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A novel antimicrobial peptide from amphibian skin secretions of *Odorrana grahami*

Qiaolin Che [a,1], Yu Zhou [a,1], Hailong Yang [b], Jianxu Li [b,c], Xueqing Xu [b,c], Ren Lai [a,b,*]

[a] Key Laboratory of Microbiological Engineering of Agricultural Environment, Ministry of Agriculture, Life Sciences College of Nanjing Agricultural University, Nanjing, Jiangsu 210095, China

[b] Biotoxin Units of Key Laboratory of Animal Models and Human Disease Mechanisms, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, 650223 Yunnan, China

[c] Graduate School of the Chinese Academy of Sciences, Beijing 100009, China

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ABSTRACT

A novel antimicrobial peptide named odorranain-NR was identified from skin secretions of the diskless odorous frog, *Odorrana grahami*. It is composed of 23 amino acids with an amino acid sequence of GLLSGILGAGKHIVCGLTGCAKA. Odorranain-NR was classified into a novel family of antimicrobial peptide although it shared similarity with amphibian antimicrobial peptide family of nigrocin. Odorranain-NR has an unusual intramolecular disulfide-bridged hexapeptide segment that is different from the intramolecular disulfide-bridged heptapeptide segment at the C-terminal end of nigrocins. Furthermore, the -AKA fragment at the C-terminal of odorranain-NR is also different from nigrocins. Three different cDNAs encoding two odorranain-NR precursors and only one mature odorranain-NR was cloned from the cDNA library of the skin of *O. grahami*. This peptide showed antimicrobial activities against tested microorganisms except *Escherichia coli* (ATCC25922). Its antimicrobial mechanisms were investigated by transmission electron microcopy. odorranain-NR exerted its antimicrobial functions by various means depending on different microorganisms.

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1. Introduction

In recent years, it has been widely recognized that many organisms use peptides as part of their host defense systems against invasion of microorganisms [3–5,9,11–13,16–18,20,21]. Amphibian skin glands are rich resources for antimicrobial peptides. Granular glands in the skin of anuran amphibians, particularly those belonging to the families Pipidae, Hylidae, Hyperoliidae, Pseudidae, and Ranidae, synthesize and secrete a remarkably diverse array of antimicrobial peptides, 10–50 residues in length, that are released onto the outer layer of the skin to provide an effective and fast-acting defense against harmful microorganisms [1,8]. Extensive studies have been conducted on amphibian antimicrobial peptides of frogs belonging to the genus *Rana*. Members of the *Rana* genus comprise more than 250 species and are distributed worldwide, except for the Polar Regions, southern South America and most of Australia. About 160 antimicrobial peptides have been identified from more than 20 ranid amphibians [1,6,8,11,15,16,19].

The skin of the diskless odorous frog, *Odorrana grahami* is rich in antimicrobial peptides. In our previous report, more than 100 antimicrobial peptides belonging to 30 different families have been identified from skin secretions of *O.*

* *Corresponding author at*: Biotoxin Units of Key Laboratory of Animal Models and Human Disease Mechanisms, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, 650223 Yunnan, China.
E-mail address: rlai72@njau.edu.cn (R. Lai).

[1] Both the authors contributed equally to this work.

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grahami [15]. In this report, we described the isolation, characterization, cDNA cloning and biological activities of a novel antimicrobial peptide with unique intramolecular disulfide-bridged hexapeptide segment from skin secretions of *O. grahami*.

2. Materials and methods

2.1. Collection of frog skin secretions

Adult specimens of *O. grahami* 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. Following 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 toad with 0.1 M NaCl solution (containing 0.01 M EDTA). The collected solutions (500 ml of total volume) were quickly centrifuged and the supernatants were lyophilized.

2.2. Peptide purification

Lyophilized skin secretion sample of *O. grahami* (3.5 g, total $OD_{280\ nm}$ of 1000) was dissolved in 10 ml 0.1 M phosphate buffer, pH 6.0, containing 5 mM EDTA. The sample was applied to a Sephadex G-50 (Superfine, Amersham Biosciences, 2.6 cm × 100 cm) gel filtration column equilibrated with 0.1 M phosphate buffer, pH 6.0. Elution was performed with the same buffer, collecting fractions of 3.0 ml. The absorbance of the eluate was monitored at 280 nm. The antimicrobial activities of fractions were determined as indicated below. The protein peak containing antimicrobial activity was pooled (30 ml) lyophilized, and re-suspended in 2 ml 0.1 M phosphate buffer solution, pH 6.0, and purified further by C_{18} reverse phase high-performance liquid chromatography (RP-HPLC, Hypersil BDS C_{18}, 30 cm × 0.46 cm) column as illustrated in Fig. 1.

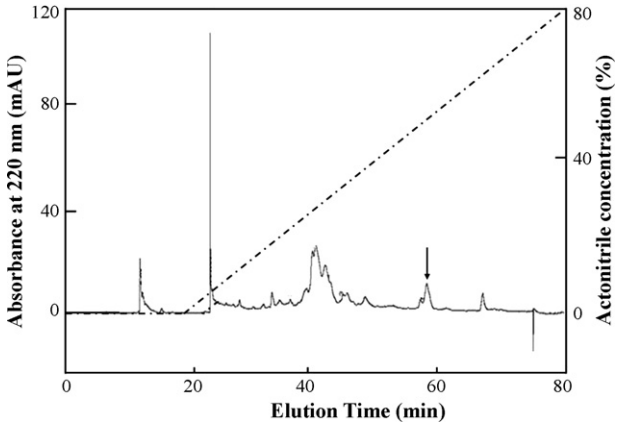

Fig. 1 – Purification of odorranin-NR from *Odorrana grahami* skin secretions. The peak with antimicrobial activity from Sephadex G-50 gel filtration was further purified on a Hypersil BDS C_{18} RP-HPLC column (30 cm × 0.5 cm) equilibrated with 0.1% (v/v) trifluoroacetic acid/water. Elution was performed with the indicated gradient of acetonitrile at a flow rate of 0.7 ml/min, and fractions were tested for antimicrobial activity. The odorranin-NR peak is indicated by an arrow in the figure.

2.3. Structural analysis

Complete peptide sequencing was undertaken by Edman degradation on an Applied Biosystems pulsed liquid-phase sequencer, model 491. Electrospray ionization (ESI) tandem mass spectrometry was performed using an Applied Biosystems API QSTAR XL quadrupole orthogonal time of-flight (QqToF)-MS/MS hybrid instrument equipped with a syringe pump capable of analyzing a mass range of *m/z* 5–10 000, with a resolution of 10 000 in the positive ion mode. ESI was performed with a Turbo Ionspray source operated at 4.5 kV at a temperature of 200 °C. The tested sample was dissolved in acetonitrile/water (1:1) to achieve a concentration of 0.1 mmol/ml. Calibration of the ToF analyzer was performed using a Mass Spectrometer Standard Kit (Applied Biosystems).

2.4. SMART cDNA synthesis

Total RNA was extracted using TRIzol (Life Technologies Ltd.) from the skin of single *O. grahami*. cDNA was synthesized by SMART™ techniques by using a SMART™ PCR cDNA synthesis kit (Clontech, Palo Alto, CA). The first strand was synthesized by using cDNA 3′ SMART CDS Primer II A, 5′-AAGCAGTGGTATCAACGCAGAGTACT (30) N-1N-3′ (N = A, C, G or T; N-1 = A, G or C), and SMART II An oligonucleotide, 5′-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3′. The second strand was amplified using Advantage polymerase by 5′ PCR primer II A, 5′-AAGCAGTGGTATCAACGCAGAGT-3′.

2.5. Screening of cDNA encoding antimicrobial peptide

The cDNA synthesized by SMART™ techniques was used as template for PCR to screen the cDNAs encoding antimicrobial peptides. Two oligonucleotide primers, S_1 (5′-CCAAA(G/C)ATGTTCACC(T/A)TGAAGAAA(T/C)-3′), in the sense direction, a specific primer designed according to the signal peptide sequences of antimicrobial peptides from ranid frogs [15,19] and primer II A as mentioned in Section 2.4 in the antisense direction were used in PCR reactions. The DNA polymerase was Advantage polymerase from Clontech (Palo Alto, CA). The PCR conditions were: 2 min at 94 °C, followed by 30 cycles of 10 s at 92 °C, 30 s at 50 °C, 40 s at 72 °C. Finally, the PCR products were cloned into pGEM®-T Easy vector (Promega, Madison, WI). DNA sequencing was performed on an Applied Biosystems DNA sequencer, model ABI PRISM 377.

2.6. Microorganism strains and growth conditions

Gram-positive bacterium *Staphylococcus aureus* (ATCC2592), Gram-negative bacteria *Escherichia coli* (ATCC25922), *Bacillus dysenteriae* and fungus *Candida albicans* (ATCC2002) were obtained from Kunming Medical College and were first grown in LB (Luria-Bertani) broth or yeast extract-peptone-dextrose broth as our previous report [19].

2.7. Susceptibility testing

Minimal inhibitory concentration (MIC) of the antimicrobial peptide against microorganisms was determined using broth dilution determination. The peptide was prepared as a stock solution in H_2O at a series of concentration. 890 μl broth, 100 μl bacterial suspension (10^8 CFU/ml) and 10 μl test peptides were put together in the test tube and shaken at 37 °C for 24 h. A tube with a corresponding volume of H_2O was used as control. The MIC was defined as the lowest concentration of test peptides inhibiting microorganism's growth.

2.8. Hemolysis assays

Hemolysis assays were undertaken using rabbit red blood cells in liquid medium as reported [2]. Serial dilutions of the peptide were used, and after incubation at 37 °C for 30 min, the cells were centrifuged and the absorbance in the supernatant was measured at 595 nm. Maximum hemolysis was determined by adding 1% Triton X-100 to a sample of cells.

2.9. Circular dichroism (CD) spectroscopy

CD data were acquired with a Jasco J-810 CD spectrophotometer using a 0.2 mm path length cylindrical cuvette. The response was measured using wavelengths from 190 to 250 nm with a 0.2 nm step resolution and a 1 nm bandwidth. The rate of 100 nm/min and a response time of 0.25 s were used, and the spectra were averaged over eight scans. Spectra were recorded at a peptide concentration of 70 μM in three different environments: water, 5 mM Tris–HCl (pH 8.0), and 5 mM Tris–HCl containing 160 μM SDS (pH 8.0). The experimental temperature was 25 °C. In each case, the circular dichroism spectrum of the solvent was subtracted from the spectrum of the peptide.

2.10. Transmission electron microscopy

Transmission electron microscopy was performed to study the possible mechanisms of action of these antimicrobial peptides on several microorganisms according to the methods described by Friedrich et al. [10] with minor modification. Exponential-phase bacteria were treated with the peptides (100 μg/ml) for 30 min at 37 °C. After treatment, the bacteria were centrifuged at 300 × *g* for 10 min, and the pellets were fixed with 2.5% buffered glutaraldehyde for 1 h. The cells were then postfixed in 1% buffered osmium tetroxide for 1 h, stained en bloc with 1% uranyl acetate, dehydrated in a graded series of ethanol washes, and embedded in white resin. The buffer used was 0.1 M sodium cacodylate, pH 7.4. Thin sections were prepared on copper grids using an LKB-V (Sweden) and stained with 1% uranyl acetate and lead citrate. The resin and grids were purchased from Marivac (Halifax, Nova Scotia, Canada). Microscopy was performed with a JEM1011 microscope under standard operating conditions.

2.11. Synthetic peptide

The peptide used for antimicrobial test and CD analysis in this paper was synthesized by solid peptide synthesizer (433A, Applied Biosystems) in AC Scientific (Xi An) Inc. (Xi An, China) and analyzed by HPLC and MALDI-ToF mass spectrometry to confirm that the purity was higher than 95%. All peptides were dissolved in water.

3. Results

3.1. Purification of antimicrobial peptides

The supernatant of *O. grahami* skin secretions was divided into six peaks by Sephadex G-50 [15], and the antimicrobial activity occurred in the peaks IV, V, and VI. The antimicrobial peptides

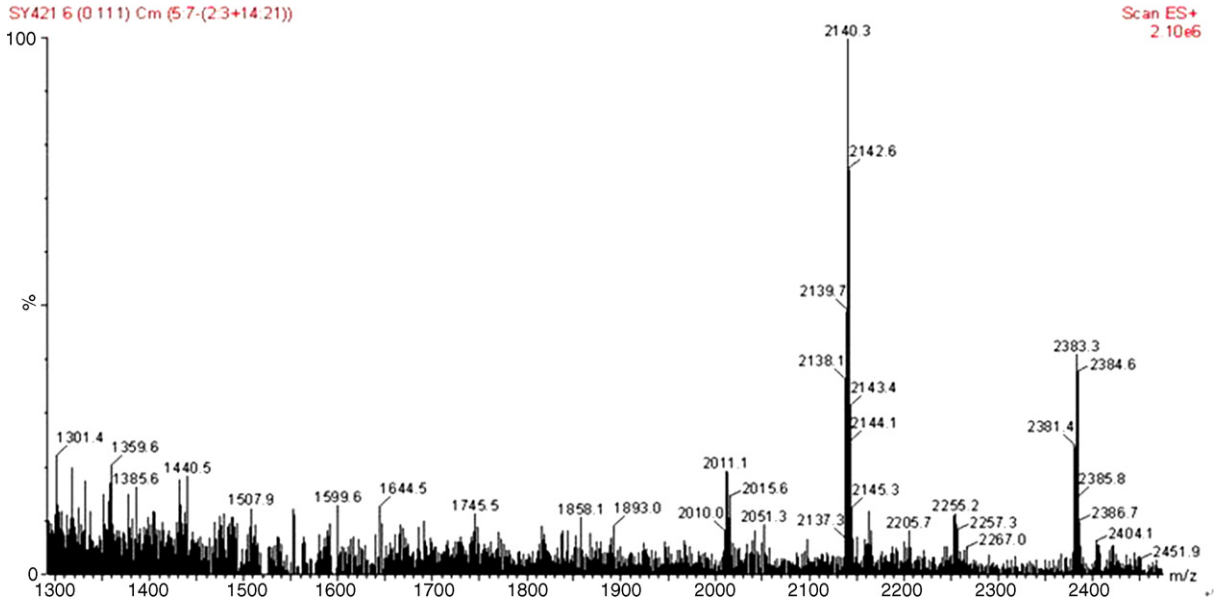

Fig. 2 – Electrospray ionization tandem mass spectrometry of synthetic odorranin-NR.

from peaks V and VI have been described in our previous reported [15]. The peak IV was applied to an RP-HPLC column, and more than 20 peaks were obtained from this separation as indicated by Fig. 1. The peak with antimicrobial activity (marked by an arrow) was collected.

3.2. Structural characterization

A purified antimicrobial peptides marked by an arrow in Fig. 1 named as odorranain-NR. It was subjected to amino acid sequence analysis by automated Edman degradation. It is composed of 23 amino acids with an amino acid sequence of GLLSGILGAGKHIVCGLTGCAKA. By ESI-MS analysis, the observed molecular weight of the synthetic odorranain-NR was 2139.3 Da that matched well with the theoretical molecular weights (2139.6 Da) (Fig. 2). There are multiple basic amino acids in the sequence of odorranain-NR as found in other antimicrobial peptides. Analysis using the ExPASy MW/pI tool (http://www.expasy.ch/tools/pi_tool.html) showed that they had the predicted pI (isoelectric point) of 8.90. By BLAST search, odorranain-NR shared similarity with amphibian antimicrobial peptide family of nigrocin (Fig. 3) [7,15].

3.3. cDNA cloning

Several clones, which contain inserts around 300-base pairs, were identified and isolated. Both strands of these clones were sequenced (Fig. 3A). Three cDNAs were found to have open reading frames that encode a polypeptide composed of 68 amino acids, including the mature odorranain-NR sequences. These three cDNAs encode two odorranain-NR precursors and only one mature odorranain-NR. The structural organization of the precursor is quite similar to other amphibian antimicrobial peptide precursors, comprising a signal peptide sequence, an N-terminal spacer peptide region containing several aspartic and glutamic acid residues, and the mature antimicrobial peptide at the C-terminus of the precursor [8,15]. There is a di-basic cutting site (-$K^{44}R^{45}$-) for trypsin-like proteases between spacer peptide and mature peptide. The amino acid sequence deduced from the cDNA sequences is the same with the amino acid sequence determined by Edman degradation.

3.4. Antimicrobial activity

Synthetic odorranain-NR exhibited antimicrobial activity against the tested strains except *E. coli* (ATCC25922) (Table 1). Of the tested strains, *S. aureus* was the most sensitive to odorranain-NR. The MIC of odorranain-NR for *S. aureus* was 9.375 μg/ml as shown in Table 1. The antibiotic activity of odorranain-NR was proved to be lethal for the sensitive strains. The sensitive strains were not capable of resuming growth on agar plates after a 6 h treatment with concentrations above the corresponding MICs.

3.5. Hemolytic activity

Some antimicrobial peptides exhibit hemolytic activities [14]. Rabbit red blood cells were used to check for hemolytic capability in our experiments. Odorranain-NR had little hemolytic activity on red blood cells even with peptide concentration up to 100 μg/ml.

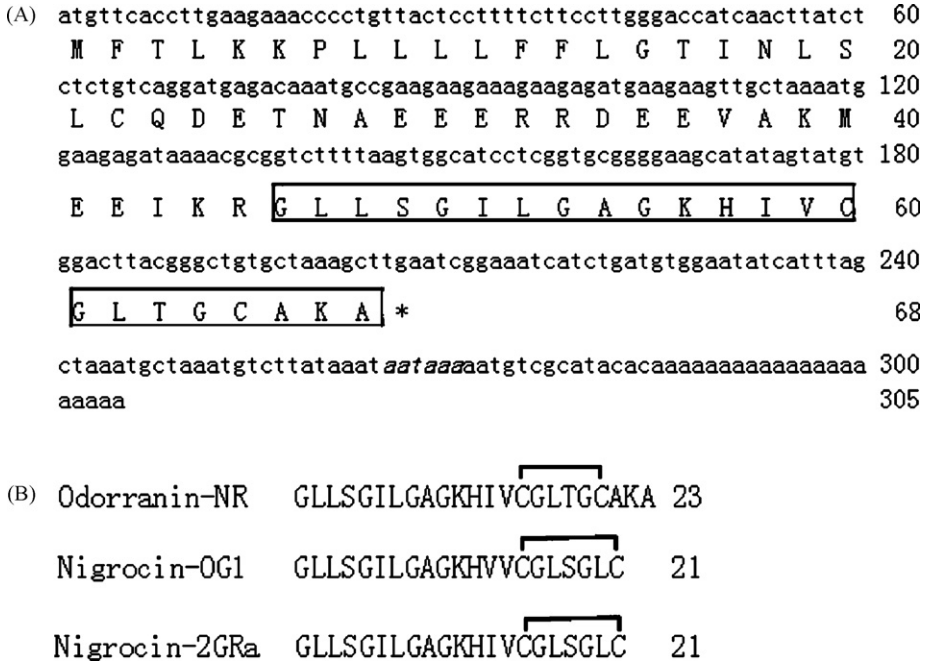

Fig. 3 – The nucleotide sequence encoding odorranin-NR peptide from *O. grahami* and the deduced amino acid sequence of the precursor polypeptide (A) and its sequence comparison with nigrocin-OG1 (B). (A) The sequence of mature odorranin-NR is boxed. The asterisk (*) indicates stop condon. The putative polyadenylation signal is italic. (B) Nigrocin-OG1 is from Ref. [14]. Nigrocin-2GRa is from Ref. [7]. The disulfide bridges are connected by a line.

Table 1 – Antimicrobial activities of odorranain-NR

Microorganism	MIC (μg/ml)[a]
Escherichia coli ATCC25922	>100
Bacillus dysenteriae	37.500
Staphylococcus aureus ATCC2592	9.375
Candida albicans ATCC2002	18.750

[a] The data represent mean values of three independent experiments performed in duplicates.

Table 2 – Second structure of odorranain-NR in different solutions

Second structure (%)	Water	Tris–HCl	Tris–HCl + SDS
α-Helix	14.7	15.3	4.6
β-Sheet	39.8	59.1	79.2
β-Turn	20.1	10.5	1.3
Random	25.4	17.1	14.9

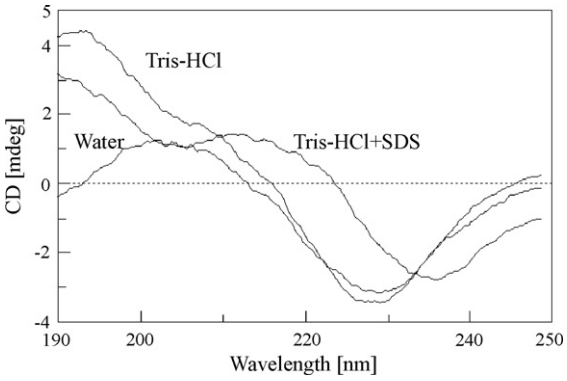

Fig. 4 – The CD spectrum of odoranin-NR in different solutions: 70 μM odoranin-NR was resolved in 5 mM Tris–HCl, pH 8.0; 5 mM Tris–HCl with SDS 160 μM, pH 8.0; water.

3.6. *Secondary structure analysis of odorranain-NR*

The conformation distribution of odorranain-NR molecule was analyzed. CD spectroscopy was studied (Fig. 4). Odorranain-NR had negative absorption at 225–240 nm in all three solutions. It suggested that odorranain-NR had a large proportion of β-sheet. Patterns of CD in Tris–HCl and in Tris–HCl + SDS were very similar. They have characteristic negative absorption at the 225–230 nm. Table 2 listed the conformation distribution of odorranain-NR in these

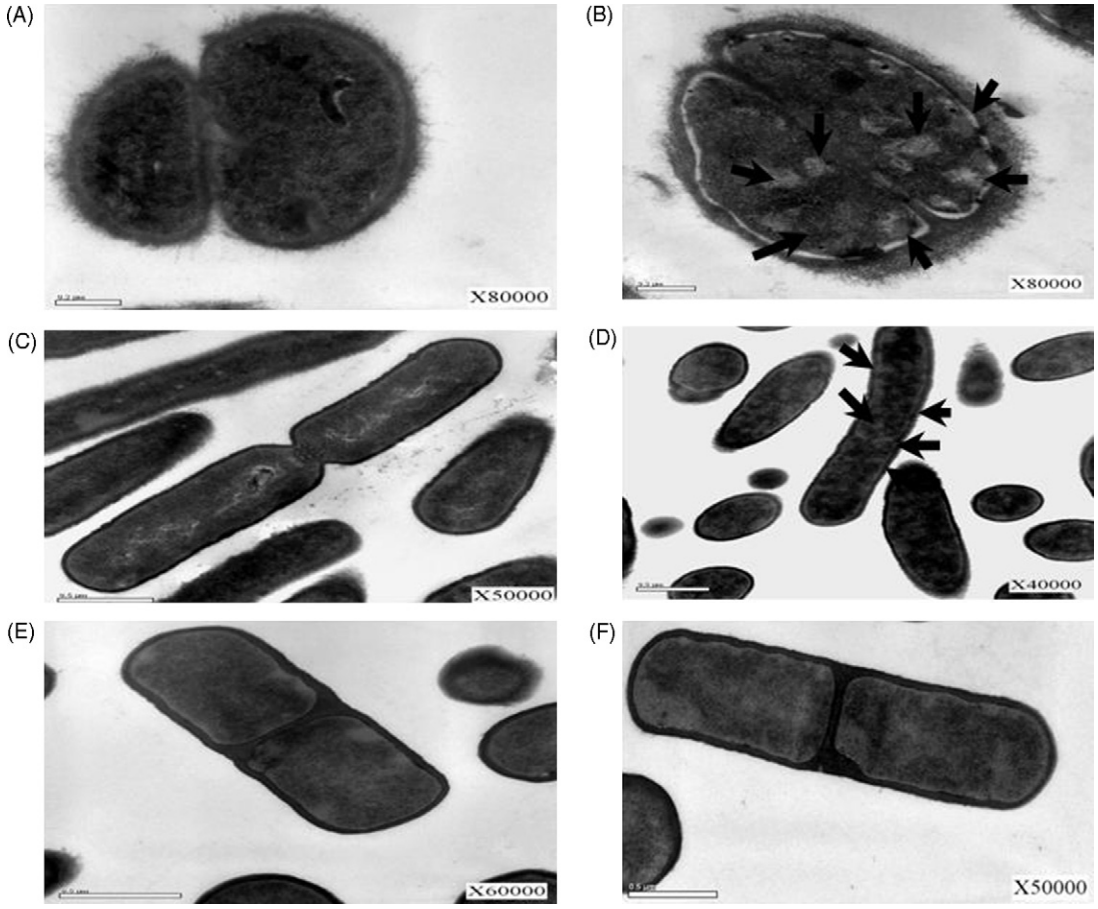

Fig. 5 – Ultrastructure of microorganisms treated by odoranin-NR (100 μg/ml): (A) control *Staphylococcus aureus* ATCC2592; (B) *S. aureus* ATCC2592 treated by odoranin-NR; (C) control *Candida albicans* ATCC2002; (D) *C. albicans* ATCC2002 treated by odoranin-NR; (E) control *Bacillus dysenteriae*; (F) *B. dysenteriae* treated by odorranin-NR. The mesosome structures are indicated by arrows.

solutions. In Tris–HCl + SDS, odorranain-NR had a major conformation of β-sheet (79.2%) and a minor conformation of β-turn (1.3%). In Tris–HCl, the β-sheet has been decreased to 57.1% and the β-turn has been increased to 10.5%. The β-sheet has been decreased to 39.8% in water.

3.7. *Variable antimicrobial mechanisms*

To search for clues to possible mechanisms of action of the antimicrobial peptide odorranain-NR on the tested microorganisms, transmission electron microscopy was performed on thin sections of bacteria that had been treated with the peptide for 30 min, according to the methods described by Friedrich et al. [10]. Odorranain-NR had different effects on different microorganisms (Fig. 5). It seems have direct bacteria-killing capability against *S. aureus* ATCC2592 (Fig. 5B) and *C. albicans* ATCC2002 (Fig. 5D) but no obvious effect on morphology of *B. dysenteriae* (Fig. 5F). Apparently, odorranain-NR severely destroyed the cell walls and membranes of bacteria *S. aureus* ATCC2592, with some mesosomes being found in the treated bacterium (Fig. 5B). The mesosomes likely led to the breaking of the cell wall. Mesosomes must be regarded as being indicative of cytoplasmic membrane alteration [10]. Small laminar mesosomes were seen arising from the septa and cell wall of treated fungi *C. albicans* ATCC2002 by odorranain-NR but the cell membrane seems intact, as indicated in Fig. 5D. As illustrated in Fig. 5F, odorranain-NR did not affect the cell membrane and cell wall of *B. dysenteriae*. No mesosome-like structure was found the treated bacterium. Odorranain-NR may exert its antibacterial function against *B. dysenteriae* by other mechanism. The current results demonstrate that the given antimicrobial peptide had different strategies to kill different microbes.

4. Discussion

Amphibians have excellent chemical defense systems composed of pharmacological and antimicrobial peptides [1,8] to adopt and survive in a variety of conditions laden with pathogenic microbes. A large amount of antimicrobial peptides with variable structure and function have been identified from amphibian skins. Frogs of the genus *Odorrana* are composed of more than 20 members. Most of members of the genus are distributed in China. Recently, we have identified 107 different antimicrobial peptides belonging to 30 different families from *O. grahami* [15]. It is the most diversity of antimicrobial peptides in a single organism. Odorranin-NR shared similarity with amphibian antimicrobial peptide family of nigrocin, particularly, the N-terminal 14-amino acid fragment is highly conserved (Fig. 3B). Odorranain-NR has an intramolecular disulfide-bridged hexapeptide segment that is different from the intramolecular disulfide-bridged heptapeptide segment at the C-terminal end of nigrocins (Fig. 3). Most of amphibian antimicrobial peptide families contain disulfide-bridged heptapeptide segment such as nigrocin, brevinin, esculentin, odorranain-C, -H, -P [7,15] except ranatuerins [8,12]. The disulfide-bridged hexapeptide segment is a new type of disulfide motif in amphibian antimicrobial peptides. Furthermore, the -AKA fragment at the C-terminal of odorranain-NR is also different from other amphibian antimicrobial peptides (Fig. 3). Therefore, odorranain-NR should be classified into a new family of antimicrobial peptide. This discovery of odorranin-NR with its novel structure confirmed further the extreme diversity of antimicrobial peptides of *O. grahami*.

Odorranin-NR exerted antrimicrobial activities against *S. aureus* ATCC2592, *C. albicans* ATCC2002, and *B. dysenteriae* (Table 1). It is not clear how antimicrobial peptides actually kill microbes, although many hypotheses have been presented. As illustrated in Fig. 5, odorranain-NR exerted its antimicrobial functions by various means depending on different microorganisms. It could target cell walls, cell membrane, cytoplasm or other sites. Odorranin-NR caused similar changes in cell morphology of *S. aureus* ATCC2592 as some other antimicrobial peptides did, such as odorranain-C1 and brevinin-1E-OG6 [15]. Different antimicrobial peptides with different structures exert their antimicrobial functions by various means, including forming lamellar mesosome-like structures, peeling off the cell walls, forming pores, and inducing DNA condensation [15]. With respect to the development of antibiotics, the current antimicrobial peptide odorranin-NR with its unusual intramolecular disulfide-bridged hexapeptide segment provides potential new template.

Acknowledgements

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