming Institute of Zoology, Chinese Academy of Sciences.

### Peptide fractionation

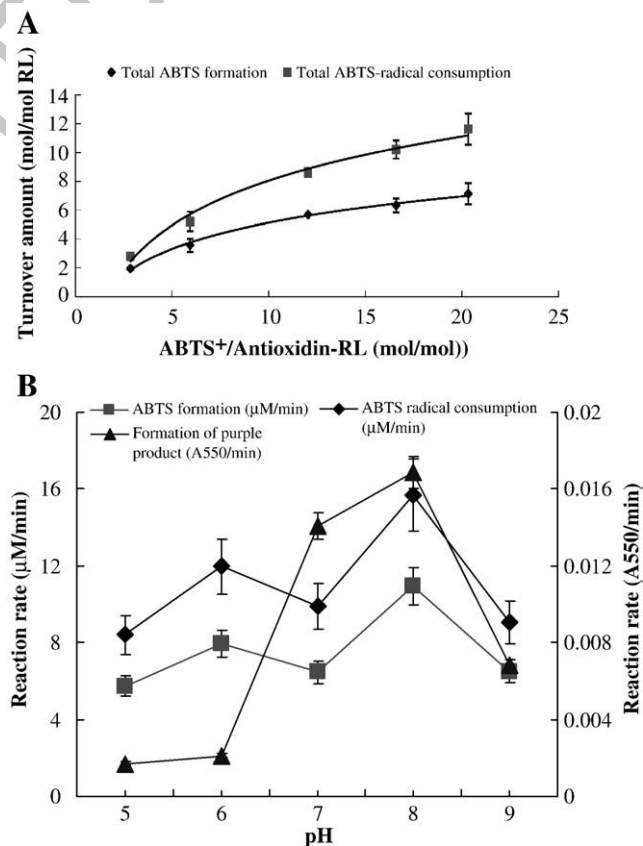
Skin secretions were dissolved with 0.1 M NaCl solution (containing protease inhibitor cocktail; Sigma). Peptide purification from the skin secretions was performed using a Sephadex G-50 (Superfine,  $2.6 \times 100$  cm; Amersham Biosciences) gel filtration column followed by  $C_{18}$  reverse-phase high-performance liquid chromatography (RP-HPLC; Hypersil BDS  $C_{18}$ ,  $30 \times 0.46$  cm) as illustrated in [Supplementary Fig. S1](#). All the purifications were traced by antioxidant testing. The purified antioxidant peptide was named antioxidant-RL.

### Structural analysis

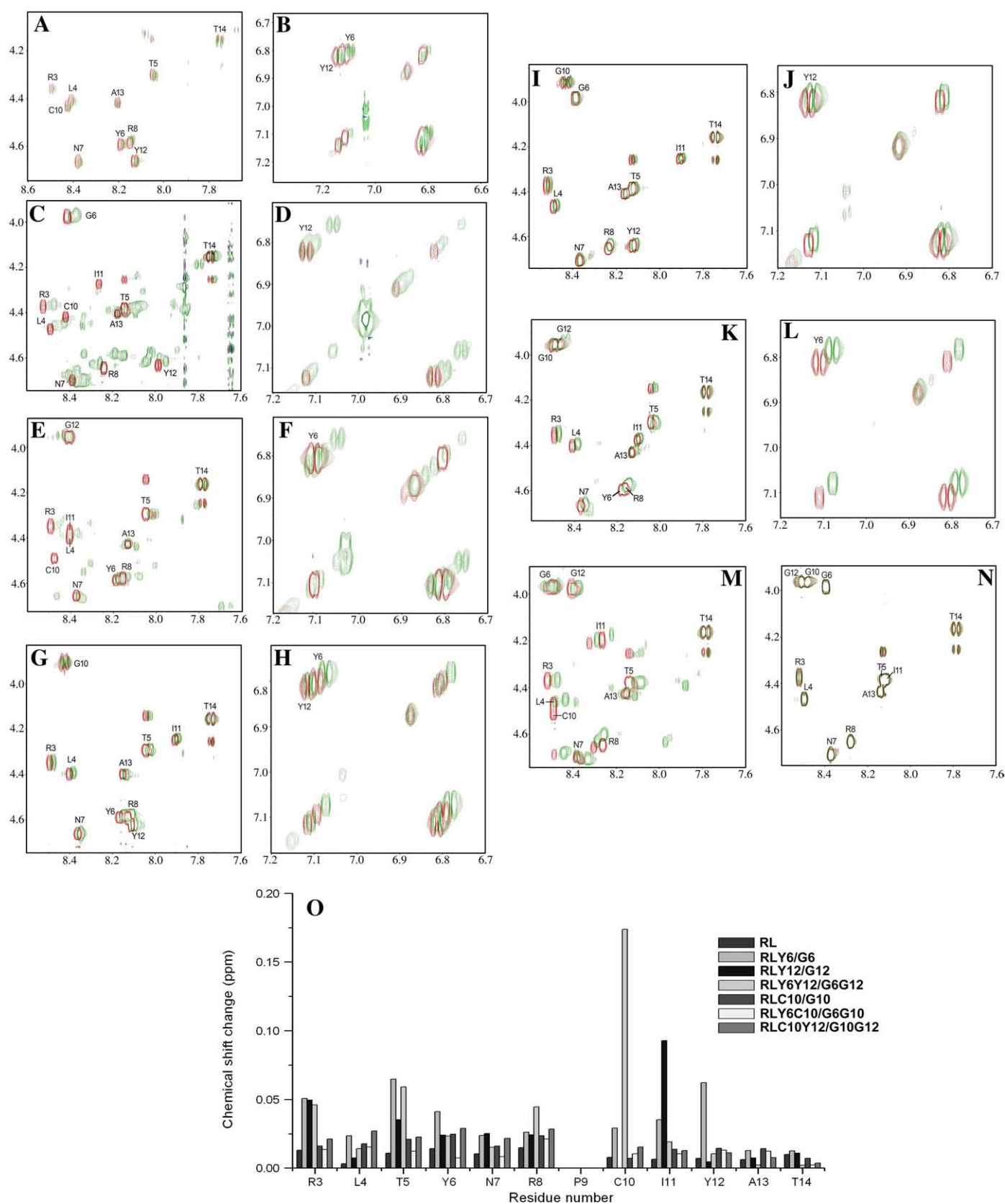
Complete peptide sequencing was undertaken by Edman degradation on an Applied Biosystems pulsed liquid-phase sequencer, Model 491. The fractions with antioxidant activities from RP-HPLC were placed in a MALDI plate (Kratos Analytical). Mass fingerprints were obtained using a matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF-MS), AXIMA CFR



**Fig. 2.** ABTS free radical scavenging kinetics of antioxidant-RL and BHT. Potassium persulfate was added to a 7 mM ABTS solution in water to a final concentration of 3 mM, allowing at least 5 h for the reaction. The stock solution was diluted 75 times with PBS. 3 μM antioxidant-RL or BHT was added for each experiment, and the reaction was monitored according to the absorbance change at (A) 415, (B) 340, and (C) 550 nm. ARL, antioxidant-RL. Each point represents the mean of triplicates  $\pm$  SD. The difference in the antioxidant properties of antioxidant-RL compared to BHT are statistically significant ( $P < 0.01$ ).

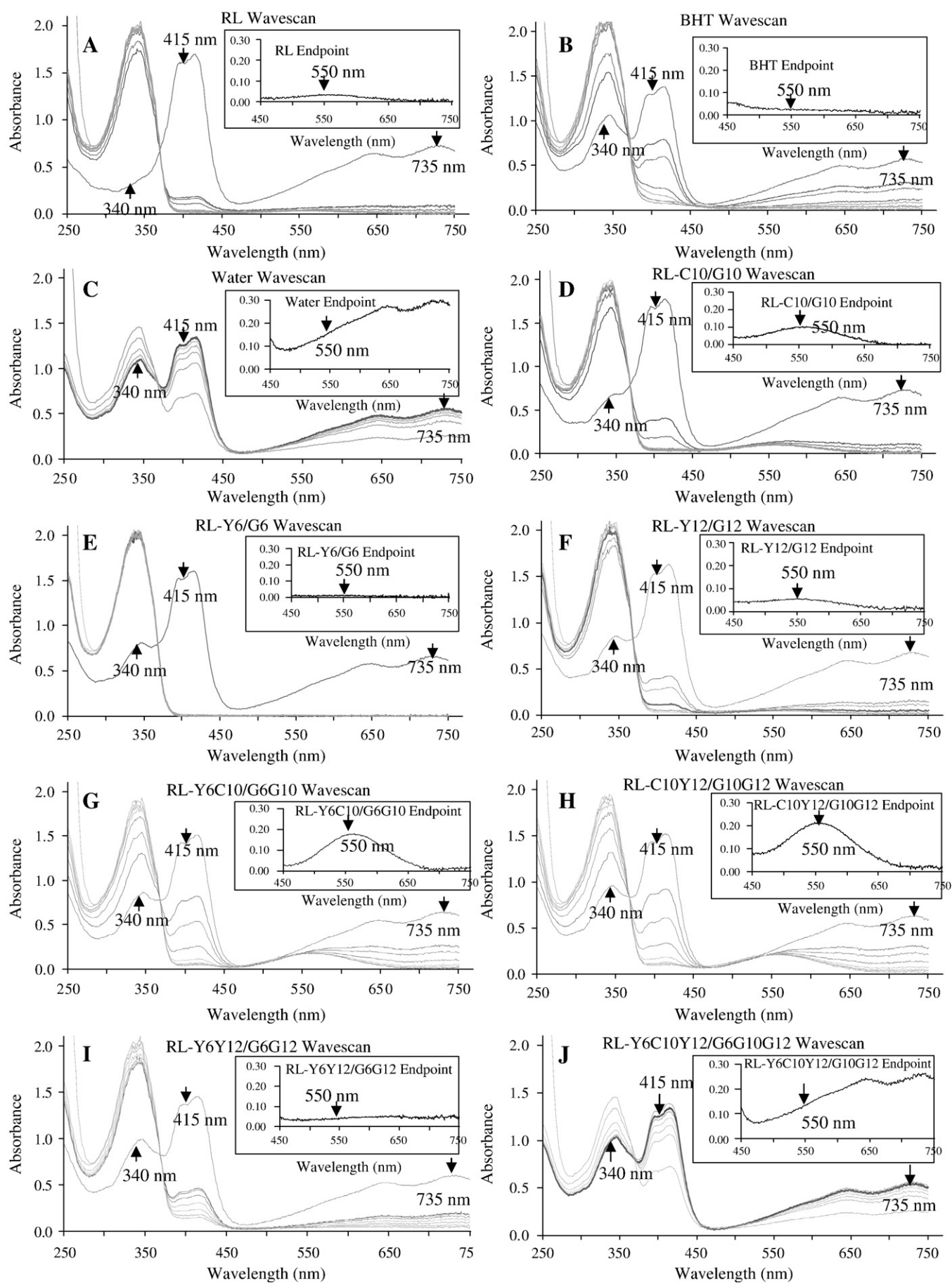


**Fig. 3.** (A) Antioxidin-RL (final concentration of 3 μM) was added to solutions with various concentrations of ABTS radical and the turnover numbers of ABTS and ABTS radical were calculated. (B) Antioxidin-RL (final concentration of 3 μM) was added to solutions with a pH of 5, 6, 7, 8, and 9. Reaction rates during the first minute were calculated. RL, antioxidant-RL. Each point represents the mean of triplicates  $\pm$  SD.



Q1

**Fig. 4.** Superposition of TOCSY spectra of RP1 and its mutants free (red) and in the complex with ABTS radicals (green). Backbone HN–H<sub>α</sub> and side-chain aromatic regions of (A, B) RL, (C, D) RL-Y6/G6, (E, F) RL-Y12/G12, (G, H) RL-C10/G10, (I, J) RL-Y6C10/G6G10, (K, L) RL-C10Y12/G10G12, (M) RL-Y6Y12/G6G12, and (N) RL-Y6C10Y12/G6G10G12. (O) Average chemical shift changes of HN and HA versus residue number in RL and six mutants. RL, antioxidant-RL.



(Kratos Analytical), in positive-ion and linear mode. The specific parameters were as follows: ion acceleration voltage was 20 kV, accumulating time of single scanning was 50 s, polypeptide mass standard (Kratos Analytical) served as external standard. The accuracy of mass determinations was within 0.1%.

#### 114 cDNA synthesis

115 Total RNA was extracted from the dorsal skin of a single amphibian  
116 using TRIzol (Life Technologies). cDNA was synthesized by SMART  
117 techniques using a SMART PCR cDNA synthesis kit (Clontech, Palo  
118 Alto, CA, USA). The first strand was synthesized using cDNA 3' SMART  
119 CDS Primer II A, 5'-AAGCAGTGGTATCAACGCAGAGTACT(30)N-1N-3'  
120 (N = A, C, G, or T; N-1 = A, G, or C), and SMART II An oligonucleotide,  
121 5'-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3'. The second strand  
122 was amplified using Advantage polymerase by 5' PCR Primer II A, 5'-  
123 AAGCAGTGGTATCAACGCAGAGT-3'.

#### 124 cDNA cloning

125 The cDNA synthesized by SMART techniques was used as  
126 template for PCR to screen the cDNA encoding antioxidant peptide.  
127 The oligonucleotide primer 5'-GC(A/T/C/G)ATG(A/C/G)(A/T/C/G)  
128 CT(A/T/C/G)AC(A/T/C/G)TA(T/C)AA(T/C)-3' in the antisense di-  
129 rection was designed according to the amino acid sequences  
130 determined by Edman degradation. Primer II A as mentioned  
131 under cDNA synthesis was used as the sense-direction primer.  
132 The DNA polymerase was Advantage polymerase from Clontech. The  
133 PCR conditions were 2 min at 94°C, followed by 30 cycles of 10 s at  
134 92°C, 30 s at 50°C, and 40 s at 72°C. Finally, the PCR products were  
135 cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA).  
136 DNA sequencing was performed on an Applied Biosystems DNA  
137 sequencer, Model ABI PRISM 377.

#### 138 Free radical scavenging test

139 A stock solution of ABTS radical (ABTS<sup>+</sup>) was prepared by  
140 incubating 2.8 mM potassium persulfate with 7 mM ABTS in water,  
141 allowing at least 5 h for the reaction in the dark, and used within  
142 24 h. The stock solution was diluted 125 times with phosphate-  
143 buffered saline (PBS; 10 mM sodium phosphate, pH 7.4, 120 mM  
144 NaCl, 3 mM KCl) to get a standard solution containing about 36 μM  
145 ABTS<sup>+</sup> and 18 μM ABTS [13]. In some experiments, PBS was added  
146 according to the various ABTS<sup>+</sup> concentrations needed. The exact  
147 concentrations of ABTS and ABTS<sup>+</sup> were determined by  
148  $\epsilon_{340} = 4.8 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$  and  $\epsilon_{415} = 3.6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ , respec-  
149 tively [14]. The absorbance values at 550 nm were corrected using  
150 the following formula:  $A_{550} = 0.5 \times (A_{550 \text{ observed}} - (0.403 \times A_{735})) +$   
151  $0.5 \times (A_{550 \text{ observed}} - (0.163 \times A_{415}))$  [15]. Antioxidin-RL and positive  
152 control butylated hydroxytoluene (BHT) [16,17] dissolved in water  
153 and methanol, respectively, with different final concentrations, were  
154 added and solvents of the same volume were used as negative  
155 controls.

156 The reaction was followed by monitoring the absorbance changes  
157 of ABTS and ABTS<sup>+</sup>. End-point scanning of the reaction products was  
158 done immediately after adding NaN<sub>3</sub> to a final concentration of 60 mM  
159 to reduce the remaining ABTS radicals [18]. Initial reaction rates and  
160  $K_m$  and  $V_{\text{max}}$  during the first 8 s and first 1 min of the reaction were  
161 obtained by analyzing the absorbance values detected by an Ultrospec  
162 2100 Pro UV/visible spectrophotometer (Amersham Bioscience) using  
163 the software Swift II Reaction Kinetics.

#### Determination of reducing power

164

Using BHT and  $\alpha$ -tocopherol dissolved in methanol as positive  
controls, the reductive potential was determined according to the  
method described by Oyaizu [19]. Briefly, 1 ml of samples of different  
concentrations was mixed with phosphate buffer (2.5 ml, 0.2 M, pH  
6.6) and potassium ferricyanide (2.5 ml, 1% w/v). The mixture was  
incubated at 50°C for 20 min. Trichloroacetic acid (10%, w/v, 2.5 ml)  
was added to the mixture, which was then centrifuged at 3000 rpm  
for 10 min. The upper layer of solution (2.5 ml) was mixed with 2.5 ml  
of distilled water and ferric chloride (0.1% w/v), and the absorbance  
was monitored at 700 nm.

#### pH studies

175

pH studies were done by dissolving antioxidant-RL into 20 mM  
sodium acetate, pH 5.0; 20 mM sodium phosphate, pH 6.0, 7.0, or 8.0;  
or 20 mM glycine-OH, pH 9.0, at the final concentration of 3 μM.  
Reaction rates during the first minute were calculated.

#### Nuclear magnetic resonance (NMR)

180

The NMR sample of free peptide was prepared by dissolving 1 mg  
of antioxidant-RL or its mutant powder in 550 μl of PBS buffer (90%  
H<sub>2</sub>O/10% D<sub>2</sub>O, pH 5.2). The purple complex was prepared by  
dissolving 0.5 mg of antioxidant-RP1 powder in 550 μl of PBS buffer  
including 1 mM ABTS<sup>+</sup> (pH 5.2). All NMR experiments were  
performed on a Varian Unity INOVA 600 MHz spectrometer equipped  
with four radiofrequency channels, pulse shaping, and z-axis pulsed-  
field gradient capabilities. Two-dimensional phase-sensitive NMR  
spectra, including total correlation spectroscopy (TOCSY; mixing time  
of 75 ms) and nuclear overhauser enhancement spectroscopy  
(NOESY; mixing time of 300 ms), were recorded at 298K. The  
Watergate approach was employed for suppression of the solvent  
peak. Quadrature detection in the F1 dimension was achieved using  
the states-TPPI approach. Data were collected with 256 and 4096  
complex data points in the  $t_1$  and  $t_2$  dimensions, respectively, and  
signals were averaged over 32 transients. Data were processed using  
the program NMRPipe/NMRDraw [20] and analyzed by Sparky. Linear  
prediction in the  $t_1$  dimension was used before the Fourier  
transformation. The average chemical shift changes of backbone  
protons were calculated according to the following formula:

$$\delta_{\text{ave}} = \sqrt{\Delta\delta_{\text{HN}}^2 + \Delta\delta_{\text{HA}}^2}$$

202

#### Alkylation of cysteine

203

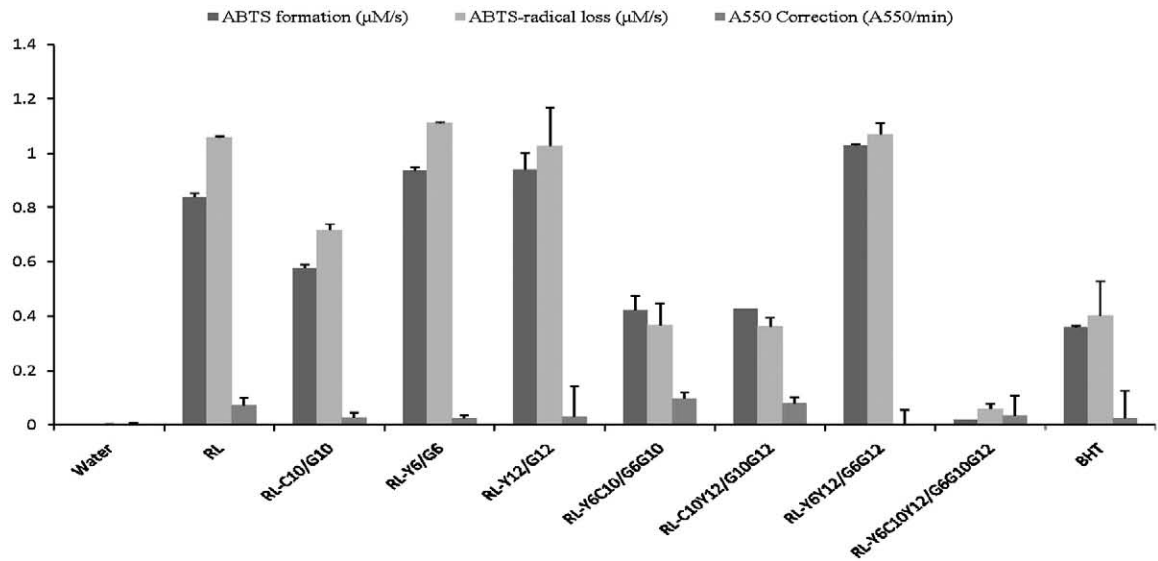
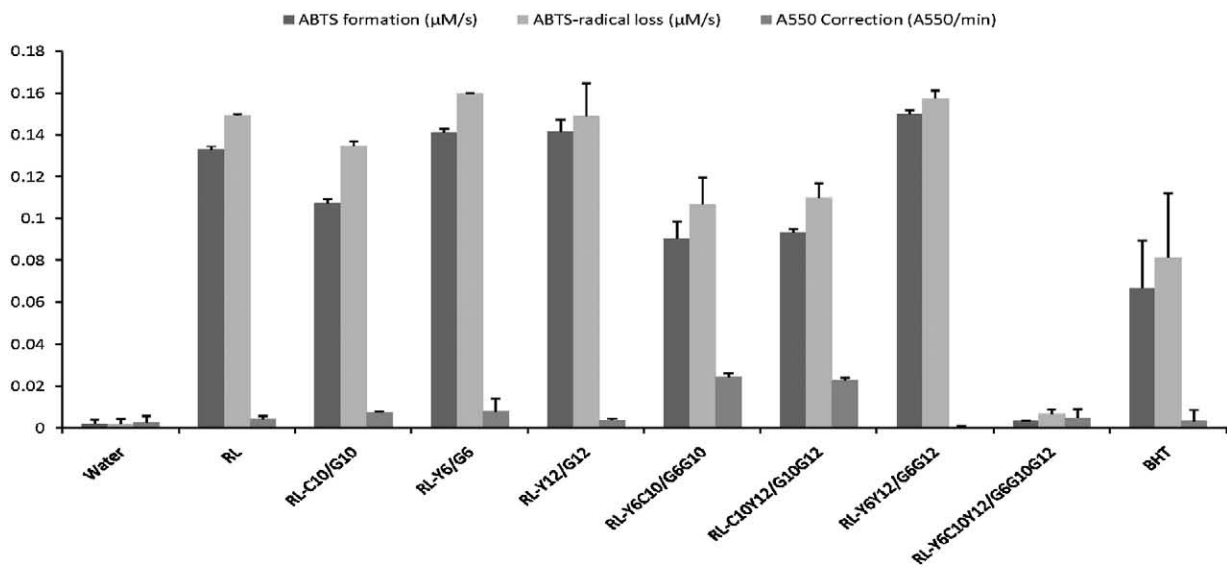
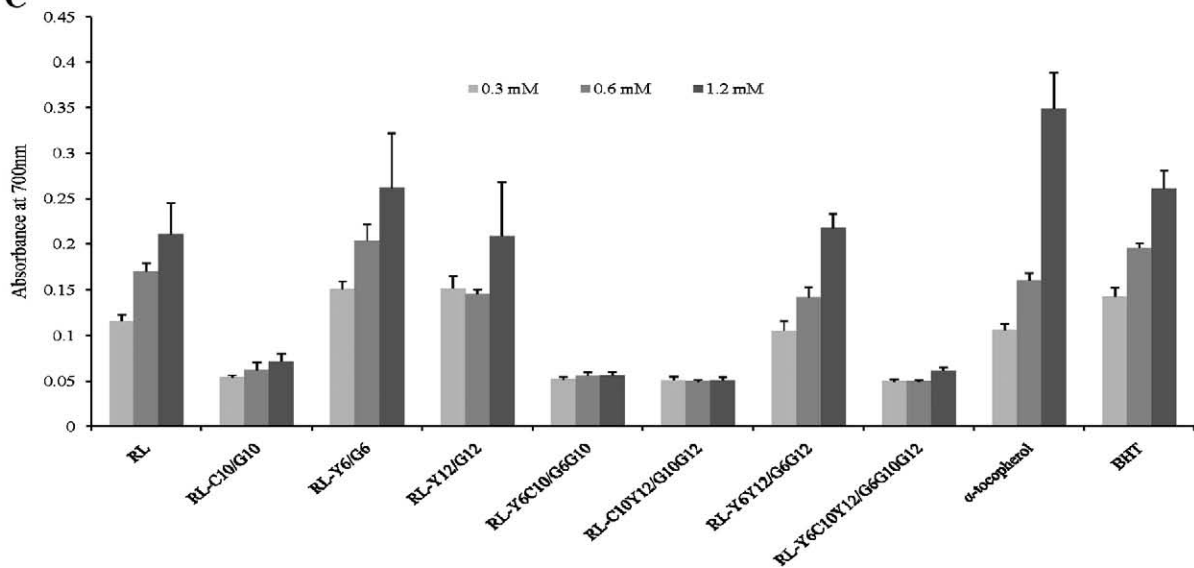
Thiol groups were alkylated according to a previous method [12].  
Peptides (0.18 mM) were incubated with 22 mM iodoacetamide (IAA)  
in PBS (10 mM sodium phosphate, pH 7.4, 120 mM NaCl, 3 mM KCl)  
for 1 h at room temperature in the dark and then desalted by  
Sephadex G-25 Fine (column volume, 30 × 1.6 cm) and eluted with  
2 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 8.5.

#### Synthetic peptides

210

All of the peptides (Supplementary Table S1) used in this work  
were synthesized by AC Scientific (Xi An), Inc. (Xi An, China), with  
confirmed purity higher than 95% by HPLC and MALDI-TOF-MS.

**Fig. 5.** Wave scanning of antioxidant-RL and its mutants during free radical scavenging. (A) Antioxidant-RL, (B) BHT, (C) water, (D) RL-C10/G10, (E) RL-Y6/G6, (F) RL-Y12/G12, (G) RL-Y6C10/G6G10, (H) RL-C10Y12/G10G12, (I) RL-Y6Y12/G6G12, or (J) RL-Y6C10Y12/G6G10G12 was added to standard ABTS<sup>+</sup> solution, to a final concentration of 3 μM, and the absorbance spectrum was read at 0 s, 2 s, 30 s, 2 min, 5 min, 10 min, and 20 min and finally an end-point scan was read immediately after the addition of sodium azide. RL, antioxidant-RL.

**A**Reaction rate of 1  $\mu\text{M}$  sample in  $1.618 \pm 0.2 \mu\text{M}$  ABTS/ $8.981 \pm 0.543 \mu\text{M}$  ABTS-radical solution in the first 8 seconds**B**Reaction rate of 1  $\mu\text{M}$  sample in  $1.618 \pm 0.2 \mu\text{M}$  ABTS/ $8.981 \pm 0.543 \mu\text{M}$  ABTS-radical solution in the first minute**C**

214 **Results**215 *Purification of antioxidant peptide*

216 The crude skin secretions of the frog were fractionated into 4 peaks  
217 by the Sephadex G-50 column as illustrated in [Supplementary Fig.](#)  
218 [S1A](#). The antioxidant activity occurred in peak III. Peak III was applied  
219 to an RP-HPLC column as described under [Materials and methods](#).  
220 [Supplementary Fig. S1B](#) shows that more than 15 peaks were eluted  
221 by the RP-HPLC separation. The eluted peak indicated by an arrow  
222 showed antioxidant activity and it was subjected to further structural  
223 and functional analysis.

224 *Structural characterization*

225 The purified antioxidant peptide was named antioxidant-RL. Its  
226 amino acid sequence is AMRLTYNRPCYAT, determined by automated  
227 Edman degradation. The 14 amino acids include 2 aromatic amino  
228 acid residues (Tyr), 2 basic amino acid residues (Arg), and a single  
229 cysteine residue. The sequence was further confirmed by chemical  
230 synthesis. The synthetic peptide showed the same elution behavior as  
231 its natural counterpart on RP-HPLC and exhibited identical mass  
232 value. By MALDI-TOF-MS analysis, the observed molecular mono-  
233 isotopic mass was 1671.53, which matched well with the theoretical  
234 molecular mass (1671.82). By BLAST search, antioxidant-RL had  
235 significant similarity to the antioxidant peptide of antioxidantins-RP  
236 [12], which were found in the frog *R. pleuraden* ([Fig. 1](#)).

237 *cDNA cloning*

238 As illustrated in [Fig. 1](#), the cDNA sequence encoding antioxidant-RL  
239 was amplified from the skin cDNA library of the frog. It has an open  
240 reading frame that encodes a polypeptide composed of 63 amino  
241 acids, including the mature antioxidant-RL sequence. The amino acid  
242 sequence deduced from the cDNA sequence is identical to the amino  
243 acid sequence determined by Edman degradation. The overall  
244 structure of the precursor encoding antioxidant-RL is composed of a  
245 predicted signal peptide sequence, an N-terminal spacer peptide  
246 region containing several aspartic and glutamic acid residues, and the  
247 mature antioxidant peptide at the C-terminus of the precursor ([Fig.](#)  
248 [1A](#)). In addition, there are two dibasic enzymatic processing sites (-  
249 KR-) flanking the mature peptide.

250 *Free radical scavenging activity*

251 Owing to its relative stability, easy measurement, good reproduc-  
252 ibility, the ABTS<sup>+</sup> radical is commonly used to evaluate antioxidant  
253 capacity [15]. Antioxidant-RL reacted with ABTS<sup>+</sup> and converted the  
254 colored stable free radicals into colorless compounds. The amount of  
255 reduced ABTS<sup>+</sup> could be quantified by measuring a decrease in  
256 absorbance at 415 nm or an increase in absorbance at 340 nm. As  
257 illustrated in [Fig. 2](#), both antioxidant-RL and the positive control BHT  
258 showed strong ABTS<sup>+</sup> free radical scavenging ability. [Fig. 2](#) shows that  
259 the radical scavenging process of antioxidant-RL could be separated  
260 into two parts: a fast reaction phase (0–5 s) and a slow reaction phase  
261 (after 5 s). At the concentration of 3 μM, antioxidant-RL had a stronger  
262 ability to quench ABTS<sup>+</sup> than BHT. Most of the ABTS<sup>+</sup> (>75%) was  
263 reduced after 5 s treatment by antioxidant-RL. There were two end  
264 products found in the antioxidant-RL–ABTS reaction system; they were  
265 reduced ABTS, represented by the 340 nm peak, and a novel peak with  
266 maximal absorbance at 550 nm (*A*<sub>550</sub>) ([Fig. 2C](#)), which gave the  
267 solution a purple color. No product with *A*<sub>550</sub> was found in the BHT–

ABTS reaction system ([Fig. 2C](#)), which indicates that BHT may have a  
radical scavenging mechanism different from that of antioxidant-RL.

[Fig. 3A](#) shows that a higher ratio of ABTS<sup>+</sup>/antioxidant-RL resulted  
in faster ABTS<sup>+</sup> consumption and ABTS formation. The ABTS<sup>+</sup>  
consumption was larger than the ABTS formation. This result  
confirmed further that ABTS<sup>+</sup> consumption by antioxidant-RL does  
not completely result from the reduction of ABTS<sup>+</sup> to ABTS. The ABTS<sup>+</sup>  
consumption also produced another product, such as the purple  
product with *A*<sub>550</sub> as mentioned above. Different pH environments had  
different effects on ABTS<sup>+</sup> consumption and ABTS and *A*<sub>550</sub> formation  
([Fig. 3B](#)). Antioxidant-RL showed the strongest ability for these  
reactions at pH 8.0. We assumed that an amino acid with a side  
chain whose p*K*<sub>a</sub> is about 8 plays an important role in this procedure.  
The only amino acid in antioxidant-RL that matches this is Cys10.

*NMR titration indicated that Tyr6, Cys10, and Tyr12 are crucial residues  
for radical elimination*

[Figs. 4A](#) and [B](#) show a superposition of the backbone HN–H<sub>α</sub> and  
side-chain aromatic regions of TOCSY spectra recorded on the peptide  
free and in complex with ABTS<sup>+</sup>. It is indicated that the residues Tyr6  
and Tyr12 experienced larger chemical shift changes in both HN–H<sub>α</sub> and  
aromatic regions in antioxidant-RL ([Figs. 4A](#) and [B](#)). Seven mutants with  
Tyr or Cys replacement (RL–Y6/G6, RL–Y12/G12, RL–Y6Y12/G6G12,  
RL–C10/G10, RL–Y6C10/G6G10, RL–C10Y12/G10G12, RL–Y6C10Y12/  
G6G10G12) were synthesized and their superpositions of the backbone  
HN–H<sub>α</sub> and side-chain aromatic regions of TOCSY spectra were studied  
by 2D NMR. Resonance assignments of all protons of antioxidant-RL and  
its seven mutants free and in complex with ABTS radical were almost  
fully completed through a combination of TOCSY and NOESY spectra  
([Figs. 4A–N](#)). The assigned chemical shifts are listed in [Supplemental](#)  
[Tables S2–9](#). NOESY spectra of antioxidant-RL and its mutants in both  
forms show few cross peaks, indicating that all the peptides do not  
adopt regular secondary structures.

All the chemical shift changes are plotted versus the residue  
numbers in [Fig. 4O](#). A large chemical shift change of Tyr12 or Tyr6 was  
found in the mutants RL–Y6/G6, RL–Y12/G12, RL–C10/G10, RL–  
Y6C10/G6G10, and RL–C10Y12/G10G12, which could form the purple  
product (*A*<sub>550</sub>) ([Figs. 4C–L](#)). In the mutant RL–Y6Y12/G6G12, without  
the ability to form *A*<sub>550</sub>, the residue Cys10 experienced the largest  
chemical shift changes compared with the other residues ([Fig. 4M](#)).  
Furthermore, the Y6C10Y12/G6G10G12 mutant in which the Tyr's  
and Cys are replaced by glycine displayed no chemical shift change in  
the free and complex forms ([Fig. 4N](#)).

These results indicate that Tyr6, Tyr12, and Cys10 in antioxidant-RL  
are required to form the complex with the ABTS radical. Both Tyr6 and  
Tyr12 play important roles in the formation of the purple product. It  
has been reported that a purple compound was formed via the  
covalent interaction between tyrosyl residues and ABTS<sup>+</sup> [15].  
Therefore, it is suggested that the purple complexes studied herein  
are formed by the covalent linking between Tyr6/Tyr12 and ABTS<sup>+</sup>,  
resulting in significant chemical shift changes for both Tyr6 and Tyr12.  
Meanwhile, Cys10 is not essential in the interaction between Tyr6/  
Tyr12 and ABTS<sup>+</sup>. Furthermore, the current results clearly indicate  
that Cys10 interacts with ABTS<sup>+</sup> in a mechanism that is different from  
that of the interaction between Tyr6/Tyr12 and ABTS<sup>+</sup>.

*Cys10 is responsible for rapid radical scavenging*

The radical scavenging processes of antioxidant-RL, its mutants, BHT,  
and water were monitored at various times as illustrated in [Fig. 5](#).  
Overlaid scans of the solution showed a decrease in the ABTS<sup>+</sup>-specific

**Fig. 6.** Effects of Tyr6, Cys10, and Tyr12 replacement on radical scavenging kinetics. Water, antioxidant-RL, its mutants, or BHT was added to standard ABTS<sup>+</sup> solution. Their reaction rates during (A) the first 8 s and (B) the first minute were measured. RL, antioxidant-RL. (C) The reductive potentials of antioxidant-RL, its mutants, BHT, and α-tocopherol. Each point represents the mean of triplicates ± SD.

415 and 735 nm peaks and a concomitant increase in the ABTS-specific peak at 340 nm. Fig. 5 directly shows that the radical scavenging process is a two-phase reaction. An initial faster phase over several seconds was followed by a second slower phase that was still ongoing after 20 min. Although ABTS<sup>+</sup> displayed a slender autoscavenging activity as found in the case of blank control (water, Fig. 5C), antioxidant-RL and its mutants could significantly catalyze this process (Figs. 5A, D–F, I, and 6). The catalysis rate is much faster than that of the commercial antioxidant factor (BHT; Fig. 5B). Other mutants (RL-Y6C10/G6G10, RL-C10Y12/G10G12), which have no Cys and only one Tyr, have much slower radical scavenging rates but they have a stronger ability to form the purple product with maximal absorbance at 550 nm ( $A_{550}$ ) than their native counterpart (antioxidin-RL; Figs. 5G, H, and 6). The mutant RL-Y6C10Y12/G6G10G12 completely lost radical scavenging ability (Fig. 5J), as did the blank control (Fig. 5C). The results indicate that Cys has an essential role for fast radical scavenging rate and that Tyr's also take part in the radical scavenging by forming  $A_{550}$ .

To compare the radical scavenging efficiencies of antioxidant-RL and its mutants, we measured their reaction rates during the first 8 s and the first minute (Fig. 6). The ABTS<sup>+</sup> consumption rates of antioxidant-RL, Y6/G6, Y12/G12, and RL-Y6Y12/G6G12 were 1.1–12  $\mu\text{M/s}$  in the first 8 s versus 0.15–0.16  $\mu\text{M/s}$  in the first minute. Antioxidin-RL had a much faster rate (approx 1.06  $\mu\text{M/s}$ ) of ABTS<sup>+</sup> consumption and ABTS formation than BHT (approx 0.4  $\mu\text{M/s}$ ). Three mutants, including RL-C10/G10, RL-Y6C10/G6G10, and RL-C10Y12/G10G12, which lack Cys, had much slower ABTS<sup>+</sup> consumption rate than antioxidant-RL. In addition, the mutant RL-Y6Y16/G6G12, which lacks Tyr, had a similar ABTS<sup>+</sup> consumption rate compared with antioxidant-RL, indicating that Tyr did not play an important role in the fast phase of ABTS radical scavenging. The reducing powers of antioxidant-RL and its mutants are shown in Fig. 6C. Antioxidin-RL and its mutants containing Cys showed strong reducing powers, whereas the mutants containing no Cys lost reducing power. All these results indicate that Cys plays a key role in the rapid ABTS<sup>+</sup> consumption.

Free Cys has a thiol group that can donate an electron to a radical. Antioxidin-RL alkylated ( $C_{10}$  alkylation) by IAA had a significant, but incomplete, decrease in the reduction rate of ABTS radical, as did the Cys-replacement mutant RL-C10/G10 (data not shown). This suggests that the  $C_{10}$  thiol group is responsible for the rapid reduction of ABTS<sup>+</sup>.

## Discussion

Amphibian skins are pharmacologically active organs displaying multiple defensive functions essential for survival. Their skin glands have rich secretions containing bioactive molecules with a large amount of structural and functional diversity [21]. Several families of amphibian skin peptides, including bombesins [22,23], protease inhibitors [24,25], antimicrobial peptides [26–31], tachykinins [32], cholecystokinin [33], bradykinins [34], and gene-encoded neurotoxin, have been identified [35]. In contrast to a large amount of investigation related to the defense against biological injuries, few investigations against nonbiological injuries have been reported. Compared with other vertebrates, amphibians have to face more nonbiological injuries because of their fragile and naked skins. ROS injury may be one of the most unavoidable.

One peptide antioxidant system has been found in the skin of an amphibian species, *R. pleuraden*, in our previous work, but it is still uncertain whether peptide antioxidant systems are a common mechanism to prevent ROS injury in amphibians. In addition, the rapid radical scavenging mechanism of amphibian skin antioxidant peptide is unknown. Here in this report, antioxidant-RL from the skin secretions of *R. (O.) livida* shows obvious sequence similarity to the antioxidant peptides found in the skin secretions of *R. pleuraden* [12]. This provides support that a peptide antioxidant system may be a common protective strategy to avoid ROS injury in amphibian skins.

It is essential that radicals in amphibian skins can be scavenged rapidly. Antioxidin-RL could swiftly (<5 s) reduce ABTS<sup>+</sup> and combine with ABTS derivatives. This activity is superstoichiometric because 1 molecule of antioxidant-RL is capable of scavenging 10–12 molecules of ABTS<sup>+</sup> (Fig. 3A). Amino acid replacement experiments indicate that Cys10 in antioxidant-RL has a crucial role in the rapid radical scavenging (Figs. 5 and 6). Akerström et al. have reported that lipocalin  $\alpha$ -microglobulin (LMG) has catalytic reductase prosperities, involving the unpaired thiol group of Cys34 in the reactive center [15]. Cys10 in antioxidant-RL has the same unpaired thiol group, which possibly has the same function as that of Cys34 in LMG.

Fig. 3A shows that the reaction rate between antioxidant-RL and ABTS<sup>+</sup> displayed saturation kinetics. ABTS<sup>+</sup> was bonded to antioxidant-RL before its reduction. The same situation was found in LMG [15]. Only 6–7 mol ABTS was formed, although 10–12 mol ABTS<sup>+</sup> was consumed per antioxidant-RL molecule (Fig. 3A), indicating that 4–6 ABTS<sup>+</sup> may bind to each molecule of antioxidant-RL. A novel purple product with maximal absorbance at 550 nm was found in this reactive system. It has been proven that phenols can react with ABTS<sup>+</sup> to form purple compounds that show a maximal absorbance around 550 nm [13]. Only Tyr in this peptide contains a phenol side chain. Two Tyr residues ( $Y_6$  and  $Y_{12}$ ) in antioxidant-RL possibly react with ABTS<sup>+</sup> to form the antioxidant-RL–ABTS purple product.

During the fast phase of the antioxidant-RL–ABTS<sup>+</sup> reaction, there are considerable rates of ABTS<sup>+</sup> consumption (1.06  $\mu\text{M/s}$ ) and ABTS formation (0.86  $\mu\text{M/s}$ ) (Fig. 6). In the investigation of LMG, these rates are 0.3 and 0.2  $\mu\text{M/s}$ , respectively [15], suggesting that antioxidant-RL has a much faster radical scavenging rate than LMG.

In summary, the current work suggests that: (1) rapid radical scavenging is a unique character of amphibian skin antioxidant peptides; (2) Cys is the determinant residue for rapid radical scavenging; and (3) antioxidant peptides in frog skins are fast-acting antioxidants containing the ability to rapidly and constantly eliminate free radicals and thus protect skins from ROS injury in real time.

## Acknowledgments

This work was supported by the Chinese National Natural Science Foundation (30830021), Chinese Academy of Sciences (KSCX2-YW-G-024), and Ministry of Science (2008AA02Z133, 2010CB529801/05) and by Yunnan Province (0803461101).

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.freeradbiomed.2010.01.036.

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