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Short communication

Modification and characterization of a new recombinant marine antimicrobial peptide N2

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ABSTRACT

NZ17074, a member of the arenicin family from *Arenicola marina*, exhibited potent antimicrobial activity against bacteria and fungi, but had some toxicity to human erythrocytes and porcine intestinal epithelial cells. To resolve this problem, one novel peptide N2 was designed based on NZ17074 by replacing Gly in position 1, 12 with Ala. N2 exhibited a typical β-sheet conformation in aqueous and almost had no hemolysis. N2 was expressed in *Pichia pastoris* using the small ubiquitin-like modifier fusion technology. The yield of N2 was approximately 6.5 mg/l, and its molecular weight was 2665.0 Da. The minimum inhibitory concentrations of N2 against Gram-negative bacteria ranged from 0.125 to 4 μg/ml. The antibacterial activity of N2 against *Escherichia coli* CVCC 195 and *Salmonella enteritidis* CVCC 3377 was enhanced up to 1–3-fold over parent NZ17074. This result provides some information that assists in the rational design of toxic antimicrobial peptides.

1. Introduction

Arenicins, cationic antimicrobial peptides (AMPs) isolated from *Arenicola marina*, have potent antimicrobial activity against bacteria and fungi, which may be potential agents for future use in the treatment of resistant bacteria. Three subtypes of arenicin (−1, −2, and −3) have been reported recently [1,2]. However, due to their high toxicity to human erythrocytes and protein binding, these areincins are not suitable for clinical treatment [3]. NZ17074, a variant of arenicin-3, belongs to the β-sheet AMPs and contains two disulfide bonds between Cys3, Cys20 and Cys7, Cys16. NZ17074 exhibits a broad spectrum activity against a series of Gram-negative bacteria and *Candida albicans* [4]. However, NZ17074 also exhibits some toxicity to human erythrocytes [4] and porcine intestinal epithelial cells (data not published).

Residues replace has been successfully used to improve the activity of AMPs and reduce cytotoxicity [5]. Yoshida et al. (2001) reported that Gly could weaken the α-helicity of peptides, replacement of the Gly residues in Ple by Ala (Gly 1, 3 Ala) increased the hydrophobicity and α-helicity, decreased the hemolysis and enhanced the antibacterial activity against *Staphylococcus aureus* [6]. Site-directed substitutions of residues in the polar face of the AMPs with more polar residues decreased the hemolytic effect without significantly affecting the antimicrobial activity [7].

In this study, to reduce toxicity of parent peptide NZ17074, a new modified peptide named N2 from this parent peptide was designed by substituting Gly with Ala in the polar face. The N2 gene was synthesized and expressed as fusion protein with small ubiquitin like modifier (SUMO) in *Pichia pastoris* X-33 to reduce its potential toxicity to host cells. The N2 peptide released from the SUMO-N2 protein by chemical cleavage was evaluated preliminarily.

2. Materials and methods

2.1. Strains, plasmids, and reagents

The strain of *Escherichia coli* DH5α (Invitrogen, Beijing, China) was used as the host strain for vector construction. *P. pastoris* X-33,

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Table 1
Sequences and physicochemical properties of NZ17074 and N2.

Peptides	Amino acid sequence	MW (Da)	PI	Charge	GRAVY	Instability index	Antibacterial activity score	Boman index (kcal/mol)
NZ17074	GFCWNVCVYRNGVRVCHRRCN	2542	9.37	+4	−0.243	52.96	0.245	2.66
N2	**A**FCWNVCVYRN**A**VRVCHRRCN	2570	9.38	+4	−0.033	60.12	0.121	2.58

MW: molecular weight; PI: isoelectric point; GRAVY: the grand average of hydropathy.

pPICZαA vectors (Invitrogen, Beijing, China) and pE-SUMO Amp vectors (LifeSensors Inc, Philadelphia, PA) were used for cloning and expression. The test strains of *E. coli* CVCC 195, *E. coli* CVCC 1515, *Salmonella choleraesuis* CVCC 503, *Salmonella enteritidis* CVCC 3377, *Salmonella pullorum* CVCC 1802, *Staphylococcus aureus* ATCC 43300, *S. aureus* ATCC 25923, and *Streptococcus suis* CVCC 606 were purchased from China Veterinary Culture Collection Center (CVCC) (Beijing, China). The strains of *E. coli* CICC 21530 (serotype O157:H7), *Pseudomonas aeruginosa* CICC 21630, *P. aeruginosa* CICC 10149, and *Listeria ivanovii* CICC 21623 were purchased from China Center of Industrial Culture Collection (CICC) (Beijing, China). DNA restriction enzymes and T4 DNA ligase were purchased from New England Biolabs Ltd. (NEB, Beijing, China). The kits for plasmid extraction and DNA purification were purchased from Tiangen Co., Ltd. (Beijing, China). All other chemical reagents used were of analytical grade.

The primers of SUMO and N2 and the nucleotide sequence of N2 were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China).

2.2. Peptide design

The NZ17074 analogue N2 was designed based on hydrophobicity by replacing Gly in positions 1 and 12 with Ala to reduce toxicity and maintain antibacterial activity. Isoelectric point (PI), values of the instability index, aliphatic index and GRAVY index of N2 were calculated with the ProtParam soft. Net charge and the Boman index were calculated with APD2 algorithm. The amino acid sequences and properties analysis were performed with the bioinformatics program ProtParam. The helical wheel projection was performed online using the Helical Wheel Projections. The three-dimensional structure projection was predicted online using the I-TASSER server.

2.3. Circular dichroism (CD) of N2

CD spectra were measured using a MOS-450 spectropolarimeter (Bio-Logic, Grenoble) to analyze the secondary structure in sodium dodecyl sulfate (SDS) solutions to mimic the hydrophobic environment of microbial membranes. The N2 peptide was dissolved at a concentration of 0.01 mg/ml in ddH_2O, 5 mM, 10 mM, 20 mM, and 40 mM SDS. The samples were loaded into a 1 mm cell path at room temperature and the spectra were recorded from 185 to 245 nm three times [8].

2.4. Construction, expression and purification of the rSUMO-N2 fusion protein in P. pastoris X-33

The synthesized N2 nucleotide sequence was amplified with primers of N2-F (5′-ACGGGAGGTGAATTC**GACCCA**GCTTTCTGTTGGAAC-3′, the formic acid cleavage site is in bold) and N2-R (5′-GGGGGGCTGCTCTAGACTATTAGTTACATC-3′, *Xba*I is underlined). The fragment of SUMO was amplified with primers of SUMO-F (5′-CCGCTCGAGAAAAGAGGTCATCA-CCATCA-3′, *Xho*I is underlined) and SUMO-R (5′-GTTCCAACAGAAAGC**TGGGTC**GAATTCACCTCCCGT-3′, the formic acid cleavage site is in bold). The DNA fragments of SUMO and N2 were fused into one fragment by overlap PCR with SUMO-F and N2-R primers [9]. After gel-purification, the SUMO-N2 products were digested with *Xho*I and *Xba*I, ligated into pPICZαA vectors, and transformed into *E. coli* DH5α. The positive cells were screened by colony PCR and confirmed by DNA sequencing.

The recombinant plasmid pPICSUMO-N2 was linearized with *Pme*I, transformed into competent *P. pastoris* X-33 cells by electroporation [9]. The linearized pPICZαA plasmid was used as a negative control. Positive transformants were selected on YPDS plates containing 100 μg/ml zeocin and confirmed by PCR amplification [9,10]. Expression and purification of the rSUMO-N2 fusion protein were performed as previously described [9]. The purified rSUMO-N2 protein was desalted and freeze-dried.

2.5. Cleavage and purification of the N2 peptide

The purified rSUMO-N2 protein was dissolved in 30%, 50% and 70% formic acid, incubated at 37 °C or 50 °C for 28 h, 36 h, 48 h, and 72 h, respectively. The optimal cleavage condition was determined using the inhibition zone assay [11]. rN2 released from the rSUMO-N2 protein was purified by gel filtration on a Sephadex G-25 column, and analyzed by Tricine-SDS-PAGE and matrix-assisted primers of SUMO and N2 and the nucleotide sequence of N2 were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) [12].

2.6. Antimicrobial activity and hemolytic assay

The minimal inhibitory concentration (MIC) and the antimicrobial activity of the purified rN2 against test strains were determined using the microtiter broth dilution method and the inhibition zone assay as reported previously [9,10]. The hemolytic activity was performed by determining hemoglobin release from fresh mouse erythrocytes as described previously [12].

3. Results and discussion

3.1. Physicochemical properties, structure and activity of N2

Similar to parent peptide NZ17074, the net charge and PI of N2 was +4 and 9.38, respectively (Table 1). The grand average of hydropathicity of N2 was increased to −0.033, indicating that N2 was more hydrophobic than NZ17074, which is correlated with the addition of a methyl group from Gly to Ala resulting in a greater hydrophobicity [13]. Instability index of NZ17074 and N2 was 52.96 and 60.12, respectively. The values of the Boman index, which is a measure of peptide affinity to proteins and its ability to establish bio-interactions, were 2.66 and 2.58 kcal/mol for NZ17074 and N2, respectively.

The helical wheel projection for N2 is shown in Fig. 1A and C. Similar to NZ17074, hydrophobic amino acids of N2 are concentrated on one side of the helix, hydrophilic amino acids on the other and the net charge is uniformly distributed hydrophobic surface. The results of three-dimensional structure prediction also showed that there was no significant difference between NZ17074 and N2, forming amphipathic β-sheet structures except the N-terminus residues 1–2 of N2 containing an α-helix (Fig. 1B and D). Similar to previous studies by Chen et al. (2005), Ala stabilized the α-helical structure when substituted in the polar face, which was caused by the enthalpy of the helical H-bond and the entropy change for the