Boosting Salt Resistance of Short Antimicrobial Peptides

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The efficacies of many antimicrobial peptides are greatly reduced under high salt concentrations, therefore limiting their use as pharmaceutical agents. Here, we describe a strategy to boost salt resistance and serum stability of short antimicrobial peptides by adding the nonnatural bulky amino acid β-naphthylalanine to their termini. The activities of the short salt-sensitive tryptophan-rich peptide S1 were diminished at high salt concentrations, whereas the activities of its β-naphthylalanine end-tagged variants were less affected.

Antimicrobial peptides have been found to play important roles in the host defense (1). Most of the antimicrobial peptides are polycationic, amphipathic, and generally interact and permeabilize microbial membranes (1). Development of antimicrobial peptides as therapeutic agents could be promising due to their special microbicidal mechanism (2).

Development of antimicrobial peptides has been hindered by several problems, such as salt sensitivity, cost of synthesis, bioavailability, and stability. Salt sensitivity is directly related to the antimicrobial peptide's microbicidal mechanism. The efficacy of human β-defensin-1 is greatly reduced in the high-salt bronchopulmonary fluids in cystic fibrosis patients (3). Similar problems were observed for the clinically active peptide P-113, indolicidins, gramicidins, bactenecins, and magainins (4–7). Studies have been reported on the design of salt-resistant antimicrobial peptides. However, most of them were focused on overall structure modifications (4, 8–13).

Previously, we developed an easy strategy to increase salt resistance of Trp- and His-rich antimicrobial peptides by replacing their tryptophans or histidines with the bulky amino acid β-naphthylalanine (14). This strategy has been applied successfully to the Trp-rich peptide Pac-525 (Ac-KWRRWVRWI-NH$_2$) and the clinically active His-rich peptide P-113 (AKRHHGYKRKFH-NH$_2$).

A short Trp-rich antimicrobial peptide, S1 (Ac-KKWRKWLAKK-NH$_2$), was designed based on studies of PEM-2-W5K/A9W (Ac-KKWRKWLKWLAKK-NH$_2$) (15). PEM-2-W5K/A9W was found to be very effective against both bacteria and fungi under high-salt conditions (15). S1 demonstrates promising antimicrobial activities in the low-salt LYM broth medium (Fig. 1). However, the antimicrobial activities of S1 were diminished at high salt concentrations (Fig. 1). Replacement of tryptophan residues of S1 with β-naphthylalanine may generate a potent peptide (Nal-S1) with improved antimicrobial activity and salt resistance. Unfortunately, Nal-S1 does not have the expected salt resistance.

Recently, Malmsten and coworkers developed a strategy to boost activities of short antimicrobial peptides by modulating their lipophilicity with the addition of tryptophan and/or phenylalanine end tags (16–18). The addition of fatty acid, vitamin E, or cholesterol to the termini of antimicrobial peptides has been shown to have similar results (19–23). It is thus hypothesized that addition of the bulky amino acid β-naphthylalanine to the termini of short antimicrobial peptides may improve their antimicrobial activity and salt resistance.

We designed and synthesized S1, Nal-S1, S1-Nal, S1-Nal-Nal, S1-Nal-Nal-Nal, S1-W, S1-WW, and S1-KKK. The tryptophan end-tagged S1-W and S1-WW and lysine end-tagged S1-KKK were used for comparisons. Bacterial strains from ATCC are listed in Fig. 1. Antibacterial activities were determined by the standard broth microdilution method of the National Committee for Clinical Laboratory Standards. The MIC was defined as the lowest concentration of peptide at which there was no change in optical density. MICs were converted to a color scale and displayed using the TreeView program (19, 24). The serum stability of the peptides was determined in 25% (vol/vol) aqueous bovine calf serum (catalog number AUE-34962; HyClone). Peptides were dissolved in serum at a concentration of 150 μg/ml and incubated at 37°C. After 45 min on ice to precipitate serum proteins, the samples were centrifuged (10 min, 12,000 × g, 4°C) and the supernatants were lyophilized. The remaining amount of the peptides was determined by reverse-phase high-performance liquid chromatography. All MIC and serum stability tests were performed in triplicate. Toxicities of the peptides were determined from lysis of human red blood cells (hRBC) (Fig. 2) and human fibroblast cells (25). Although S1-Nal-Nal and S1-Nal-Nal-Nal had higher cell lytic activities than the other peptides, all of the peptides exhibited <5% cell lytic activity at their effective MIC.

All of the peptides studied demonstrated promising activities in the LYM broth medium (Fig. 1). However, the activities of S1, Nal-S1, S1-W, S1-WW, and S1-KKK were reduced or diminished in Mueller-Hinton broth or under high-salt conditions. The MICs of S1-Nal, S1-Nal-Nal, and S1-Nal-Nal-Nal were found to be more potent than S1 in both Mueller-Hinton and modified LYM broth medium. S1-Nal-Nal and S1-Nal-Nal-Nal still retained their antibacterial activities with 300 mM NaCl added. The N-terminal-tagged variant had similar MICs and hemolytic activities as the C-terminal-tagged S1-Nal-Nal. Results from fluorescence quenching experiments indicated that β-naphthylalanine end
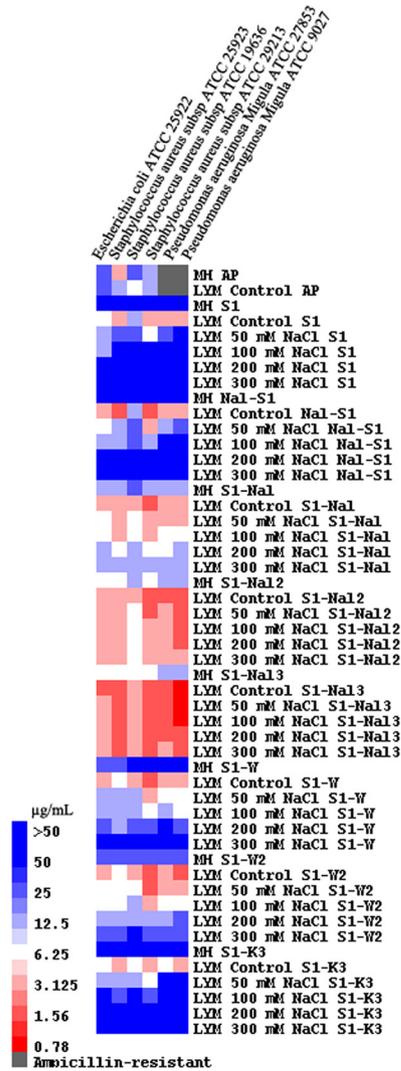
FIG 1 MIC values displayed on a color scale for ampicillin (AP), S1, Nal-S1, S1-Nal, S1-Nal-Nal, S1-Nal-Nal-Nal, S1-W, S1-WW, and S1-KKK with different concentrations of NaCl.

FIG 3 Serum stability results for S1, Nal-S1, S1-Nal, S1-Nal-Nal, S1-Nal-Nal-Nal, S1-W, S1-WW, and S1-KKK.

tagging may help these peptides penetrate deeper into the membranes, hence making them more efficient at disrupting the membranes.

Additionally, β-naphthylalanine end tagging was found to protect S1 from degradation in serum. S1-Nal-Nal-Nal retained almost 100% of its integrity after 8 h in bovine calf serum (Fig. 3). The degrees of protection of these peptides from degradation in bovine calf serum were found to be S1-Nal-Nal-Nal > S1-Nal-Nal > S1-Nal > S1-KKK > S1 > Nal-S1 = S1-W > S1-WW.

There are several advantages of using β-naphthylalanine rather than tryptophan as end tags. Five tryptophan end tags were needed to provide salt resistance to the antimicrobial peptide KNK10 in 150 mM NaCl (17). Only one β-naphthylalanine end tag was needed to provide substantial salt resistance in the present study. This is particularly important for design and development of short antimicrobial peptides for clinical use and to lower the cost of synthesis. Furthermore, β-naphthylalanine end tagging provides superior serum stability compared to tryptophan end tagging, probably because of its nonnatural and bulky characteristics.
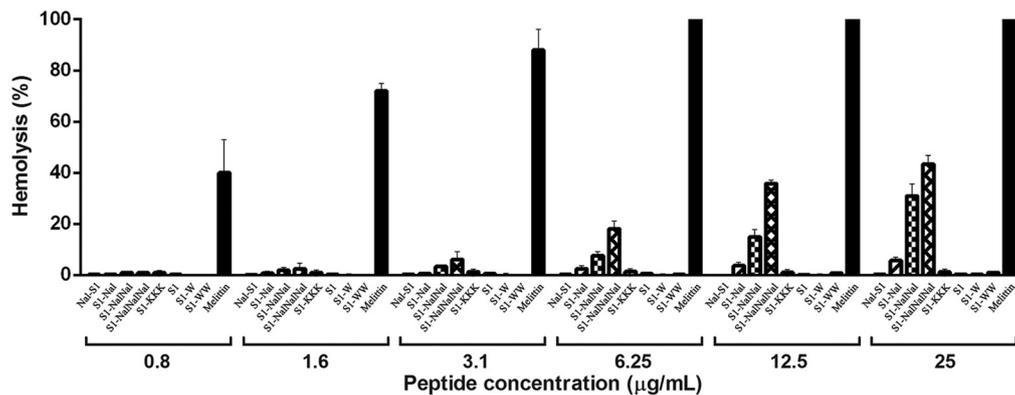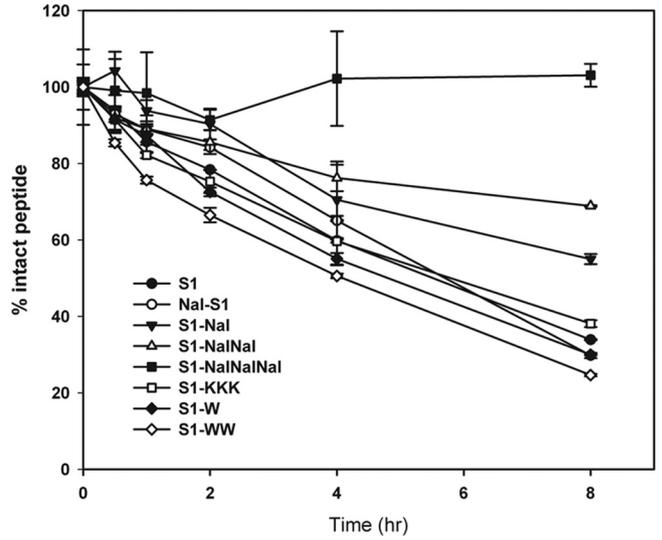

FIG 2 Hemolytic assay results for S1, Nal-S1, S1-Nal, S1-Nal-Nal, S1-Nal-Nal-Nal, S1-W, S1-WW, and S1-KKK. Melittin (black bar) was used as a control. The antibacterial activities of S1, Nal-S1, S1-W, S1-WW, and S1-KKK were reduced (MICs, >25 μg/ml) under the salt concentration used in the hemolytic assay, whereas S1-Nal (MIC, ~6.25 μg/ml), S1-Nal-Nal (MIC, ~3.1 μg/ml), and S1-Nal-Nal-Nal (MIC, ~1.6 μg/ml) still retained antibacterial activities.

Tryptophan or β-naphthylalanine end tagging can also be complementary. The advantage of tryptophan end tagging is the relatively low hemolytic activity compared to β-naphthylalanine end tagging. However, β-naphthylalanine end tagging demonstrates better salt resistance and serum stability. A peptide could be generated to have both tryptophan and β-naphthylalanine end tags. This peptide might exhibit lower hemolytic activity and still possess salt resistance and serum stability.

In addition to S1, we studied β-naphthylalanine end tagging on an ultrashort peptide, KWWK. The ultrashort peptide KWWK-Nal-Nal has a MIC of 1.6 μg/ml at 100 mM NaCl, while its parent peptide, KWWK, has no antimicrobial activity.

Here, we have described a strategy to boost salt resistance and serum stability of short antimicrobial peptides by adding β-naphthylalanine to their termini. This strategy has been applied successfully to S1 and the ultrashort peptide KWWK. We have also added S1-Nal and S1-Nal-Nal into a mouthwash solution formulation and tested their antiplaque and antigingivitis effects. We have not noticed any adverse results. *In vivo* tests of efficacy and toxicity of these modified peptides are ongoing in our laboratory.

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