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Engineering a Potent Anti-MRSA Agent: Development and Characterization of Chimeric Endolysin ZAM-MSC

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ABSTRACT

Background: The increasing prevalence of *S. aureus*, especially methicillin-resistant strains, poses a major healthcare threat due to limited therapies. To address this challenge, we engineered the chimeric endolysin ZAM-MSC as a potent antibiotic alternative, using domain-fusion strategies to enhance antibacterial activity. We designed ZAM-MSC by integrating the catalytic (M23) and cell wall-binding (SH3b) domains of lysostaphin with the catalytic domain (CHAP) from endolysin SAL-1. Structural optimization was performed using AlphaFold2 prediction, AutoDock Vina docking, and GROMACS simulations to evaluate domain interactions, protein stability, and binding dynamics.

Methods: The chimeric construct was cloned into pCold I, expressed in *E. coli*, and purified under solubility-optimized conditions. Purified ZAM-MSC, at a minimum concentration of 3 µg, reduced bacterial optical density within 15 minutes, demonstrating potent lytic activity. Thermal stability assays indicated that ZAM-MSC retained its enzymatic activity over 80–90% across 4-37 °C, with only a 10–20% decrease at 25-37 °C after 30 minutes. NaCl stability tests revealed maximal activity in the absence of NaCl, with a gradual reduction in enzyme activity by increasing NaCl concentrations.

Results: Cytotoxicity evaluation using the MTT assay on L929 fibroblast cells demonstrated cell viabilities of up to 90% even at the highest enzyme concentrations tested, indicating no significant cytotoxic effects compared with untreated control cells. Hemolysis assays confirmed nearly 100% red blood cell integrity across all tested enzyme concentrations, supporting its biocompatibility with mammalian cells.

Conclusion: Our findings establish ZAM-MSC as a highly promising therapeutic candidate, combining computational precision with robust experimental validation. ***DOI: 10.61882/ibj.5200***

Keywords: Endolysin, Lysostaphin, Methicillin-resistant *Staphylococcus aureus*

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List of abbreviations

BLAST: Basic Local Alignment Search Tool; **CBD:** Cell wall binding domain; **Csp:** cold shock proteins; **DAB:** 3,3'-Diaminobenzidine; **DMSO:** dimethyl sulfoxide; ***E. coli*:** *Escherichia coli*; **EDTA:** ethylenediaminetetraacetic acid; **GRAVY:** grand average of hydropathicity; **HRP:** horseradish peroxidase; **IPTG:** isopropyl β-d-1-thiogalactopyranoside; **LDDT**: local distance difference test; **MMPBSA**: Molecular Mechanics Poisson-Boltzmann Surface Area; **MRSA*:*** methicillin-resistant *Staphylococcus aureus*; **MSSA*:*** methicillin-sensitive *Staphylococcus aureus*; ***MTT*:** 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide; **Ni-NTA:** nickel-nitrilotriacetic acid; **PAE**: predicted aligned error; **PGS:** proline-enhanced glycine-serine; **ProDA:** protein design assistant; **PTCC**: Persian Type Culture Collection; **RBC**: red blood cell; **RMSD:** root mean square deviation; ***S. aureus:*** *Staphylococcus aureus*; **SDS-PAGE*:*** sodium dodecyl sulfate–polyacrylamide gel electrophoresis; **TSA:** tryptic soy agar; **TSB:** tryptic soy broth

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INTRODUCTION

Staphylococcus aureus is an opportunistic human pathogen that can cause a range of infections, from mild skin and soft tissue to severe acute infections and fatal septicemia. The risk of developing MRSA and other antibiotic-resistant strains has made *S. aureus* a serious concern[1]. Resistance to methicillin in *S. aureus* appeared shortly after penicillin resistance was first reported in 1940, with antibiotic-resistant colonies observed in 1950, leading to severe hospital-acquired infections[2]. Once resistance emerged, the process of developing resistance to other antibiotics accelerated, with methicillin resistance becoming particularly critical[2]. The success of antibiotics has thus proven temporary, and the long-term challenge of antibiotic resistance emphasizes the urgent need for alternative antimicrobial methods.

Phage lytic enzyme is called endolysin or lysin, which is a protein expressed during the terminal stage of the phage lytic cycle to destroy the bacterial cell wall (peptidoglycan) and release viral progeny. Endolysins targeting Gram-positive hosts typically contain a catalytic domain (known as the enzyme active domain or the enzyme catalytic domain) at the N-terminus and a cell wall-binding domain at the C-terminus[3]. Their high specificity enables them to target particular bacterial species or strains, thereby minimizing disruption of beneficial bacteria and reducing collateral damage to the microbiota[4]. Resistance to endolysins is rare due to their protected target sites and the limited bacterial resistance mechanisms against cell wall-acting enzymes[4]. Generally, endolysin exhibits low toxicity toward mammalian cells, as they specifically target bacterial cell wall components absent in mammalian cells, thereby minimizing the risk of side effects[4]. Despite promising alternatives to traditional antibiotics, native endolysins have notable disadvantages. Their efficacy can be compromised by instability under physiological conditions, potential loss of functional conformation when expressed as inclusion bodies, and narrow antibacterial spectra that restrict activity against diverse *S. aureus* strains[5-7]. In contrast, chimeric endolysins offer several advantages[8]. By combining domains from different endolysins, their activity[9], stability[10], and solubility[7,11] are enhanced; however, their antibacterial spectrum[5,11] is broadened, and the likelihood of resistance is reduced[12]. This modular approach not only enhances enzymatic activity but also increases the potential to overcome bacterial defenses, including biofilm formation[12,13], which is a significant barrier to effective treatment. The ability of these engineered proteins to penetrate and degrade biofilms further establishes them as valuable tools against antibiotic-resistant strains[14]. Among endolysins effective against *S. aureus*, eight specific enzymes, 80a, phi11, LysK, P68, 2638A, Twort, phiSH2, and WMY, along with the bacteriocin lysostaphin, have exhibited strong bactericidal activity, broad-spectrum efficacy, and biofilm removal capacity[5,15]. Recently, the endolysin SAL-1 has been isolated from phage SAP-1. Compared to LysK, SAL-1 shows approximately two-fold lower minimum inhibitory concentration, despite its highly similar amino acid sequences and protein structure. These two endolysins differ only in three amino acids: isoleucine instead of valine (position 26), glutamine instead of glutamic acid (position 114), and histidine instead of glutamine (position 486)[16].

Lysostaphin, first extracted from *Staphylococcus simulans* in 1960, is a potent antibacterial protein against *S. aureus*. It is a pre-protein containing a guide sequence, inhibitory region, catalytic domain, linker sequence, and cell wall-targeting sequence[17]. The catalytic domain of lysostaphin is located at the N-terminal end, belongs to the M23 family of peptidases, whereas the cell wall-targeting sequence is located at the C-terminus[17].

Lysostaphin exhibits strong antimicrobial activity; however, resistance has been reported in certain cases[18-20]. Studies have suggested that combining lysostaphin with endolysins, or incorporating its domains into chimeric constructs, can enhance efficacy and reduce resistance development[14]. Given these considerations, the present study focused on developing a chimeric endolysin, ZAM-MSC, that combines the M23 and SH3b domains of lysostaphin with the CHAP domain of SAL-1. Our objective was to enhance the antibacterial efficacy of the chimeric endolysin ZAM-MSC against antibiotic-resistant *S. aureus* strains. After its production and purification, we evaluated ZAM-MSC under different conditions to assess its antibacterial activity. This approach aims to provide a new antibacterial enzyme that mitigates the risks posed by antibiotic resistance and its associated infections.

MATERIALS AND METHODS

Design and bioinformatics analysis of chimeric ZAM-MSC

The chimeric endolysin ZAM-MSC was designed by incorporating the M23 and SH3b domains from lysostaphin, along with the CHAP domain from SAL-1. The overall structure of the CHAP domain in SAL-1 is highly similar to that of LysK; however, previous research has shown that due to three different amino acid residues in SAL-1 compared to LysK, the catalytic activity of SAL-1 is significantly higher than that of LysK[16]. Therefore, the CHAP domain sequence used in this study was sourced from the CHAP domain of

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SAL-1. A flexible PGS linker was employed to connect the domains, ensuring proper spatial orientation and functional integrity. To design the ZAM-MSC structure, we obtained the nucleic acid sequences of the SAL-1 CHAP domain and mature lysostaphin from relevant patents[21-23] and the GenBank database (accession nos. YP_009041293.1 and WP_013012297.1, respectively; www.ncbi.nlm.nih.gov/genbank/). The amino acid sequence of the chimeric endolysin was analyzed using the BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Protein modeling was conducted using Nanome virtual reality software (https://nanome.ai/) and AlphaFold2 software[24]. To evaluate the residue polarity, secondary structure, and residue type of the designated linker, ProDA[25] was used. ProtParam (https://web.expasy.org/protparam/) was employed to analyze the physicochemical properties. ProtParam (References/Documentation) is a tool that computes various physical and chemical parameters for specified proteins deposited in Swiss-Prot or TrEMBL or for user-entered protein sequences.

Modeling, docking, and molecular dynamics simulation

We utilized the AlphaFold2 software[24] within the ColabFold platform for protein modeling. To determine optimal binding interactions between ZAM-MSC and the components of the peptidoglycan cell wall, we performed protein-ligand docking analyses. In this context, penta-glycine was selected as the ligand due to its significance in targeting the bacterial cell wall, while lysostaphin served as the receptor protein in docking studies. The AutoDockVina software (AutoDockVina 1.2.7 version) was employed for molecular docking, and the Gromacs software (GROMACS 2022.6 version) was utilized to simulate molecular dynamics and evaluate the stability of the designed protein.

Construction of the ZAM-MSC coding gene

Prior to gene synthesis, the nucleotide sequence encoding ZAM-MSC was optimized using GenScript software (http://www.genscript.com/). Restriction sites for the NdeI and BamHI enzymes were identified at the 3' and 5' sites of the coding sequence, respectively. The designed gene was subsequently synthesized by ShineGene Biotechnologies (Shanghai, China) and cloned into a pUC57 backbone.

Bacterial strains, culture, subcloning, and protein expression

The ZAM-MSC coding sequence (GenBank accession no. PP784250) was subcloned from pUC57 into the pCold I expression vector using NdeI and BamHI restriction enzymes. The recombinant pCold I vector was introduced into the bacterial host, *E. coli* (DH5α). Subsequently, digestion and colony PCR were performed to confirm successful cloning. The pColdI expression vector was then transformed into *E. coli* BL21(DE3) Gold. The recombinant strain was cultured at 37 °C on Luria-Bertani agar medium supplemented with 100 µg/mL of ampicillin (Merck, Germany) for colony screening. Selected colonies were incubated in Terrific Broth medium, consisting of tryptone, yeast extract, glycerol, and salts KH_2PO_4 and K_2HPO_4 (all from Merck), enriched with ampicillin at 100 µg/mL. The process of protein expression was performed according to a previously published cold-shock protocol[26]. The bacterial cells were incubated until the OD_{600} of the cultures reached 0.9. Following a 30-minute cold shock at 15 °C, protein expression was induced by adding IPTG (Thermo Scientific™, USA) to a final concentration of 0.5 mM. Cells were incubated at 15 °C and harvested after 24 hours. Protein expression was evaluated by 12% SDS-PAGE gel.

Confirmation of recombinant protein expression by Western blot

The lysate of ZAM-MSC-expressing cells was separated by 12% SDS-PAGE (Bio-Rad, USA). After transferring the protein bands to a nitrocellulose membrane, the membrane was placed in a 5% blocking solution at 4°C overnight. The membrane was washed three times in TBS-T buffer (1×) for 10 minutes. Then, the nitrocellulose membrane was incubated at room temperature for 2 hours with gentle shaking in a 3% blocking solution containing anti-polyhistidine antibody-HRP (Sigma, A7058, USA) conjugate at a dilution of 1:2000. Afterwards, the membrane was washed three times for 10 minutes each in TBS-T buffer (1×) and then incubated with the HRP substrate solution, and DAB for 5-15 minutes until the bands became visible.

Protein purification

Cells expressing recombinant ZAM-MSC were isolated from the culture medium by centrifugation. Sonication was used to lyse bacteria, and soluble proteins were loaded onto a Ni-NTA chromatography column (Qiagen, Valencia, CA, USA) to purify the His-tagged recombinant protein. The Ni-NTA column was washed in four consecutive steps, each with 1 mL of a buffer containing 500 mM NaCl, 50 mM NaH_2PO_4, 20-30 mM imidazole, 20 mM 2-mercaptoethanol, 5% glycerol (pH 8). The bound protein was then eluted with a buffer containing 500 mM NaCl, 50 mM NaH_2PO_4, 250 mM imidazole, 20 mM 2-mercaptoethanol, and 5% glycerol (pH 8). To remove excess imidazole from the protein buffer, a dialysis process was performed using a

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buffer consisting of 500 mM NaCl, 50 mM NaH_2PO_4, and 10% glycerol at 4°C for 4 hours. Following dialysis, the glycerol concentration was increased from 10% to 25%. The concentration of purified protein was then determined using a bicinchoninic acid protein quantification kit (Parstous, Khorasan, Iran).

Determination of antibacterial activity against MRSA and MSSA

The lytic effects of ZAM-MSC were evaluated against the MRSA strain ATCC 33591 and the MSSA strain ATCC 29213. The bacterial strains were obtained from the PTCC of the Iranian Research Organization for Science and Technology (IROST, Tehran, Iran) and cultured on TSA medium.

Plate lysis assay

After transferring precultures of *S. aureus* (MRSA, ATCC 33591, and MSSA, ATCC 29213) to fresh TSB at a 1:50 dilution overnight, the bacterial cultures were incubated at 37 °C until an OD_{600} of 0.3-0.6 (exponential phase) was reached. After centrifugation (6654 × g, 10 min), the cell pellets were resuspended in 50 mM Tris buffer, pH 8.5, and the suspension was adjusted to an OD_{600} of 0.2. The bacterial suspension was then spread evenly onto the TSA plates using a sterile cotton swab. The purified recombinant protein was diluted in phosphate buffer containing 500 mM NaCl and 50 mM NaH_2PO_4. Next, 10 µL of enzyme solutions at (final concentrations of 1550, 775, 387, and 193 µg/mL) were spotted onto the freshly plated bacterial cells on TSA plates and dried under a laminar flow hood for 30 min. To ensure complete drying of the plate surface, the spotted plate was incubated for 15 min without a lid in a laminar flow hood. The plates were then incubated at 37 °C for 24 h, after which the clear (lysis) halos around the enzyme spots were measured to assess antibacterial activity.

Turbidity reduction assay

The lytic activity of the chimeric endolysin ZAM-MSC was investigated using the turbidity reduction assay method. Overnight cultures of *S. aureus* (MRSA, ATCC 33591, and MSSA, ATCC 29213) were transferred (1:50) into fresh TSB. The bacterial culture was incubated at 37 °C until an OD_{600} of 0.3-0.6 (exponential phase) was reached. After centrifugation (6654 × g, 10 min), the cell pellets were resuspended in 50 mM Tris buffer, pH 8.5, and the suspension was adjusted to an OD_{600} of 0.4. Turbidity reduction assay was performed in a 96-well plate (SPL, Korea), in which 100 µL of the bacterial suspension was mixed with 100 µL of enzyme solution (diluted in Tris buffer) at concentrations ranging from 1550 µg/ml to 3 µg/mL. The decrease in absorbance at 600 nm was monitored using a multimode microplate reader (Cytation 5 Cell Imaging Multimode Reader, BioTek, United States) at 37 °C for 30 minutes, and OD_{600} readings were recorded every two minutes. Before each reading, the plate was automatically shaken for 20 seconds. The buffer without endolysin served as the negative control.

Thermal stability

To evaluate the thermal stability of the designed enzyme, we incubated ZAM-MSC at different temperatures (4-55 °C) for 30 minutes. After treatment, 100 µL of enzyme solution was added to a 96-well plate containing 100 µL of a bacterial suspension. Turbidity reduction was then monitored at 37 °C for 30 minutes, with OD_{600} measurements recorded every two minutes.

Effect of NaCl on protein activity

To study the effect of different NaCl concentrations on the activity of the designed chimeric endolysin ZAM-MSC, we dissolved ZAM-MSC in 50 mM Tris buffer containing NaCl at different concentrations, ranging from 0 to 1000 mM. Subsequently, 100 µL of this solution was combined with 100 µL of bacterial suspension in a 96-well plate to analyze the enzyme activity. Turbidity reduction was observed at 37 °C for 30 min.

Cytotoxicity assay

The viability of L929 cells treated with ZAM-MSC was assessed using the MTT assay to determine the potential cytotoxic effect. For the MTT assay, which measured the impact of ZAM-MSC with different concentrations on the L929 cell line viability, cells were cultured in RPMI medium (Gibco, Germany) supplemented with serum and maintained at 90% humidity and 5% CO_2 at 37 °C. Different concentrations of ZAM-MSC (500-3.9 µg/mL) were prepared by diluting the stock solution in RPMI medium. After seeding into 96-well plates, the cells were treated with various concentrations of ZAM-MSC in triplicate, along with control wells that did not receive ZAM-MSC treatment. After incubating the cells with ZAM-MSC for 24 hours, MTT solution was added to each well and incubated at 37 °C for 3 hours. Formazan crystals formed by viable cells were dissolved in DMSO (Sigma-Aldrich, pro. No. D4540), and the absorbance of the resulting solution was measured at 570 nm using a microplate reader. The absorbance values obtained from the MTT assay were used to calculate cell viability. The viability of the L929 cells was estimated as follows:

$$\text{Viability (\%)} = (OD_{\text{Sample}} - OD_{\text{Positive control}})/(OD_{\text{Negative control}} - OD_{\text{Positive control}}) \times 100$$

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All experiments were performed in triplicate to ensure reproducibility. Triton X-100 as the positive control and RPMI medium as the negative control were included in each experiment.

Hemolytic assay

Human blood was collected in a tube containing EDTA anticoagulant solution. RBCs were isolated by centrifuging the blood at 500 ×g at 4°C for 10 min, washing three times with 0.9% NaCl solution, and centrifuging after each wash. Finally, the RBC pellet was resuspended in 0.9% NaCl to prepare a 2% RBC suspension. To assess binding/hemolytic activity, ZAM-MSC (500-0.976 µg/mL in 0.9% NaCl) was incubated with 50% RBC suspension at 37°C for 1 h with gentle shaking. After centrifugation, supernatant absorbance was measured at 490 nm using a spectrophotometer (Thermo ND-2000). The negative control consisted of a RBC solution containing 0.9% NaCl, and the positive control consisted of a RBC solution comprising Triton X-100. Finally, the percentage of hemolysis was calculated using the following formula:

$$\text{Hemolysis (\%)} = (OD_{\text{Sample}} - OD_{\text{Negative control}})/(OD_{\text{Positive control}} - OD_{\text{Negative control}}) \times 100$$

Statistical Analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) to compare differences between study groups. All analyses were conducted using GraphPad Prism version 9 (GraphPad Software, Inc., Chicago, IL, USA). Data are presented as mean ± standard error of the mean, and differences were considered statistically significant at $p < 0.05$.

RESULTS

Computational design and bioinformatics assessment of the chimeric ZAM-MSC protein

According to the M23 domain cleavage site within the pentaglycine chain of peptidoglycan, one glycine residue in the native linker between M23 and SH3b was converted to proline to prevent endolysin self-digestion. In our engineered endolysin, we substituted valine (position 26) and glutamic acid (position 113) in the CHAPk domain of LysK with isoleucine and glutamine, respectively, to enhance catalytic activity[16]. The M23 and SH3b domains were linked via a native linker, while the C-terminal of the SH3b domain was connected to the N-terminal of the CHAP domain via a designed synthetic linker. The selection of a flexible linker derived from human proteins was essential for this design. Using the ProDa database, we identified the sequence GSSSGSG, which is commonly found in human proteins (PDB codes: 1R79, 1UFW, 1UF0, 1UEM, 1UDL, and 1UCV). We implemented two copies of this sequence separated by a proline residue. Although the GGGGS linker is frequently employed for its flexibility, it was avoided herein due to its similarity to the M23 substrate recognition sequence. The final designed linker sequence was GSSGSSGPGSSGSSG. The engineered ZAM-MSC chimeric endolysin (Fig. S1) combines functional domains from lysostaphin and the SAL-1 CHAP domain to optimize lytic activity. In the refined ColabFold model (Fig. S1A), the lysostaphin component, comprising the M23 peptidase and SH3b domains, was connected to the CHAP domain via a synthetic linker, with a critical glycine substitution at position 392. The schematic structure of ZAM-MSC (Fig. S1C) shows a fusion protein in which mature lysostaphin (M23 and SH3b domains linked natively) is C-terminally fused to the SAL-1 CHAP domain through a flexible PGS linker (Fig. S1B). Functionally, the M23 domain provides catalytic activity targeting bacterial cell walls, the SH3b domain facilitates specific binding to the bacterial surface, and the CHAP domain contributes to an additional catalytic function, altogether enhancing the enzyme's antibacterial efficacy. Physicochemical parameters were calculated using ProtParam and included protein length, extinction coefficient, molecular weight (Da), chemical formula, secondary structure composition, amino acid composition (total number of negatively charged residues (Asp + Glu), and total number of positively charged residues (Arg + Lys), instability index, theoretical pI, GRAVY, and aliphatic index. As shown in Table S1, the aliphatic index increased slightly, suggesting potential effects on the thermo-stability of modular proteins. Proteins with higher aliphatic indices generally contain more hydrophobic amino acids, which can contribute to stabilizing protein structures. These hydrophobic interactions play a crucial role in maintaining protein folding and conformational stability. The negative GRAVY index of ZAM-MSC indicates a predominantly hydrophilic interaction of this protein with water.

Modeling of the chimeric ZAM-MSC

To obtain the optimal structure, we utilized AlphaFold2 and then selected the model with the highest confidence score from the generated predictions[27]. Figure 1A displays the alignment error estimated for each residue, where the blue color indicates the lowest error. As can be seen in the Figure, across all five top-ranked models, the three domains of the structure are modeled with the lowest error. Figure 1B estimates the local distance difference test values for each position, where the high score of this value means that the designed model is accurate.

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Molecular Docking of Penta-Glycine Binding to Lysostaphin and ZAM-MSC

Molecular docking of penta-glycine, as a ligand, was performed against both native lysostaphin and our designed chimeric protein using AutoDock Vina software[28]. The protein-ligand binding sites are clearly visible in Figure 2A. The optimal binding conformation demonstrates a binding energy of -4.2 kcal/mol. Figure 2B illustrates the protein-ligand binding sites, with the optimal binding conformation exhibiting a binding energy of -4.6 kcal/mol. The molecular docking results reveal a strong agreement with crystallographic data (PDB: 5LEO), showing that the chimeric endolysin CBD maintains the native binding conformation of lysostaphin SH3b domain.

Molecular dynamics simulation

Following molecular docking, we focused on optimizing the structure of the compound and precisely calculating the protein-ligand binding affinity. For this purpose, we used the complex file—containing information about the ligand optimal binding site on the protein—as input for molecular dynamics simulations in GROMACS software (Fig. S2). The simulation was conducted for 100 nanoseconds.

Results of RMSD analysis

RMSD analysis was employed to assess protein structural equilibrium. As depicted in Figure S3A, the protein reached equilibrium rapidly, stabilizing in approximately 40 nanoseconds. Furthermore, Figure S3B shows that the radius of the lysostaphin molecule remains relatively constant. Similarly, the radius of the ZAM-MSC molecule did not change significantly after 40 nanoseconds, indicating that the system has reached equilibrium.

Results of MMPBSA analysis

The results from both simulation systems indicate that the binding affinity of penta-glycine in the designed structure is similar to that of lysostaphin. This observation suggests that the affinity of the lysostaphin domains used in the designed structure has not diminished. The MMPBSA analysis results are presented in Table S2.

Confirming the subcloning of the constructed sequence into pColdI

The successful insertion of the ZAM-MSC gene into the pCold I vector was verified by colony PCR followed by 1% agarose gel electrophoresis in 1× TAE buffer and staining with safe stain. Multiple transformed colonies

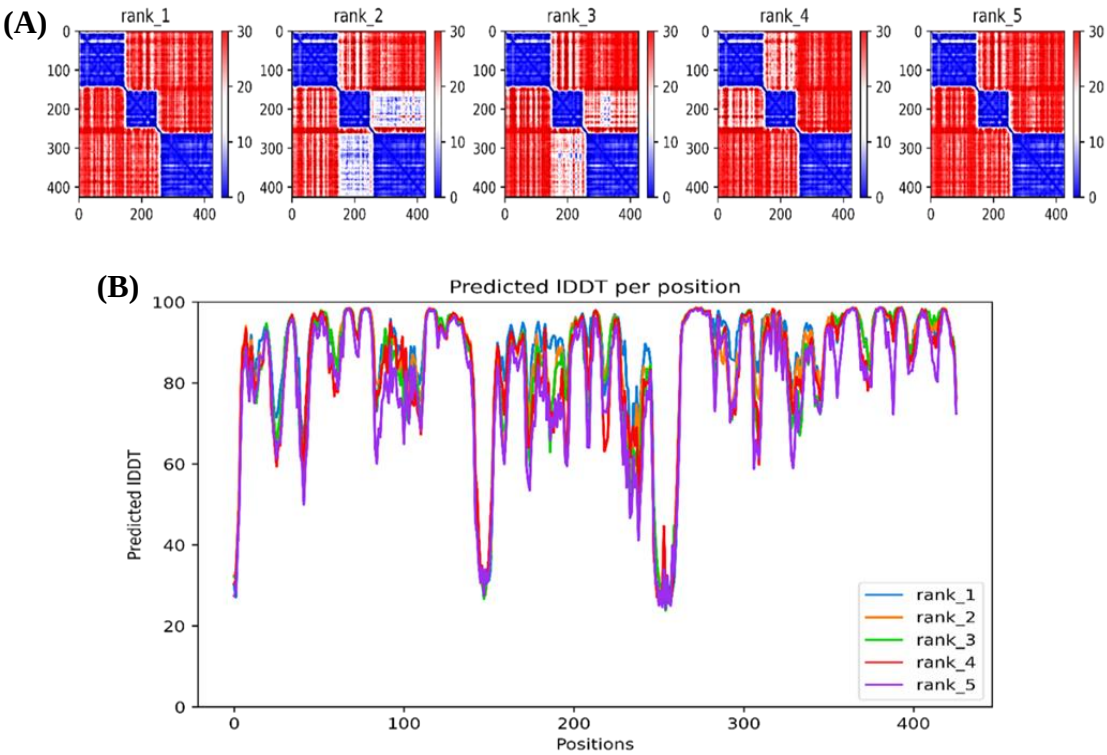

Fig. 1. Structural and molecular dynamics analysis of lysostaphin and chimeric protein interactions with penta-glycine ligand. (A) Predicted aligned error matrix, colored from blue (low error) to red (high error); (B) predicted local distance difference test as a function of residue number.

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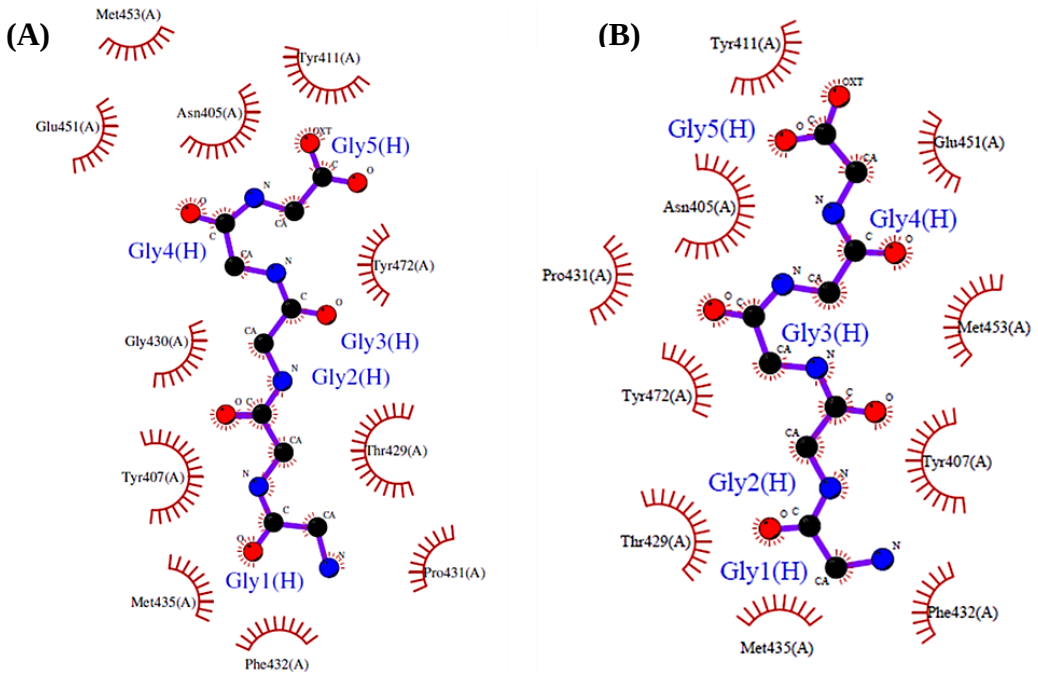

Fig. 2. Molecular docking. Amino acid interactions with pentapeptide ligand in (A) lysostaphin docking and (B) chimeric protein docking.

were randomly selected for PCR analysis. Agarose gel analysis revealed the amplified target sequence, indicating that the ZAM-MSC gene was successfully inserted into the pCold I vector. An empty pCold I vector was included as a negative control. The presence of a 1293 bp band confirmed the accuracy of the cloning of the ZAM-MSC gene into the pCold I vector (Fig. S4).

Expression and production of the chimeric ZAM-MSC protein

Protein expression was evaluated using cold-shock induction, followed by comparison of the induced and non-induced cell lysates (Fig. S5A). SDS-PAGE analysis demonstrated that cold-shock induction combined with the addition of IPTG to the medium successfully induced the expression of ZAM-MSC protein (46 kDa). The produced recombinant protein was detected and confirmed by Western blotting (Fig. S5B).

Recombinant ZAM-MSC purification

The pColdI vector contains a hexa-His tag (His6) sequence, downstream of the translation-enhancing element. Hence, Ni-NTA affinity chromatography was employed to isolate the recombinant protein product. The quality of protein purification was analyzed by running the samples on an SDS-PAGE gel (Fig. 3). Based on the observations from the gel, the purified ZAM-MSC protein appeared in the soluble phase as a single band of 46 kDa.

Evaluating the antibacterial activity of ZAM-MSC against MRSA

The lytic activity of endolysin ZAM-MSC was verified by the plate lysis method. The clear zones formed on the plate demonstrate the antibacterial activity of endolysin at different concentrations (1550, 775, 387, and 193 µg/ml; Fig. S6). To confirm

Fig. 3. Protein purification using Ni-NTA affinity chromatography. M: protein molecular marker (protein with a known molecular weight of 45 kDa); E1-E9: different elution fractions.

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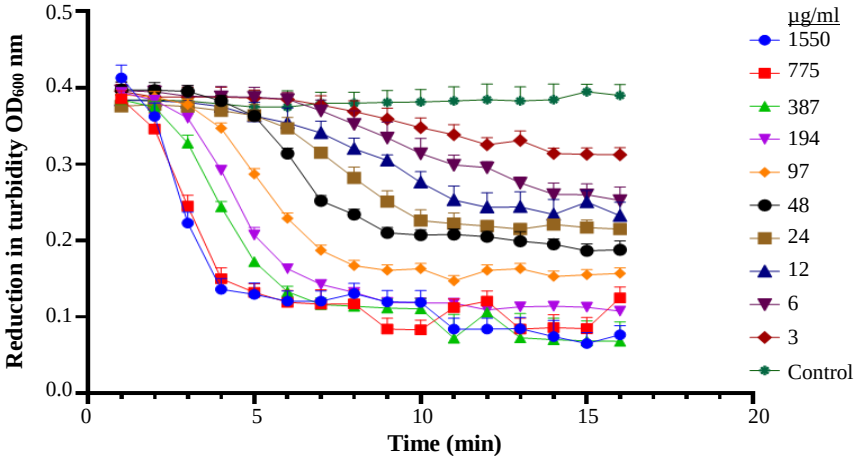

Fig. 4. Assessment of the lytic potential of varying ZAM-MSC concentrations against MSSA in TSB medium. Turbidity reduction assays (OD_{600} nm) show that ZAM-MSC at 1550–194 µg/mL rapidly decreased bacterial density within 7 minutes, regardless of concentration. At lower concentrations (<194 µg/mL), the lysis rate was concentration-dependent. Even very low amounts (3 µg/mL) eventually caused complete lysis over a longer period. All data are presented as mean ± standard error of the mean.

the antibiotic resistance phenotype of the MRSA strain and highlight the efficacy of ZAM-MSC against it, a cephalexin antibiogram disk was included as a negative control. According to the data obtained from the turbidity reduction assays (Fig. 4), concentrations ranging from 1550 to 194 µg/ml of ZAM-MSC decreased the OD_{600} nm of *S. aureus* PTCC1917 within seven minutes, independent of the enzyme concentration. However, at concentrations lower than 194 µg/ml, the rate of reduction was dependent on the enzyme concentration. Notably, bacterial lysis was observed even at very low concentrations, such as 3 µg/ml, though a longer time was required for complete bacterial lysis at these low concentrations.

Evaluation of the thermal stability of ZAM-MSC

ZAM-MSC was incubated with buffer at temperatures ranging from 4 °C to 55 °C for 30 minutes, after which the treated enzymes were used for turbidity reduction assays. A combination of endolysin and buffer without thermal incubation was used as a control. Figure 5 reveals that endolysin activity decreases with increasing temperature. These results indicate that temperatures beyond the optimal range (4-37 °C) could reduce endolysin efficacy.

Effect of NaCl on ZAM-MSC activity

The effect of different NaCl concentrations on ZAM-MSC activity was evaluated to determine its salt tolerance and optimal conditions for enzymatic function. As shown in Figure 6, the highest enzymatic activity was observed in the absence of NaCl, indicating that salt negatively affects enzyme function. Increasing NaCl concentrations likely led to a gradual reduction in activity, suggesting that ZAM-MSC is sensitive to ionic interactions of NaCl that may alter its structure and catalytic efficiency.

Cytotoxic effect of ZAM-MSC on L929 fibroblast cell line

The MTT assay was performed to evaluate the cytotoxic effect of ZAM-MSC at different concentrations on the L929 fibroblast cell line. Cells were exposed to varying concentrations of ZAM-MSC, ranging from 1 to 500 µg/ml in triplicate, with untreated samples

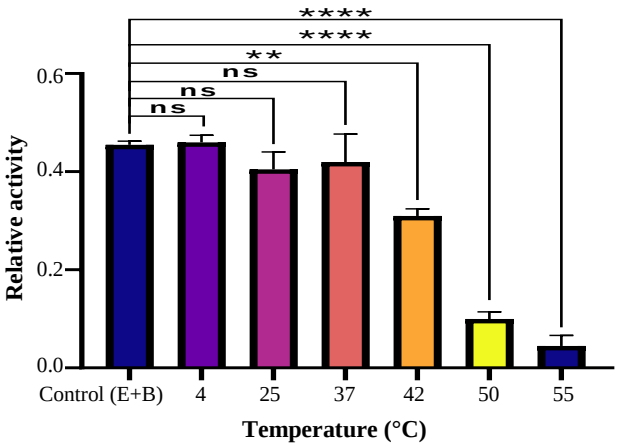

Fig. 5. Thermal stability of ZAM-MSC. ZAM-MSC endolysin activity was evaluated across a temperature range of 4-55 °C. The enzyme was most effective at 4 °C and 37 °C, with reduced activity observed at higher temperatures. All data are presented as mean ± standard error of the mean. Statistical significance is indicated as $^{**}p < 0.01$; $^{****}p < 0.0001$; ns: not significant.

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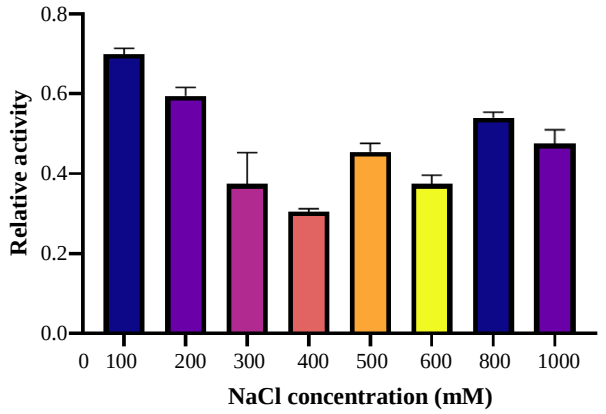

Fig. 6. NaCl tolerance of ZAM-MSC. ZAM-MSC activity was evaluated across different NaCl concentrations ranging from 0 to 1000 mM. The highest activity was observed in the absence of NaCl, with activity steadily declining as salt concentration increased. All data are presented as mean ± standard error of the mean.

serving as controls. After incubation, the absorbance of the formazan solution was measured at a specific wavelength. The results showed a high cell viability across all tested concentrations, with no significant decrease in cell viability compared to the control group. The control group exhibited a viability rate of nearly 100%, while ZAM-MSC concentrations between 3.9 µg/ml and 500 µg/mL maintained viability rates above 50%. Notably, statistical analysis indicated a significant difference ($p < 0.0001$) between the control and the highest concentration of ZAM-MSC (500 µg/ml), suggesting that while ZAM-MSC does not adversely affect L929 cell viability, the highest concentration may warrant further investigation for potential cytotoxic effects (Fig. 7).

Hemolytic effect of ZAM-MSC on RBC

The hemolytic assay was performed to evaluate the effect of ZAM-MSC on RBCs. RBCs were exposed to varying concentrations of ZAM-MSC, ranging from 1 µg/mL to approximately 500 µg/mL, in triplicate. Control samples without ZAM-MSC treatment were also included. After incubation, the absorbance of the released hemoglobin was measured at a specific wavelength. The results demonstrate that RBCs exposed to any concentration of ZAM-MSC endolysin did not exhibit lysis, with almost 100% of RBCs remaining intact. These observations indicated that the chimeric endolysin has no adverse effects on human RBCs (Fig. 8). This is a promising indication of the safety profile of ZAM-MSC, which is essential for its potential use in medical treatments.

DISCUSSION

Due to the resistance of *S. aureus* to most available antibiotics, there is an urgent need for effective MRSA treatments, particularly for life-threatening hospital-acquired infections. In recent years, several alternative antibacterial approaches have emerged, with endolysins demonstrating exceptional target specificity compared to conventional antibiotics.

In this research, we performed in silico analysis and successfully expressed and purified a novel three-domain chimeric endolysin containing the catalytic (M23) and cell wall-binding (SH3b) domains of lysostaphin, along with the CHAP domain of SAL-1. Notably, while the overall structure of the CHAP domain of SAL-1 closely resembles that of LysK, previous studies[16] have demonstrated that three distinct amino acid residues in SAL-1 confer dramatically higher catalytic activity compared to LysK. Therefore, we specifically selected the SAL-1 CHAP domain for our construct and evaluated its antimicrobial activity against *S. aureus*. For this study, we exclusively used the CHAP domain of SAL-1 (comprising the first 165 amino acids) while omitting the amidase-2 domain. This truncated version showed significantly enhanced activity and solubility compared to the full-length protein[29,30].

Our approach builds on previous research showing that strategic amino acid substitutions within catalytic domains can substantially improve endolysin activity[31-33]. Accordingly, valine at position 26 and glutamic acid at position 113 of the CHAPk domain

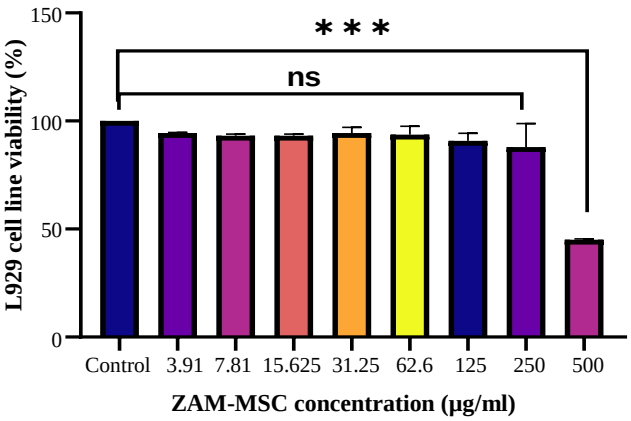

Fig. 7. The effect of ZAM-MSC concentrations on the viability of L929 cell lines. The L929 cell line was treated with increasing concentrations of ZAM-MSC (3.91-500 µg/ml). All tested concentrations maintained cell viability above 50%, with significant differences observed at higher concentrations. Data are presented as mean ± standard error of the mean. Statistical significance is indicated as $^{***}p < 0.001$; ns: not significant.

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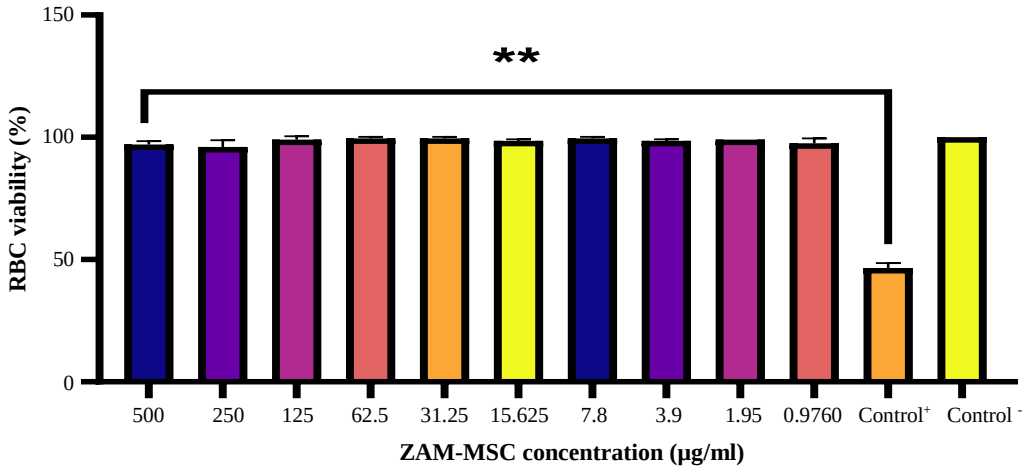

Fig. 8. Hemolytic Assay on human red blood cells, a range of ZAM-MSC concentrations (500 to 0.976 µg/ml) exposure to RBCs, and the cell lysis rate was calculated. Each column is related to a certain concentration. ZAM-MSC endolysin showed no hemolytic activity across all tested concentrations, with red blood cell integrity maintained at nearly 100%. All data are presented as mean ± standard error of the mean. Statistical significance is indicated as $^{**}p < 0.01$.

were replaced with isoleucine and glutamine, respectively[16]. The SH3b domain used in ZAM-MSC was derived from lysostaphin, based on prior experimental evidence demonstrating its superior ability to enhance bactericidal activity compared to the binding domain of LysK[34]. A synthetic PGS linker was designed to fuse the CHAP domain to lysostaphin. This flexible linker serves two key functions: (1) it maximizes the freedom of movement for the catalytic domains while preventing interdomain interference[31], and (2) its composition makes it resistant to human serum proteases. In addition, by substituting proline with glycine in the native linker sequence between M23 and SH3b domains, we reduced the risk of self-digestion. Furthermore, incorporating a sufficient number of glycine residues into the linker enhances its flexibility, whereas proline residues promote extended conformations[35].

By comparing the binding pose energies of native lysostaphin and the chimeric protein, this study provides a detailed analysis of their interaction landscapes. These energy values are critical as they represent the binding energy between each protein and its corresponding ligand, indicating the stability and strength of these interactions. The results demonstrated that both proteins achieved favorable energy values, establishing that native lysostaphin and the chimeric protein form energetically favorable and make stable interactions with their cognate ligands. However, it is worth noting that the chimeric protein exhibited slightly stronger binding affinity for its ligand than native lysostaphin. In addition, the study indicated that the binding affinity of penta-glycine in the newly designed structure was comparable to that of lysostaphin. This finding indicates that the designed structure successfully retains lysostaphin key binding characteristics, which are essential for its potential applications in drug development and biotechnology. These results provide valuable insights that could guide future research advances in these fields. The key design innovation of this endolysin lies in the unique arrangement of its catalytic domains relative to the CBD. While most characterized endolysins against *S. aureus*, whether native or chimeric, have their catalytic domains at the N-terminus and the CBD at the C-terminus, the recombinant chimeric endolysin ZAM-MSC arranges the M23 and CHAP catalytic domains on either side of the SH3b domain[6,36,37]. Unlike previous chimeric endolysins such as CHAPk-SH3bk, LYZ2-SH3b, and ClyC, our designed ZAM-MSC endolysin features a unique domain arrangement in which the M23 and CHAP catalytic domains flank the SH3b CBD[8,38,39]. This arrangement of domains also showed improved activity in in vitro assays, in which the enzyme reduced the initial OD_{600} in a shorter period of time, demonstrating rapid bacteriolytic action.

The challenges associated with recombinant production of endolysins include low expression levels, aggregation into inclusion bodies, and limited solubility. However, designing and expressing chimeric endolysins can partially address these concerns[7,40,41]. To overcome these challenges, we utilized the pCold I vector in this study. In pCold vectors, protein expression is tightly regulated by the *E. coli* cspA promoter. This cold-inducible promoter enables efficient protein production at lower temperatures, which benefits proteins prone to aggregation or instability at higher temperatures. Protein expression levels and solubility under this promoter

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system are significantly higher than those achieved with the T7 system[42-47]. To enhance translation initiation, the pCold vector incorporates a translation-enhancing element consisting of a five-codon sequence, positioned after the cspA 5′-UTR[26]. The downshift of temperature from 37 °C to 15 °C induces high expression of cspA, and these levels during temperature reduction are particularly suitable for soluble protein expression[45]. Using ImageJ software, 55% of the expressed endolysin was in the soluble phase relative to the total protein content. Although solubilization of endolysins from inclusion bodies may appear advantageous for improving activity, it can be ineffective due to loss of functional conformation during aggregation. Therefore, direct production of soluble endolysins is preferred for maximizing activity[48-50].

Our results demonstrated a potent antibacterial activity of purified chimeric endolysin ZAM-MSC against both MRSA and MSSA. First, various concentrations of ZAM-MSC produced distinct inhibition zones on agar plates, confirming its bacteriolytic capability. Second, turbidity reduction assays revealed a strong antimicrobial activity even at minimal concentrations, with OD_{600} values decreasing rapidly from an initial 0.2 to near-zero within seven minutes. These observations provide robust evidence for ZAM-MSC effectiveness. For comparative analysis, we included cephalexin. While cephalexin showed limited efficacy against MRSA, ZAM-MSC showed potent bactericidal activity against this resistant strain, highlighting its therapeutic potential.

ZAM-MSC chimeric endolysin remained active across a range of NaCl concentrations, though its antibacterial efficacy was inversely correlated with salt concentration. Maximal activity was observed in the complete absence of NaCl, but the enzyme retained its activity even in the presence of NaCl up to 500 mM, which was comparable to the stability observed for ClyC and $CHAP_K$-$SH3b_K$[8,38]. This stability, especially under saline conditions, indicates ZAM-MSC potential for use in diverse environments.

The MTT assay evaluated the impact of ZAM-MSC on L929 fibroblast viability with ZAM-MSC concentrations from 0 to 500 µg/ml, showing no significant cytotoxicity. These findings hold particular importance for developing novel antimicrobial therapies. The ability of the ZAM-MSC endolysin to target specific bacterial cells without affecting eukaryotic cells and human RBCs is a desirable characteristic for clinical applications. This selectivity presents a targeted approach to treating bacterial infections while minimizing potential harm to human cells.

This research introduces ZAM-MSC, an innovative chimeric endolysin with a unique domain architecture that differs fundamentally from conventional designs. Through strategic amino acid substitutions, its catalytic efficiency and structural stability were significantly enhanced. We evaluated ZAM-MSC antibacterial activity against MRSA and optimized its operating conditions, including temperature stability and suitable salt concentration. These collective findings presented ZAM-MSC as a potent antibacterial agent, offering a promising alternative to conventional antibiotics in the near future. Despite these remarkable advancements, further studies are ongoing to elucidate additional facets of this novel endolysin.

CONCLUSION

In this research, we designed a chimeric endolysin, ZAM-MSC, which has a new domain arrangement distinct from all previously reported chimeric endolysins. Its catalytic performance and structural stability were improved by replacing some amino acids. The antibacterial activity of this endolysin was evaluated against MRSA. Also, the optimum conditions for the activity of this enzyme, including temperature stability and suitable salt concentration, were determined. In sum, the findings and observations of this research indicate that ZAM-MSC has high potential as an antibacterial drug and a promising alternative to antibiotics in the near future. However, more studies and experiments are being conducted to reveal the different aspects of this endolysin.

DECLARATIONS

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Ethical approval

Not applicable.

Consent to participate

Not applicable.

Consent for publication

All authors reviewed the results and approved the final version of the manuscript.

Authors' contributions

AN: methodology and writing–original draft; ZAB: conceptualization, supervision, funding acquisition, analysis and interpretation of data, writing–review, and editing; SM: writing–review and editing; FA: methodology; SSA: bioinformatic analysis; MMJ: review and editing.

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Data availability

All data produced or examined in the course of this study are contained within this published article.

Competing interests

The authors declare that they have no competing interests.

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Supplementary information

The online version contains supplementary material.

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