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Recombinant Tandem Repeated Expression of S3 and SΔ3 Antimicrobial Peptides

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Abstract

Background: Antimicrobial peptides (AMPs) are promising candidates for new generations of antibiotics to overcome the threats of multidrug-resistant infections as well as other industrial applications. Recombinant expression of small peptides is challenging due to low expression rates and high sensitivity to proteases. However, recombinant multimeric or fusion expression of AMPs facilitates cost-effective large-scale production of AMPs. In This project, S3 and SΔ3 AMPs were expressed as fusion partners. S3 peptide is a 34 amino acid linear antimicrobial peptide derived from lipopolysaccharide (LPS) binding site of factor C of horseshoe crab hemolymph and SΔ3 is a modified variant of S3 possessing more positive charges.

Methods: Two copy tandem repeat of the fusion protein (named as SΔ3S3-2mer-GS using glycine- serine linker was expressed in *E. coli*. BL21 (DE3). After cell disruption and solubilization of inclusion bodies, the protein was purified by Ni -NTA affinity chromatography. Antimicrobial activity and cytotoxic properties of purified SΔ3S3-2mer-GS were compared with a previously produced tetramer of S3 with the same glycine-serine linker (S3-4mer-GS) and each of monomeric blocks of S3 and SΔ3.

Results: SΔ3S3-2mer-GS was successfully expressed with an expression rate of 26%. The geometric average of minimum inhibitory concentration (MIC_{GM}) of SΔ3S3-2mer-GS was 28%, 34%, and 57% lower than SΔ3, S3-4mer-GS, and S3, respectively. SΔ3S3-2mer-GS had no toxic effect on eukaryotes human embryonic kidney cells at its MIC concentration.

Conclusions: tandem repeated fusion expression strategy could be employed as an effective technique for recombinant production of AMPs.

Keywords: Antimicrobial Peptide, S3, SΔ3 Fusion Expression, Tandem Repeat Expression.

Introduction

Microbial resistance to antibiotics has been reported annually even monthly with a gradient increasing which made a need for approaching more effective antimicrobial treatments (1, 2). Antimicrobial peptides as parts of the host defense system of many organisms have a climactic role in modulating immune response and protecting against infectious pathogens (3, 4). Despite the great structural diversity, AMPs are common in net positive charges and amphiphilic structures due to the presence of cationic and hydrophobic residues in their sequences (1, 2).

Increasing net positive charges of AMPs by adding positively charged (lysine or arginine) residues(5-9), expelling or replacing negatively charged (glutamic acid or aspartic acid) residues(10), increasing amphipathicity by adding hydrophobic residues (11-16) or AMPs hybridization (17) may enhance antimicrobial activity and/or lipopolysaccharide (LPS) binding affinity of AMPs without considerable intensification of their cytotoxicity. Lipopolysaccharide binding affinity and high selectivity of AMPs have made them good choices as ligands for LPS removal affinity

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chromatography matrices (18, 19), biosensors (20), and antifouling agents (21). However, the high cost of peptide synthesis has restricted growing applications of AMPs.

There are several reports about the recombinant expression of AMPs as a fused protein to overcome this problem. Although fusion expression is an effective technique to shield or protect small peptides from the proteolytic degradation by host proteases, due to the low mass ratio of peptides to carrier proteins, simple fusion expression does not improve the production yield of AMPs extremely (22, 23). Expression of tandem multimers and/or hybrids form are special features of fusion expression to achieve more amount of target AMPs (22, 24, 25).

The effect of aspartic acid- proline (DP) and glycine -serine [(GGGGS)3 linkers on the biological activity of tetramer of sushi S3 peptide was studied, previously. It was observed that glycine serine linker improved antimicrobial activity of S3-tetramer approximately 25% and 86% in comparison to tetramer with aspartic acid - proline linker and S3 monomer, respectively, without any significant effects on its cytotoxicity (25). S3 peptide, a serine-protease- 34 amino acid linear peptide derived from LPS binding site of factor C of horseshoe crab's hemolymph, is one of the antimicrobial peptides which eradicate Gram-negative bacteria via binding to LPS of the bacterial membrane (26). Low cytotoxic and hemolytic effect on eukaryotic cells introduces S3 peptide and its modified variant as convenient candidates on antimicrobial agents (9) or ligands to be immobilized on chromatography resin for LPS removal from biopharmaceuticals (18, 19). S3 peptide has 3 positively charged (lysine) and 3 negatively charged (glutamic acid) residues and at neutral pH, it has weak cationic charges due to possessing 2 histidine residues (27). SΔ3 peptide is a modified variant of S3 peptide which has 3 cationic charges more than S3 due to replacing glycine (G 276) and glutamic acid (E278) with lysine (9).

In this study, SΔ3 AMP was used as a fusion partner for recombinant expression of S3 AMP and a tetramer fusion form of S3 and SΔ3 was produced by two copy tandem repeat the expression of antimicrobial peptides in *E. coli* BL21 (DE3). Glycine- serine was used as a linker to connect each of the monomeric peptides and the antimicrobial activity and cytotoxicity properties of resulted protein were studied.

Materials and methods

The hybrid protein (named as SΔ3S3-2mer-GS) was designed as two copy of SΔ3 (HAEHKVKIKVKQKYGQFPQGTEVTYTC SGNYFLM) and S3 (HAEHKVKIGVEQKYGQFPQGTEVTYTCS GNYFLM) with $(GGGGS)_3$ linker ($SΔ3(GGGGS)_3S3(GGGGS)_3SΔ3(GGGGS)_3S3$). A 10 His-tag tail and an enterokinase-cleaving site were added at the N-terminal of the SΔ3S3-2mer-GS sequence for purification. A Kanamycin resistance gene was added and designed gen were synthesized in PET 26b (+) vector for *E. coli* based expression (Biomatik, Inc. Canada). The vector was transferred to *E. coli* Top10 to amplify the plasmid. The amplified plasmids were purified by plasmid purification kit (GeneAll, Germany) and the qualification of extracted plasmid were evaluated by gel electrophoresis on 1% agarose gel. The purified plasmids were transferred to *E. coli* BL21 (DE3) strain as an expression system (25). Luria-Bertani (LB) broth media containing 30 ppm kanamycin was used for culturing the screened colonies at 37 ˚C and 170 rpm. At optical density at λ600 nm (OD_{600}) of 0.5, the cells were induced by adding 0.5 mM Isopropyl β-d-1-thiogalactopyranoside (IPTG) and 4 hours after induction, the biomass was harvested by centrifugation at 3380 G for 10 minutes. The tetramer form of S3 (named as 4merS3-GS) was previously produced by expression of 4 copies of S3 peptide with (GGGGS)3 linker (25).

Cell disruption and protein purification

The isolated cells were mixed with Tris-HCl 20 mM, pH 7.5 at a 1: 5 w/v ratio and were disrupted using an ultrasonic system (MISONIX, USA). After centrifugation at 7600 G for 10 minutes for isolating inclusion bodies (IBs). Then IBs were washed with 2 M urea and then solubilized with 6 M urea. Sodium dodecyl sulphate-polyacrylamide

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gel electrophoresis (SDS-PAGE) 18% (28) and western blot test using an anti-HIS-tag antibody (29) were conducted for evaluating target protein expression.

SΔ3S3-2mer-GS was purified according to the explained protocol of the previous work (25). Briefly, the Nickel-nitrilotriacetic acid (Ni-NTA) matrix (Qiagen, Sweden), equilibrated with 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, Urea 6 M, pH 8 was used for purifying target proteins containing His- tag by immobilized metal affinity chromatography (IMAC) method. Absorbed proteins were eluted by 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, Urea 6 M, pH 8 buffer. SΔ3S3-2mer-GS protein was refolded by removing urea (denaturing agent) using a dialysis bag with a 10 kDa cut-off. EndoBind-R™ affinity chromatography columns (Badti, England) was used for LPS removal and Limulus amebocyte lysate (LAL) assay (Bioendo, China) was conducted to determine LPS contamination. The protein concentration was determined at 280 nm wavelength by Nano-drop spectrophotometer (Thermo Fisher, USA) considering extinction coefficients 0.1% of SΔ3S3-2mer-GS as 0.887 (30). The tetramer S3 (named as 4merS3-GS) was previously produced by expression purification of four copies of S3 peptide with (GGGGS)3 linker (25).

Biological activity

The effect of hybridization on antimicrobial activity and cytotoxicity properties of the resulted protein (SΔ3S3-2mer-GS) were evaluated and compared with those of the recombinant tetramer form of S3 with the same linker (named as 4merS3-GS), S3 and SΔ3 monomers. The tetramer 4merS3-GS was previously produced by expression purification of four copies of S3 peptide with (GGGGS)3 linker (25).

Antimicrobial activity

Antimicrobial activities of serial dilutions of SΔ3S3-2mer-GS, 4merS3-GS, S3 and SΔ3 on 5×10^5 CFU/ml of *Pseudomonas aeruginosa* (ATCC 27853), *E. coli* (ATCC 25922), and clinically isolated strains of *Pseudomonas aeruginosa* and *E. coli* were evaluated by determining the minimum concentrations of proteins that inhibited bacterial growth rates (MIC) (25). Relative Growth was determined by dividing the optical density λ600 nm of samples by the control according to the following equation (31):

$$\text{Growth (\%)} = \frac{OD_{sample}}{OD_{\ growth\ control}} \times 100$$

MTT assay

Cytotoxic properties of SΔ3S3-2mer-GS, 4merS3-GS, S3, and SΔ3on human embryonic kidney cells (HEK-293 cell line, ATCC: CRL-1573, Pasteur Institute of Iran) were studied by 3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide (MTT) assay. The cells were freshly cultured in a 5% CO2 humidified atmosphere in Dulbecco's modified eagle's medium (GIBCO™, USA) containing 10% FBS, 100 U/ml of penicillin, and 100 µg/ml of streptomycin in a 25 -ml T tissue culture flask. The toxicity of serially diluted peptides on 3×103 cells/100 µL of HEK-293 cells was evaluated after 24 and 48 hours of exposure at 37 ˚C and 5% CO2. Formerly, after four hours of incubation with 0.5 mg/ml of MTT reagent, Dimethyl sulfoxide (DMSO) was used for dissolving MTT crystals and absorbance was read at λ595 nm by ELISA reader (ACCU reader, Taiwan). The viability (%) of cells in the samples was calculated according to the following equation (25):

$$\text{Cell Viability (\%)} = \frac{OD_{sample} - OD_{blank}}{OD_{\ untreated\ cell} - OD_{blank}} \times 100$$

Statistical analysis

The SPSS 16 software was used for statistical analysis of the results. The Box- plot was used for the recognition of outlier data. The normality test and homogeneity of variances were evaluated by performing Shapiro-Wilk and Levene tests, respectively. One-way analysis of variance (ANOVA) was conducted for comparing the means of MTT and MIC tests results (10).

Results

Protein expression and purification

The designed plasmid was amplified at *E. coli* Top 10 and after extraction and purification, was

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transferred to *E. coli* BL21 (DE3) strain. Figure 1a presents the purified plasmid on 1% agarose gel under UV light. The strains entailing target plasmids were isolated on the LB agar medium containing kanamycin. The isolated colonies were grown on LB medium and induced by IPTG. The SDS-PAGE and western blot were performed for evaluating the expression of SΔ3S3-2mer-GS protein (Figs. 1b and 1c). The band related to SΔ3S3-2mer-GS protein was located at approximately 20 kDa, which was in line with the 20680.59 Da predicted molecular weight (27).

The expression rate was estimated by GelQuant.NET software as approximately 36%. The target protein was isolated as inclusion bodies from harvested cells after cell disruption. SΔ3S3-2mer-GS protein was purified by IMAC chromatography by the aid of his- tag. The intermediate product of IBs solubilization step was applied to the thoroughly equilibrated NTA column. Figure 2 presents the chromatogram of Ni-NTA chromatography. All buffers contained 6 M urea to avoid undesired refolding and aggregation and the target protein was eluted by increasing the imidazole concentration at the elution step (25).

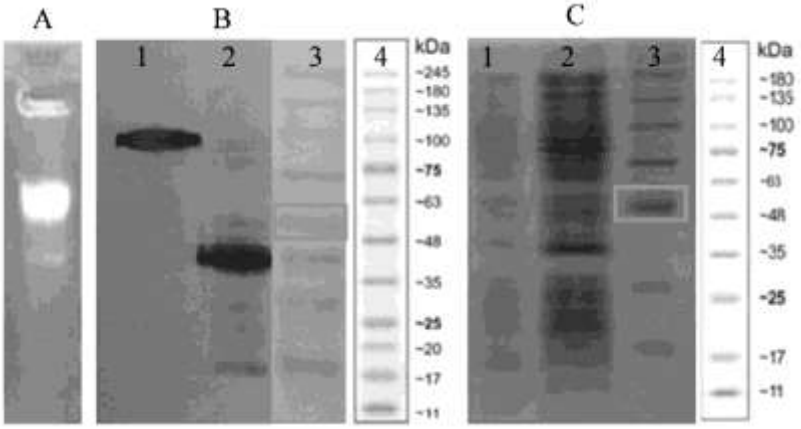

Fig. 1. Evaluation of SΔ3S3-2mer-GS expression. A: The purity of amplified plasmid on 1% agarose gel. B: Western blot of expressed protein by using anti- His tag antibody. Lane 1: His- tagged protein control. Lane 2: expressed SΔ3S3-2mer-GS protein in *E. coli* BL21 (DE3) strain. Lane 3: 11-245 kDa pre-stained ladder. Lane 4: Molecular weight Legends of the ladder. C: SDS-PAGE 18% analysis of expressed SΔ3S3-2mer-GS protein in *E. coli BL21 (DE3)*. Lane 1 and 2: Before and after induction with IPTG. Lane 3: 11-180 kDa pre-stained ladder. Lane 4: Molecular weight Legends of the ladder. At Lane B-3 and C-3, the bands related to 25 kDa were labeled with rectangles.

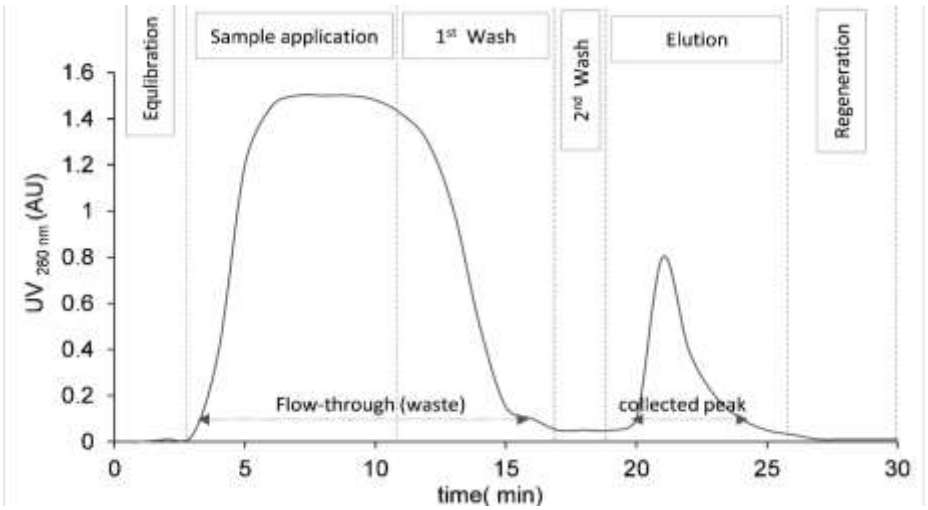

Fig. 2. The Chromatogram of IMAC purification step. During sample application, un-absorbed proteins, without His- tag, left the column. The column was washed with equilibration buffer (1st wash) until the UV reached the baseline, to ensure removing all un-absorbed protein from column. Then the column was washed with 2 column volumes (CVs) washing solution containing 20 mM imidazole to eliminate any un-specific weak binding of contaminant proteins from the column, but any peak was detected at this step. The absorbed target protein was eluted from the column by elution buffer containing 250 mM imidazole. The peak was collected and the column was regenerated. Dashed lines determine explained steps.

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The purified unfold SΔ3S3-2mer-GS protein was refolded by removing the denaturing agents by dialysis and the concentrated protein was further purified by LPS removal affinity chromatography for depleting endotoxin. Lipopolysaccharide was absorbed into the column and the protein was eluted in the flow-through effluent of the column. Gel clot assay with 0.25 EU/ml sensitivity was conducted for confirming efficient LPS removal. Lysate gel formation at 2 times diluted samples indicated the LPS concentration of samples between 0.25- 0.5 EU/ml.

Minimum inhibition concentration (MIC 50)

The relative growth of *Pseudomonas aeruginosa* (ATCC 27853), *E. coli* (ATCC 25922), antibiotic-resistant (isolated from a medical clinic) *Pseudomonas aeruginosa* and *E. coli* strains incubated with a serial dilution of AMPs were assessed (Fig. 3) and MIC values were calculated by interpolating the minimum concentrations of AMPs that inhibited bacterial growth 90% (10) (Fig. 4). For each of the stains, one-way ANOVA analysis was conducted. All p- values of the Shapiro-Wilk test for repeated MIC values were > 0.05 indicating that all MIC data had normal distributions. According to the p-value of ANOVA, except MIC values of S3-4mer-GS and SΔ3 against *Pseudomonas aeruginosa* (ATCC 27853), and resistance *Pseudomonas aeruginosa*, the differences among other MIC values were significant.

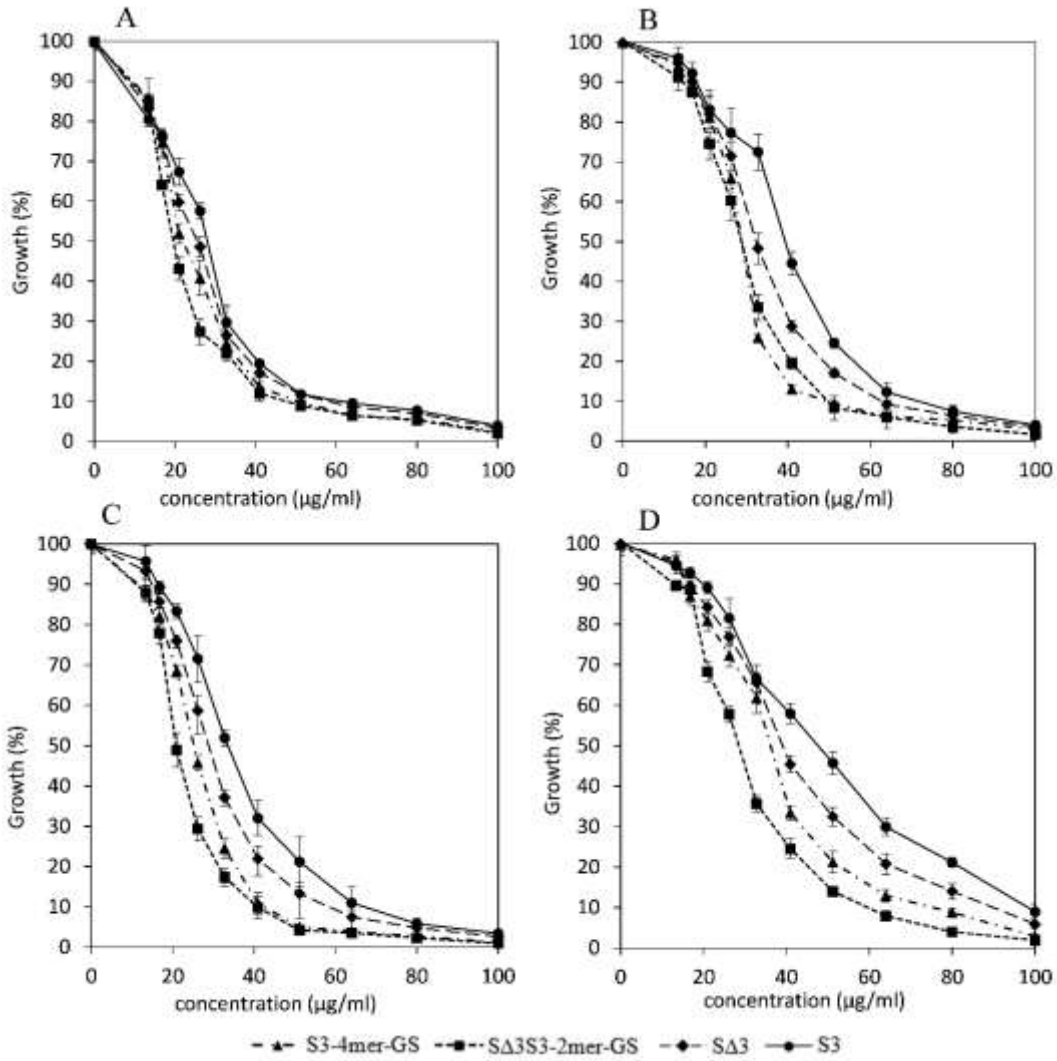

Fig. 3. Bacteria incubated with different concentrations of hybrid, tetramer, monomer of S3 and SΔ3. A: *E. coli* (ATCC 25922), B: resistant *E. coli*. C: *Pseudomonas aeruginosa* (ATCC 27853). D: resistant *Pseudomonas aeruginosa*.

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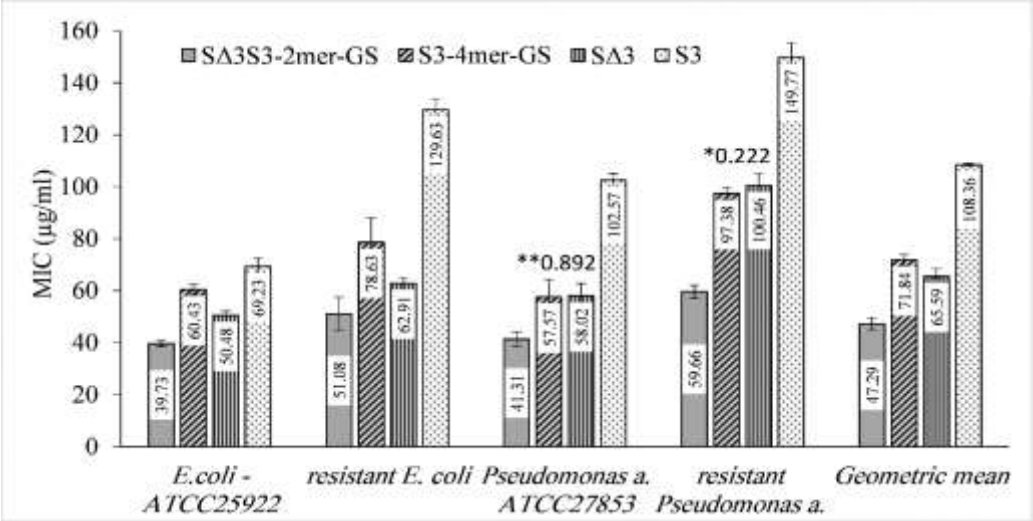

Fig. 3. Minimum inhibitory concentration (MIC) values of SΔ3S3-2mer-GS, 4-mer-S3-GS protein, SΔ3, and S3 against Gram-negative bacteria. Comparison Geometric mean of MICs revealed that MIC of SΔ3S3-2mer -GS was 28%, 34%, and 57% less than MICs of SΔ3, S3-4mer-GS, and S3, respectively.

Cytotoxicity

The cytotoxic effects of serially diluted SΔ3S3-2mer -GS with 4-mer-S3-GS protein, S3, and SΔ3 monomers on HEK-293 cell line of the human kidney were estimated (Fig. 5). All data possessed normal distribution (all p- values Shapiro- Wilk test were > 0.05) and no outlier data was detected by Box plots. At both 24 and 48 hours of exposure time, at high concentrations, Δ3S3-2mer -GS with 4-mer-S3-GS protein were more toxic than their monomers (Fig. 6).

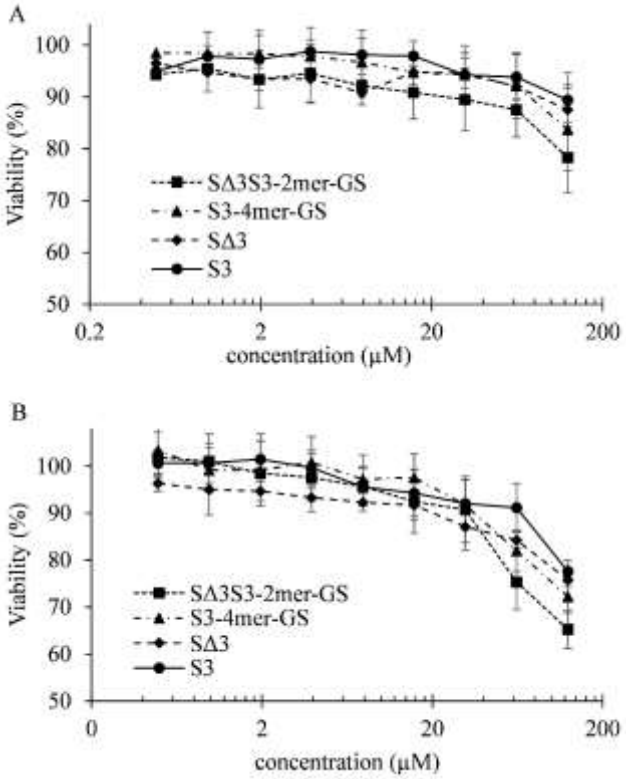

Fig. 4. The viability of HEK-293 cell: After 24 (A) and 48 (B) hours incubation with SΔ3S3-2mer-GS, 4-mer-S3-GS, SΔ3, and S3 AMPs. Each point of data are the average results of four repeats and the standard deviations were presented as error bars. The viability percentage decreased by increasing AMPs concentrations and time of exposure.

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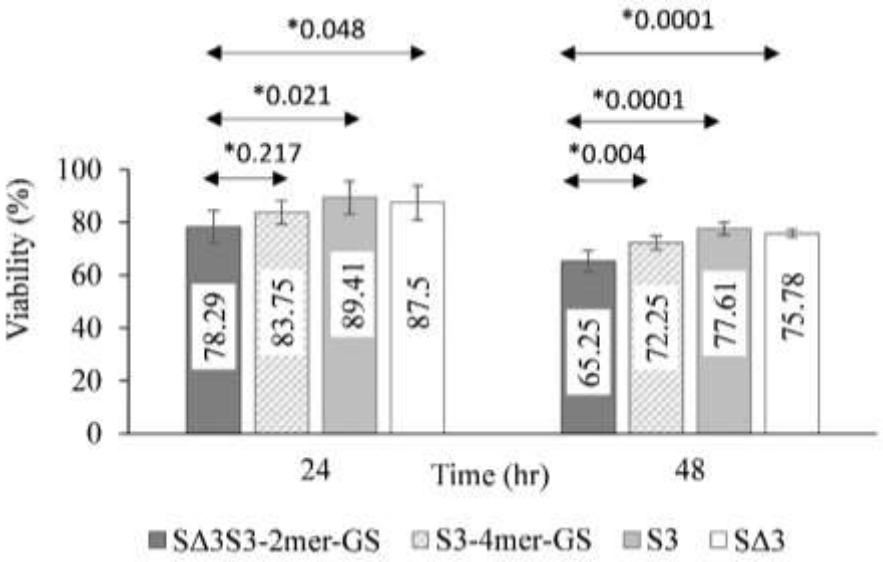

Fig. 6. The viability of HEK-293 cells after 24 and 48 hours' incubation with 125 µM of AMPs. The p- value of homogeneity tests for viability at 24 and 48 hours were 0.363 and 0.401, respectively. According to the LSD post hoc of one-way ANOVA analysis, the differences between the viability of Δ3S3-2mer -GS, and S3-4mer-GS after 24 hours of exposure, was not significant (p-value 0.217). However, after 48 hours of exposure, the viability of SΔ3S3-2mer -GS was approximately 11%, 14%, and 16% less than those of S3-4mer-GS, SΔ3, and S3, respectively and all differences were significant (p-value< 0.05).

The viability of cells at the active concentrations of AMPS against studied Gram-negative bacteria (geometric means of MICs) was calculated by interpolation of data series of Figure 5 and was listed in Table 1. Both SΔ3S3-2mer-GS and S3-4mer-GS were not toxic and the toxicity of S3 and SΔ3 were negligible.

Table 1. The viability of HEK-293 cells after 48 hours' incubation with active concentrations of AMPs.

AMP	M_W (Da)	MIC_{GM} (µg/ml)	MIC_{GM} (µM)	Viability 48 hours (%)
SΔ3S3-2mer-GS	20680.59	47.29	2.29	98.3
S3-4mer-GS	20540.23	71.84	3.5	99.2
S3	3891.42	108.36	27.85	92.5
SΔ3	3961.5	65.59	16.56	91.4

AMP: Antimicrobial peptide

MIC_{GM}: Geometric mean of minimum inhibitory concentration

Discussion

The recombinant expression has received a lot of interest in cost-effective large-scale production of AMPs. Monomeric form expression of AMPs encounters with proteolytic degradations and many of AMPs have been expressed as tandem repeat multimeric forms (22). Previously, S3 peptide was expressed as a tetrameric form in *E. coli* with two different aspartic acid-proline (S3-4mer-DP) and glycine-serine linker (S3-4mer-GS). Baghbeshti and co-workers reported that using glycine-serine linker resulted in a 25% higher antimicrobial activity without any significant increase in its cytotoxicity (25). In the present study, two S3 peptides of S3-4mer-GS protein were substituted with SΔ3 peptide. SΔ3 has more LPS neutralization activity (9) and more hemolytic property than S3 peptide (26). According to the microbial susceptibility test of the present study, the geometric mean of MIC values (MIC_{GM}) of SΔ3 was 40% less than S3 peptide indication 40% higher antimicrobial activity. However, the expression of tandem repeat of SΔ3 seems to be problematic due to the toxicity of high positively charged AMPS for expression host organisms (22). Thus, we focused on tandem repeat two copies of SΔ3 and S3 peptides as fusion partners for each other, using glycine-serine linker (SΔ3S3-2mer-GS).

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An expression rate of 26% estimated by analyzing the target band intensity of SDS-PAGE result after induction with IPTG (Fig. 1C), proved successful expression of SΔ3S3-2mer-GS protein**.** This proper expression rate indicated that the toxic effect of highly positively charged SΔ3S3-2mer-GS (+6) on the *E. coli* host was compensated by the S3 fusion partner and glycine-serine linker. This result was in parallel with fusion expression of MSI-344 AMP (an analog of Magainin AMP) by the aid of a neutral fusion partner which led to the expression of fusion MSI-344 as inclusion bodies and with a rate of approximately 30% expression (32).

Statistical analysis of antimicrobial activity tests revealed that both SΔ3S3-2mer-GS and S3-4mer- GS proteins had lower MIC_{GM} values in comparison to their monomeric building blocks (S3 and SΔ3). The lower MIC_{GM} of SΔ3S3-2mer-GS (47.29 µg/ml) than S3-4mer- GS (71.84 µg/ml) indicated that using S3 and SΔ3 AMPs as fusion partners, enhanced the antimicrobial activity of tandem repeated fusion form about 28% (Fig. 4). According to the results of MTT assay, SΔ3S3-2mer-GS and S3-4mer-GS at concentrations of their MIC_{GM} values had no toxic effects on HEK-293 eukaryote cells (Table 1).

In this research, SΔ3 and S3 peptides were used as fusion partners and two copies tandem repeat of resulting fused protein was expressed in *E. coli* BL21. According to proper expression rate and enhanced antimicrobial activity and negligible cytotoxicity, fusion tandem repeated expression could be considered as an effective production strategy for obtaining large amounts of AMPs.

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Authors declare no conflict of interests.

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