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# Antimicrobial peptides from the skin of the Asian frog, *Odorrana jingdongensis*: *De novo* sequencing and analysis of tandem mass spectrometry data

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ABSTRACT

Eight intact antimicrobial peptides were identified from the skin of *Odorrana jingdongensis* by *de novo* sequencing following low energy ESI CID Q-TOF MS/MS in positive-mode with the help of Edman degradation and structural similarity analysis. We devised exact mass measurements to discriminate the K/Q amino acid residue in the peptides between 2.0 kDa to 3.8 kDa. Moreover, the cleavage at the C–S bond at the side chain of Met was observed in all the spectra of the peptides containing Met residue. And we found unusual cleavages within the intramolecular disulfide loop with high frequency. Our data revealed that the cleavage pathways are significantly different from those reported previously which are similar to the cycle peptide cleavage mode followed by the secondary cleavage at the C–S bond on oxidized Cys. Thus, our results highly suggest that ion series generated from the cleavages within the intramolecular disulfide loop should be considered in both the top-down sequencing and the disulfide bridge location with the presence of a relatively high intensity of $MH^+$–28 ion marker. Furthermore, our activity data implied that different AMPs may use different strategies to kill microbes.



## 1. Introduction

Antimicrobial peptides (AMPs) are important reagents that can kill microbes, alternative to conventional antibiotics. The AMP field is advancing rapidly in response to the demand of the novel antimicrobial agents for microbes resistant to antibiotics. A large number of AMPs have been identified from almost all the groups of organisms, including bacteria, fungi, plants and animals [1–3]. The Antimicrobial Peptide Database (APD, updated in June 2012, http://aps.unmc.edu/AP/) has collected 1992 AMPs with the validated antimicrobial activities, and the majority of them contain less than 100 amino acid residues [2]. In Collection of Anti-Microbial Peptides database (CAMP, updated on 29th April 2010, http://www.bicnirrh.res.in/antimicrobial/), there are 2867 validated AMPs and 1153 predicted AMPs with antimicrobial sequences [4]. Many AMPs have been identified from skin [5], brain [6] and stomach [7] of Anurans (frogs and toads). Skin secretions from Anurans contain a wide range of compounds with the biological activity. Peptides are the major components of the secretions in many species, and the predominant peptides are often those with antimicrobial activity [5,8].

A large number of studies have been conducted on AMPs from the skins of *Odorrana* genus (Anura: Ranidae), which comprises 53 species [9]. AMPs sequences (identified from venom samples or predicted from mRNA sequences) from 14 species of this genus such as *Odorrana andersonii* [10], *Odorrana grahami* [11–16], *Odorrana hainanensis*, *Odorrana hejiangensis* [10,17], *Odorrana hosii* [18], *Odorrana ishikawae*

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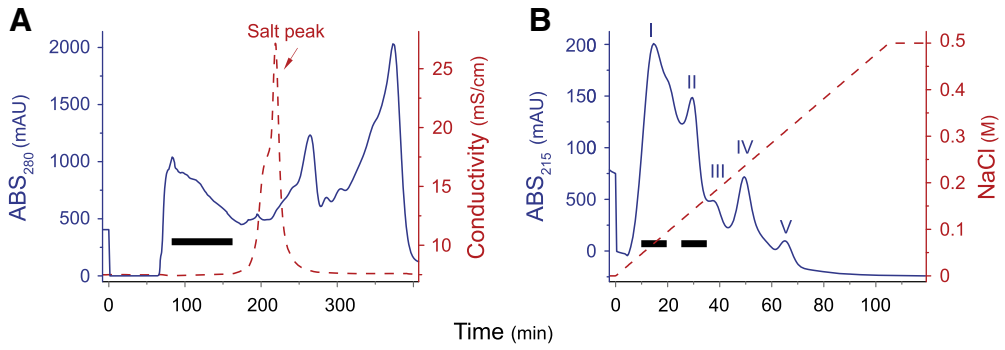


**Fig. 1 – Gel filtration chromatography and successively cation exchange chromatography of the *O. jingdongensis* skin venom sample. The antimicrobial fractions, which were eluted before the salt peak (arrowhead), from HiPrep 16/60 Sephacryl S100 HR column (A) were pooled, diluted to 3.5 times and loaded on XK 16/20 CM Sepharose FF column. Five peaks were separated, and peak I and peak II displayed antimicrobial activity (B). The bar indicates where the antimicrobial fractions were collected. The dashed line shows the conductivity (A) and concentration of NaCl in the eluting solvent (B).**

[19,20], *Odorrana livida* [10,21], *Odorrana macrotympana* [10], *Odorrana margaretae* [10], *Odorrana rotodora* [10], *Odorrana schmackeri* [10,17,22–24], *Odorrana tiannanensis* [10,25], *Odorrana versabilis* [10,17,23,24,26] and *Odorrana wuchuanensis* [10] have been reported or are available in the databases of NCBI, UniPort or ADP. *Odorrana* species frogs might represent the most extreme AMP diversity in nature. Among all those AMPs sequences reported in the genus *Odorrana*, there are at least 800 different peptides, of which 66 AMPs containing 28 different sequences are found in two or more species [10,14]. Furthermore, there are two AMPs in this genus even distributed in other genus [10,17].

Although the theoretical mass accuracy (normally within ±5 ppm [27]) of Q-TOF MS/MS is dedicated enough to discriminate the quasi-isobaric K/Q residue to the peptides over 3.6 kDa [28], the distinguishment of K/Q is still difficult in the practice of *de novo* sequencing to the peptides over 1.5 kDa. As the molecular mass of the peptides increases, a large number of fragmentation pathways lead to a concomitant decrease in signal-to-noise ratio of the fragment ion peaks, which is the main reason that the observed second stage mass accuracy frequently exceeds the theoretical value [29]. Nonetheless, it could be alleviated by introducing the modified amino acid residues, such as acetylated K [30] or by employing the higher accuracy and resolution instruments, such as Orbitrap or FT-ICR MS [29]. Here, we described exact mass measurements that use low energy CID ESI Q-TOF MS/MS in positive-ion mode mass spectrometry, which is typically applied to *de novo* sequencing of peptides, to elucidate the mature AMPs components in crude venom sample from *Odorrana jingdongensis*. Ambiguity I/L and partial sequence order were elucidated by Edman degradation or/and structural similarity analysis to known sequence in other species of *Odorrana* genus. The sequences of eight intact AMPs with molecular mass range between 2.0 and 3.8 kDa were identified. The unusual cleavages at the internal amide bonds and at the C–S bonds on the oxidized Cys within the intramolecular disulfide loop were observed with high frequency. And the cleavage pathways are significantly different from those reported previously.

## 2. Material and methods

### 2.1. *Tissue collection and extraction*

The animal protocols were approved by the Animal Care and Use Committee of the Chengdu Institute of Biology, Chinese Academy of Sciences. The specimens of the adult frog *O. jingdongensis* (n=9; 5 males, 4 females; average weight 30.1 g) were collected under permit in July, 2009 in Wuliang mountain, Jingdong county, Yunnan province, China. The animals were anesthetized and sacrificed by using chlorobutanol, and the skin was collected immediately. After air drying, the skin was sealed and transported to the lab at 4 °C. The dried skin was cut into small pieces with scissors and immersed in ethanol/0.7 M HCl (3:1 v/v, 200 mL) at 4 °C. The suspension was stirred for 3 h using a magnetic agitator at room temperature. Ethanol and HCl were removed from the solution using a rotary evaporator (60 °C, 8 min). After adding 200 mL deionized water, the suspension was centrifuged (6000 *g* for 20 min at 4 °C), and the supernatant was freeze-dried and stored at −70 °C.

### 2.2. *Antimicrobial, hemolytic, and bacterial killing assays*

The minimum inhibitory concentrations (MICs) of gram-positive bacterial strain *Staphylococcus aureus* (ATCC 25923) and its resistant strain methicillin-resistant *S. aureus* (MRSA, CIB 85462); gram-negative bacterial strain *Escherichia coli* (ATCC 25922) and its resistant strain extended-spectrum ß-lactamases *E. coli* (ESBL, CIB 84492); fungal strain *Candida albicans* (CIB 45437) were determined in this work. The MIC was measured by the standard microdilution methods [31,32] in 96-well microtiter cell-culture plates as the lowest concentration of peptide without the visible growth. In order to monitor the validity and reproducibility of the assays, the incubations were carried