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SNAC-Mediated Antimicrobial Peptide Production: Comparison with Enterokinase Digestion

Meihereayi Mutailifu
Kashi University
Danni Chen
Kashi University
Xutong Wang
Kashi University
Maierhaba Sailaijiang
Kashi University
Yuning Zhang
Kashi University
Kun Xie
xk_biology2@126.com

Kashi University

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Abstract

Antimicrobial peptides (AMPs) are promising alternatives to conventional antibiotics, but their small size and host cytotoxicity hinder direct recombinant production. In this study, six AMP genes (LK-24, GSHHW-LK-24, ZY4, GSHHW-ZY4, Mast-MO and GSHHW-Mast-MO) were synthesized by primer-complementary PCR and cloned into pET32a, generating two series of vectors that carry either the enterokinase recognition site DDDDK or the sequence-specific nickel-assisted cleavage (SNAC) tag GSHHW. The fusion proteins were expressed in *Escherichia coli* BL21(DE3) by IPTG induction and auto-induction, purified from the soluble fraction by Ni-NTA affinity chromatography, and cleaved by enterokinase digestion or SNAC-mediated chemical cleavage. Antibacterial activity was evaluated by zone-of-inhibition and minimum inhibitory concentration (MIC) assays against *E. coli* ATCC 8739 and *Staphylococcus aureus* ATCC 25923. All six AMPs were active, with inhibition zone diameters of 6.0-9.5 mm and MIC values of 4–32 μg/mL. The peptides released by SNAC cleavage gave slightly larger inhibition zones (7.0-9.5 mm) and two-fold lower MIC values (4–16 μg/mL) than those released by enterokinase digestion (6.0-8.5 mm; 8–32 μg/mL), although the differences were not statistically significant. SNAC-tag chemical cleavage therefore provides a cost-effective, protease-free strategy for the efficient preparation of recombinant AMPs.

Introduction

Antimicrobial peptides (AMPs) are a class of innate immune effector molecules that occur ubiquitously in living organisms. They typically comprise 12–50 amino acid residues and exhibit multifunctional properties, including broad-spectrum antimicrobial activity, low susceptibility to resistance development, and immunomodulatory functions (Chen and Jiang 2023; Moretta et al. 2021). With the escalating global crisis of antibiotic resistance, AMPs have emerged as leading candidates for the replacement of conventional antibiotics (Bucataru and Ciobanasu 2024; Rima et al. 2021). Nevertheless, the large-scale application of AMPs is constrained by two major bottlenecks: the extremely low yield and prohibitive cost associated with natural extraction, and the high expense of chemical synthesis, which renders it impractical for industrial-scale production (Kordi et al. 2024; Lou et al. 2025). Genetic engineering technology-encompassing gene cloning, expression vector construction, recombinant protein induction in host cells, and downstream purification-offers a feasible pathway for the cost-effective and scalable production of AMPs (Deo et al. 2022; Zhang et al. 2022).

The heterologous expression of AMPs in *Escherichia coli* presents several challenges. Due to their small molecular weight (typically 2–5 kDa), AMPs are highly susceptible to degradation by host proteases. Moreover, cationic AMPs exert membrane-disruptive effects on the host bacterium, and their direct expression frequently results in host cell death (Sampaio de Oliveira et al. 2020; Oliveira Júnior et al. 2025). To circumvent these limitations, fusion expression strategies are widely employed, in which the target AMP is fused to a larger carrier protein to enhance expression level and solubility, followed by specific cleavage to release the mature peptide (Ning et al. 2025; Tariq et al. 2026). Commonly utilized fusion tags include thioredoxin (Trx), SUMO, glutathione S-transferase (GST), and the small metal-binding

protein SmbP (Kaur et al. 2020). In recent years, several novel small-molecule fusion tags have been developed, such as the 1 kDa acidic fusion tag and the C-tag (EPEA), which improve expression yields while minimizing the impact of the tag itself on product recovery (Zhang et al. 2021; Ojima-Kato 2024).

Conventional methods for fusion tag removal include enzymatic and chemical cleavage approaches. Enterokinase is the most established proteolytic tool; it specifically recognizes the DDDDK sequence and cleaves at its C-terminus without leaving any residual amino acids, and it has been extensively applied in commercial vectors such as pET32a (Shafiee et al. 2024). The SUMO fusion system exploits the structure-specific recognition by SUMO protease, enabling scarless cleavage (Xu et al. 2023). TEV protease is another commonly employed tool enzyme that recognizes a specific sequence and cleaves efficiently (Li and Sousa 2012). However, enzymatic cleavage methods are limited by the high cost of the protease, the necessity for additional removal of the enzyme after digestion, and suboptimal cleavage efficiency for certain substrates (Yao et al. 2025). Recently, Dang et al. developed the SNAC (sequence-specific nickel-assisted cleavage) tag technology, which achieves site-specific cleavage of fusion proteins through Ni^{2+}-mediated sequence-specific chemical hydrolysis (Dang et al. 2019). The core sequence of the SNAC tag is -GSHHW-, which undergoes efficient cleavage between the glycine (G) and serine (S) residues under mild conditions (1 mM $NiCl_2$, 0.1 M CHES buffer, pH 8.2, room temperature), yielding -G and SHHW- fragments with cleavage efficiencies exceeding 90% (Huang et al. 2020). Compared with conventional enzymatic approaches, SNAC chemical cleavage offers the advantages of operational simplicity, low cost, and the elimination of exogenous protease addition, making it particularly amenable to large-scale production (Qi et al. 2026).

In the present study, three representative engineered AMPs-LK-24, ZY4, and Mast-MO-were selected as model peptides. LK-24 is a novel AMP developed in our laboratory through molecular redesign based on SAAP-148, and exhibits antimicrobial activity far superior to that of the parent peptide (unpublished data). ZY4, designed by Lai et al., is a cationic peptide comprising 17 amino acid residues whose two cysteine residues can form an intramolecular disulfide bond; it demonstrates potent and rapid bactericidal activity against multidrug-resistant (MDR) *Acinetobacter baumannii* and *Pseudomonas aeruginosa* (Mwangi et al. 2019). Mast-MO is a rationally redesigned AMP derived from wasp venom that displays antimicrobial activity against clinically relevant Gram-negative bacteria (e.g., *E. coli*, *P. aeruginosa*) and Gram-positive bacteria (e.g., *S. aureus*), with efficacy comparable to that of standard antibiotics such as gentamicin and imipenem in both *in vitro* and *in vivo* assays (Silva et al. 2020), rendering it a highly promising antibiotic candidate. Based on the primary structures of LK-24, ZY4, and Mast-MO, codon-optimized nucleotide sequences were designed according to the codon usage preferences of *E. coli*. Six fusion expression vectors harboring either the enterokinase recognition site (DDDDK) or the SNAC tag (GSHHW) were constructed, and the efficiency of enterokinase digestion versus SNAC chemical cleavage, as well as the antibacterial activities of the resulting AMPs, were systematically compared in order to provide methodological guidance for the scalable production of antimicrobial peptides (Gao et al. 2015; Wu et al. 2022). The aim of this work was to determine whether SNAC-mediated chemical cleavage can replace enterokinase digestion without compromising peptide yield or antibacterial activity.

Materials and methods

Strains, plasmids and reagents

Escherichia coli BL21(DE3) was used as the host strain for recombinant AMP expression. *E. coli* ATCC 8739 and *Staphylococcus aureus* ATCC 25923 were used as indicator organisms for antibacterial activity assays. All strains were maintained in our laboratory. The expression vector pET32a (containing the N-terminal His-Trx tag and the enterokinase recognition site DDDDK) was also preserved in our laboratory. Primers were synthesized by Beijing Genomics Institute Biotechnology Co., Ltd., Beijing, China, and restriction enzymes, T4 DNA ligase, and DNA purification kits were purchased from TAKARA Biotechnology (Beijing) Co., Ltd., Beijing, China, unless stated otherwise.

Acquisition of AMP genes and primer design

AMP genes were amplified using a primer-complementary PCR strategy. Given the short length of AMP gene sequences (typically < 100 bp), three partially complementary primers were designed for each peptide so that the complete target gene fragment could be generated by primer annealing and extension. Codon-optimized nucleotide sequences for the three AMPs were deduced from their amino acid sequences according to the codon usage preferences of *E. coli*. Three complementary primers were designed for each AMP, with P1 containing a *Kpn*I restriction site and P3 containing a *Hin*dIII restriction site; adjacent primers overlapped by 18–22 nt (Additional file 1: Table S1). For the SNAC series, the sequence encoding GSHHW was appended to the 5'-end of the AMP gene so that the expressed fusion protein carried the motif His-Trx-DDDDK-GSHHW-AMP.

Amplification of AMP genes by primer-complementary PCR

AMP genes were amplified by primer-complementary PCR in the absence of an external DNA template. The reaction mixture (50 μL total volume) contained 25 μL of 2× PrimeSTAR Max Premix (TaKaRa, R045Q, China), 1 μL each of primers P1, P2, and P3 (10 μM), and 22 μL of nuclease-free water. PCR cycling conditions were as follows: initial denaturation at 98°C for 3 min; 30 cycles of 98°C for 5 s, 55°C for 5 s, and 72°C for 5 s; and a final extension at 72°C for 5 min. PCR products were analyzed by 3% agarose gel electrophoresis to confirm the expected fragment size, and the target gene fragments were recovered using a DNA gel extraction kit.

Construction of recombinant expression vectors

A total of six recombinant expression vectors were constructed. The pET32a empty vector and the six PCR products (LK-24, GSHHW-LK-24, ZY4, GSHHW-ZY4, Mast-MO, and GSHHW-Mast-MO) were individually digested with *Kpn*I and *Hin*dIII, and the digestion products were purified. The linearized pET32a vector and the AMP gene fragments were ligated using T4 DNA ligase, and the ligation mixtures

were transformed into *E. coli* BL21(DE3) competent cells. Transformants were selected on LB agar plates supplemented with ampicillin (100 μg/mL). Ten single colonies were randomly picked from each plate and inoculated into LB liquid medium containing ampicillin for overnight culture. Positive recombinants were identified by colony PCR using T7 primers, and one positive clone from each construct was subjected to Sanger sequencing to verify sequence integrity. The six expression vectors were categorized into two series.

pET32a series for enterokinase cleavage

This series was designed for AMP release via enterokinase digestion. The resulting recombinant plasmids were pET32a-LK-24, pET32a-ZY4, and pET32a-Mast-MO, and the corresponding fusion proteins were His-Trx-DDDDK-LK-24, His-Trx-DDDDK-ZY4, and His-Trx-DDDDK-Mast-MO. Following enterokinase cleavage, the released AMPs were LK-24, ZY4, and Mast-MO (Table 1).

pET32a-GSHHW series for SNAC chemical cleavage

This series was designed for AMP release via SNAC-mediated chemical cleavage. The resulting recombinant plasmids were pET32a-GSHHW-LK-24, pET32a-GSHHW-ZY4, and pET32a-GSHHW-Mast-MO. Because the multiple cloning site of pET32a already encodes the enterokinase recognition sequence, the corresponding fusion proteins were His-Trx-DDDDK-GSHHW-LK-24, His-Trx-DDDDK-GSHHW-ZY4, and His-Trx-DDDDK-GSHHW-Mast-MO. Chemical cleavage was performed in 1 mM $NiCl_2$ and 0.1 M CHES buffer (pH 8.2) at room temperature, yielding SHHW-LK-24, SHHW-ZY4, and SHHW-Mast-MO (Table 1).

Table 1
Construction of pET32a expression vectors and nomenclature of the released AMPs

Vector	Recombinant fusion protein	Target AMP	Cleavage method	Recognition site	Cleavage product
pET32a-LK-24	His-Trx-DDDDK-LK-24	LK-24	Enterokinase	DDDDK↓	LK-24
pET32a-ZY4	His-Trx-DDDDK-ZY4	ZY4	Enterokinase	DDDDK↓	ZY4
pET32a-Mast-MO	His-Trx-DDDDK-Mast-MO	Mast-MO	Enterokinase	DDDDK↓	Mast-MO
pET32a-GSHHW-LK-24	His-Trx-DDDDK-GSHHW-LK-24	LK-24	SNAC	G↓SHHW	SHHW-LK-24
pET32a-GSHHW-ZY4	His-Trx-DDDDK-GSHHW-ZY4	ZY4	SNAC	G↓SHHW	SHHW-ZY4
pET32a-GSHHW-Mast-MO	His-Trx-DDDDK-GSHHW-Mast-MO	Mast-MO	SNAC	G↓SHHW	SHHW-Mast-MO

Expression of fusion proteins

IPTG induction

A single positive colony confirmed by sequencing was inoculated into LB medium supplemented with 100 μg/mL ampicillin and cultured at 37°C with shaking at 220 rpm until the OD_{600} reached approximately 0.6. IPTG was then added to a final concentration of 1.0 mM, and cultivation was continued at 37°C. Cells were harvested at 0, 1, 2, 3, 4, 5, and 6 h post-induction, and fusion protein expression was analyzed by SDS-PAGE.

Auto-induction

Auto-induction was performed according to the ZYM-5052 medium formulation (Studier 2005). A single positive colony confirmed by sequencing was inoculated into auto-induction medium supplemented with 100 μg/mL ampicillin and cultured at 37°C with shaking at 220 rpm until the OD_{600} reached approximately 0.6. The culture was then shifted to 25°C and incubated for an additional 16 h. Samples were subsequently collected at 2 h intervals from 16 h to 26 h (i.e., at 16, 18, 20, 22, 24, and 26 h). Fusion protein expression was monitored by SDS-PAGE. Cells from the time point exhibiting the highest expression level were harvested and disrupted by ultrasonication, followed by centrifugation to separate the soluble supernatant and the insoluble pellet. The distribution of the fusion protein between the two fractions was assessed by SDS-PAGE.

Purification by Ni-NTA affinity chromatography

The soluble fraction containing the fusion protein was loaded onto a Ni-NTA (Ni^{2+}-nitrilotriacetic acid) affinity chromatography column. Purification was achieved through the specific interaction between the His-tag and the immobilized Ni^{2+} ions. Non-specifically bound contaminant proteins were removed by sequential washing with binding buffer(20 mM Tris-HCl, 150 mM NaCl, 10 mM imidazole, pH 8.0) and wash buffer(20 mM Tris-HCl, 150 mM NaCl, 20 mM imidazole, pH 8.0), and the target fusion protein was eluted with elution buffer containing a high concentration of imidazole(20 mM Tris-HCl, 150 mM NaCl, 250 mM imidazole, pH 8.0). Purification efficiency was evaluated by SDS-PAGE.

Cleavage of fusion proteins and recovery of AMPs

Enterokinase digestion

Purified fusion proteins were incubated with enterokinase at a substrate-to-enzyme ratio of 100:1 (w/w) at 25°C for 20 h in the reaction buffer recommended by the manufacturer (20 mM Tris-HCl, 50 mM NaCl, 2 mM $CaCl_2$, pH 8.0). Following digestion, enterokinase and the cleaved His-Trx tag were removed using a 10 kDa molecular weight cut-off (MWCO) ultrafiltration device, and the filtrate containing the target AMP was collected.

SNAC chemical cleavage

Purified fusion proteins were dissolved in cleavage buffer (0.1 M CHES, pH 8.2, 0.1 M NaCl, 0.1 M acetone oxime), and $NiCl_2$ was added to a final concentration of 1 mM. The reaction was incubated at room temperature (approximately 22°C) for 20 h. Following cleavage, the His-Trx tag was removed using a 10 kDa MWCO ultrafiltration device, and the filtrate containing the target AMP was collected.

Antibacterial activity assays

Zone-of-inhibition assay

Indicator organisms (*E. coli* ATCC 8739 and *S. aureus* ATCC 25923) were cultured overnight and diluted to approximately 1 × 10^6 CFU/mL. Bacterial suspensions were evenly spread onto LB agar plates. Wells (6 mm diameter) were punched into the agar, and equal volumes of the AMP solutions obtained by the two cleavage methods were added after normalization to the same peptide concentration peptide concentrations were determined using a BCA protein assay kit (R21250-500T, Shanghai Yuanye Bio-Technology Co., Ltd., Shanghai, China) according to the manufacturer's instructions, with bovine serum albumin (BSA) as the standard. Sterile water served as the negative control and ampicillin sodium as the positive control. Plates were incubated at 37°C for 12–16 h, and the inhibition zone diameters were measured.

Minimum inhibitory concentration determination

MIC values were determined by the broth microdilution method in 96-well microtiter plates. AMPs obtained by the two methods were subjected to two-fold serial dilutions and mixed with indicator bacteria at a final density of approximately 5 × 10^5 CFU/mL. Plates were incubated at 37°C for 16–20 h. The MIC was defined as the lowest concentration at which no visible bacterial growth was observed. Ampicillin sodium was included as a positive control, and sterile water as a negative control. All assays were performed in triplicate, and three independent biological replicates were conducted.

Statistical analysis

All experiments were performed with at least three independent biological replicates, and the data are expressed as the mean ± standard deviation (SD). Differences between the enterokinase and SNAC groups were analyzed by using an unpaired two-tailed Student’s t-test with GraphPad Prism version 9.5.0 (GraphPad Software, San Diego, CA, USA). A p-value < 0.05 was considered statistically significant.

Results

Amplification of AMP genes by primer-complementary PCR

Using the primer-complementary PCR strategy, the six AMP genes-LK-24, GSHHW-LK-24, ZY4, GSHHW-ZY4, Mast-MO, and GSHHW-Mast-MO-were successfully amplified. Agarose gel electrophoresis (3%)

revealed specific amplification bands of the expected sizes for all six genes, confirming the successful synthesis of the target gene fragments (Fig. 1).

Construction and verification of recombinant expression vectors

The six AMP genes were individually cloned into the pET32a vector, yielding six recombinant plasmids. For each construct, ten single colonies were randomly selected and screened by colony PCR using T7 primers. All positive clones produced insertion-specific bands of the expected size. Sanger sequencing confirmed that the inserted sequences of all recombinant plasmids were identical to the theoretical sequences, with no mutations detected (Fig. 2).

Expression and purification of fusion proteins

The six recombinant plasmids were individually transformed into *E. coli* BL21(DE3), and the positive clones confirmed by colony PCR were subjected to both IPTG induction and auto-induction. SDS-PAGE analysis demonstrated that all six fusion proteins were successfully expressed under both induction conditions and were predominantly present in the soluble fraction of the cell lysate supernatant. Notably, auto-induction yielded substantially higher recombinant protein levels than IPTG induction, and expression levels increased progressively with extended auto-induction time (Fig. 3). Subsequent Ni-NTA affinity chromatography yielded fusion proteins of high purity, providing sufficient substrate for the subsequent cleavage experiments (Fig. 4).

Comparison of cleavage efficiency between the two methods

Enterokinase digestion

Fusion proteins from cells harboring pET32a-LK-24, pET32a-ZY4, and pET32a-Mast-MO were expressed via the auto-induction system for 24 h. Following ultrasonic lysis, the soluble fraction was purified by Ni-NTA affinity chromatography. Enterokinase digestion followed by 10 kDa MWCO ultrafiltration yielded target AMP bands at the expected positions (approximately 2–5 kDa) on SDS-PAGE (Fig. 4).

SNAC chemical cleavage

Fusion proteins from cells harboring pET32a-GSHHW-LK-24, pET32a-GSHHW-ZY4, and pET32a-GSHHW-Mast-MO were expressed via the auto-induction system for 24 h. Following ultrasonic lysis, the soluble fraction was purified by Ni-NTA affinity chromatography. SNAC chemical cleavage in 1 mM $NiCl_2$ and 0.1 M CHES buffer (pH 8.2), followed by 10 kDa MWCO ultrafiltration, yielded target AMP bands at the expected positions (approximately 2–5 kDa) on SDS-PAGE. Compared with enterokinase digestion, SNAC cleavage was operationally simpler and did not require the addition of exogenous proteases

(Fig. 4). Cleavage efficiency was assessed visually by SDS-PAGE, and both enterokinase digestion and SNAC cleavage achieved efficient cleavage, with the fusion protein bands largely disappearing after cleavage (Fig. 4).

Antibacterial activity of the AMPs released by the two methods

Zone-of-inhibition assay

The AMPs released by both methods inhibited *E. coli* ATCC 8739 and *S. aureus* ATCC 25923 (Fig. 5A, A'; Table 2). The inhibition zone diameters of the enterokinase-released peptides were 6.5 ± 0.5 mm (LK-24), 8.5 ± 0.5 mm (ZY4) and 8.0 ± 0.5 mm (Mast-MO) against *E. coli*, and 6.0 ± 0.5, 8.0 ± 0.5 and 7.5 ± 0.5 mm against *S. aureus*. The corresponding SNAC-cleavage products, SHHW-LK-24, SHHW-ZY4 and SHHW-Mast-MO, produced slightly larger zones, of 7.5 ± 0.5, 9.0 ± 0.5 and 9.5 ± 0.5 mm against *E. coli* and 7.0 ± 0.5, 8.5 ± 0.5 and 9.0 ± 0.5 mm against *S. aureus*. Ampicillin sodium, used as the positive control, gave zones of 10.0 ± 0.5 mm (*E. coli*) and 11.0 ± 0.5 mm (*S. aureus*), whereas the sterile water control produced no detectable zone (0.0 ± 0.0 mm). These results indicate that the introduction of the SNAC tag and the subsequent chemical cleavage have minimal impact on the antibacterial activity of the AMPs.

Minimum inhibitory concentration

The MIC results were consistent with the zone-of-inhibition data (Fig. 5B, B'; Table 2). The MIC values of the enterokinase-released peptides were 16, 8 and 8 μg/mL for LK-24, ZY4 and Mast-MO against *E. coli*, and 32, 16 and 16 μg/mL against *S. aureus*. The SNAC-derived peptides SHHW-LK-24, SHHW-ZY4 and SHHW-Mast-MO showed two-fold lower MIC values, of 8, 4 and 4 μg/mL against *E. coli* and 16, 8 and 8 μg/mL against *S. aureus*, indicating marginally superior *in vitro* potency. Nevertheless, the differences between the two groups were not statistically significant ($p > 0.05$).

Table 2
Antibacterial activity of the AMPs released by enterokinase digestion and SNAC chemical cleavage

AMP (cleavage method)	Inhibition zone diameter (mm) — *E. coli*	Inhibition zone diameter (mm) — *S. aureus*	MIC (μg/mL) — *E. coli*	MIC (μg/mL) — *S. aureus*
LK-24 (enterokinase)	6.5 ± 0.5	6.0 ± 0.5	16	32
SHHW-LK-24 (SNAC)	7.5 ± 0.5	7.0 ± 0.5	8	16
ZY4 (enterokinase)	8.5 ± 0.5	8.0 ± 0.5	8	16
SHHW-ZY4 (SNAC)	9.0 ± 0.5	8.5 ± 0.5	4	8
Mast-MO (enterokinase)	8.0 ± 0.5	7.5 ± 0.5	8	16
SHHW-Mast-MO (SNAC)	9.5 ± 0.5	9.0 ± 0.5	4	8
Ampicillin sodium (positive control)	10.0 ± 0.5	11.0 ± 0.5	N.D.	N.D.
Sterile water (negative control)	0.0 ± 0.0	0.0 ± 0.0	—	—

Data are the mean ± standard deviation of three independent biological replicates. Inhibition zone diameters include the 6 mm well diameter. N.D., not determined. No statistically significant difference was found between the enterokinase and SNAC groups ($p > 0.05$).

Discussion

In this study, six AMP genes were rapidly synthesized via primer-complementary PCR, and six recombinant expression vectors belonging to the pET32a and pET32a-GSHHW series were successfully constructed using restriction enzyme digestion and ligation. The efficiency of enterokinase digestion and SNAC chemical cleavage for AMP preparation was systematically compared. The primer-complementary PCR strategy eliminates the requirement for template DNA, enabling the direct synthesis of short genes from primers alone. This approach is operationally simple and time-efficient, making it particularly suitable for the cloning of short genes such as those encoding AMPs (Tao et al. 2020). This strategy is complementary to other recently reported AMP gene synthesis methods and provides an efficient technical platform for the rapid genetic engineering of antimicrobial peptides (Höpfinger et al. 2023).

Both IPTG induction and auto-induction effectively drove fusion protein expression; however, auto-induction offered superior operational convenience, eliminated toxic inducer residues, and achieved higher recombinant protein yields, rendering it more suitable for large-scale production (Studier 2005).

Enterokinase digestion represents the most mature and well-established cleavage method, producing scarless products without residual amino acids and thereby preserving the native sequence of the

AMPs. However, cleavage efficiency is influenced by the substrate sequence context, and the high cost of enterokinase, coupled with the necessity for additional steps to remove the protease after digestion, limits its applicability for large-scale operations (Zheng et al. 2025). SNAC chemical cleavage is an emerging tag removal technology whose principal advantages lie in operational simplicity and cost-effectiveness-requiring only Ni^{2+} ions to achieve sequence-specific cleavage under mild conditions without the addition of expensive proteases (Dang et al. 2019). In the present study, the AMPs obtained by SNAC cleavage exhibited robust antibacterial activity that was comparable to that of the peptides released by enterokinase digestion, indicating that this method exerts minimal impact on peptide bioactivity. The slightly larger inhibition zones and marginally lower MIC values observed for the SNAC-derived peptides suggest that the residual SHHW sequence at the N-terminus does not compromise antimicrobial potency (Lu et al. 2026; Han and Dang 2025).

Several limitations of the present work should be acknowledged. First, the peptides released by SNAC cleavage retain a four-residue SHHW extension at their N-terminus; although this extension did not detectably reduce antibacterial activity in the assays performed here, its influence on peptide stability, cytotoxicity, and immunogenicity remains to be evaluated. Second, ZY4 is a disulfide-bonded peptide, and no dedicated oxidative refolding step or direct confirmation of disulfide bond formation was included in this work; the oxidation state of the recombinant ZY4 and its contribution to antibacterial activity therefore require further investigation. Third, cleavage efficiency and final peptide yield were not quantified in absolute terms (e.g., mg of peptide per liter of culture), which should be addressed in future scale-up studies. Finally, activity was evaluated against only two indicator strains; testing a broader panel of clinically relevant and multidrug-resistant isolates would strengthen the conclusions.

Collectively, the results of this study demonstrate that the two methods possess complementary advantages: enterokinase digestion is mature, reliable, and produces scarless products, making it suitable for applications requiring maximal preservation of native peptide activity; SNAC chemical cleavage is operationally simple and cost-effective, making it particularly amenable to large-scale production. In practical applications, the most appropriate method can be selected based on specific requirements, cost considerations, and available infrastructure.

Conclusions

Six AMP genes-LK-24, GSHHW-LK-24, ZY4, GSHHW-ZY4, Mast-MO, and GSHHW-Mast-MO-were successfully synthesized by primer-complementary PCR, and six recombinant expression vectors belonging to the pET32a and pET32a-GSHHW series were constructed.

Both IPTG induction and auto-induction effectively expressed the recombinant fusion proteins, with auto-induction yielding substantially higher recombinant protein levels, and Ni-NTA affinity chromatography afforded fusion proteins of high purity. Both enterokinase digestion and SNAC chemical cleavage efficiently released recombinant AMPs with retained antibacterial activity. The AMPs obtained by SNAC chemical cleavage exhibited bactericidal activity against *E. coli* and *S. aureus* that was comparable to,

and in several cases marginally greater than, that of the peptides released by enterokinase digestion. These findings provide a methodological reference for the scalable production of antimicrobial peptides and support SNAC-tag chemical cleavage as a cost-effective, protease-free alternative to enzymatic digestion.

Abbreviations

AMP
Antimicrobial peptide
AMPs
Antimicrobial peptides
ATCC
American Type Culture Collection
bp
Base pair
CFU
Colony-forming unit
CHES
N-cyclohexyl-2-aminoethanesulfonic acid
GST
Glutathione S-transferase
IPTG
Isopropyl β-D-1-thiogalactopyranoside
LB
Luria-Bertani
MDR
Multidrug-resistant
MIC
Minimum inhibitory concentration
N.D.
Not determined
MWCO
Molecular weight cut-off
Ni-NTA
Nickel-nitrilotriacetic acid
OD600
Optical density at 600 nm
PCR
Polymerase chain reaction
SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SNAC
Sequence-specific nickel-assisted cleavage
SUMO
Small ubiquitin-like modifier
TEV
Tobacco etch virus
Trx
Thioredoxin.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Author Contribution

Conceptualization, K.X.; methodology, M.M. and M.S.; software, M.M.; validation, M.M., D.C. and X.W.; formal analysis, M.M. and M.S.; investigation, X.W. and Y.Z.; data curation, M.M. and D.C.; writing-original draft preparation, M.M. and D.C.; writing-review and editing, K.X.; supervision, K.X.; project administration,

K.X.; funding acquisition, K.X. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

All data generated or analysed during this study are included in this published article and its additional file. The nucleotide and amino acid sequences of the six antimicrobial peptides are provided in Additional file 1: Table S1.

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33. Additional file
34. Additional file 1 Table S1. Sequences of the six antimicrobial peptides and the primers used for primer-complementary PCR (XLSX)

Figures

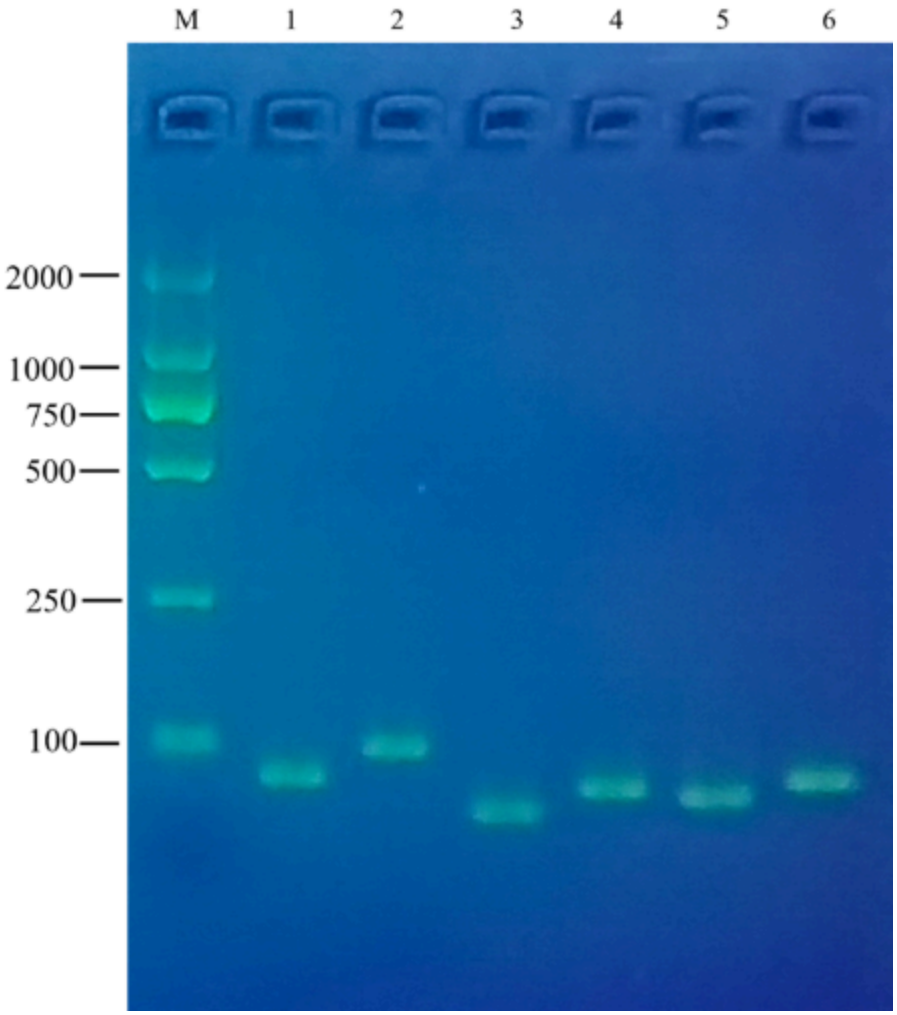


Figure 1

Agarose gel electrophoresis of PCR amplification products. The six AMP genes (LK-24, GSHHW-LK-24, ZY4, GSHHW-ZY4, Mast-MO and GSHHW-Mast-MO) were amplified by primer-complementary PCR and analysed on a 3% agarose gel. M, DL2000 DNA marker; lane 1, LK-24; lane 2, GSHHW-LK-24; lane 3, ZY4; lane 4, GSHHW-ZY4; lane 5, Mast-MO; lane 6, GSHHW-Mast-MO.

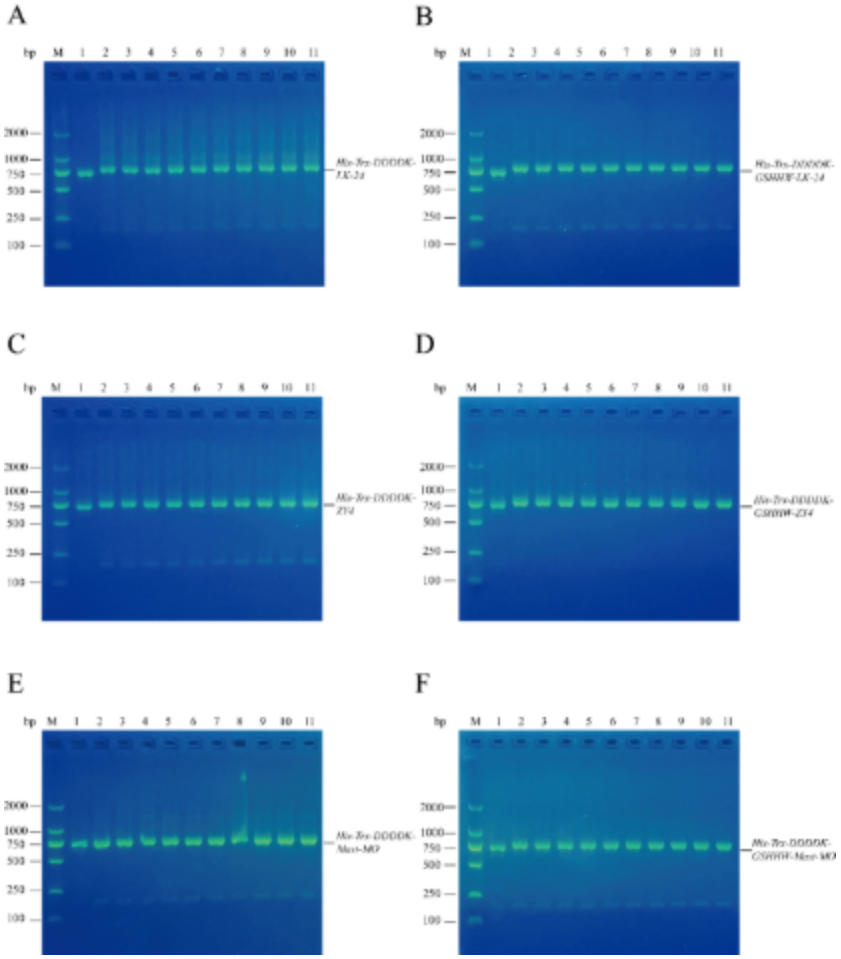


Figure 2

Colony PCR verification of the recombinant plasmids. Ten single colonies were randomly picked and cultured overnight in LB medium supplemented with 100 μg/mL ampicillin sodium, and vector construction was verified by colony PCR with T7 primers. (A) pET32a-LK-24; (B) pET32a-GSHHW-LK-24; (C) pET32a-ZY4; (D) pET32a-GSHHW-ZY4; (E) pET32a-Mast-MO; (F) pET32a-GSHHW-Mast-MO. M, DL2000 DNA marker; lane 1, pET32a empty vector control; lanes 2-11, ten randomly selected single colonies.

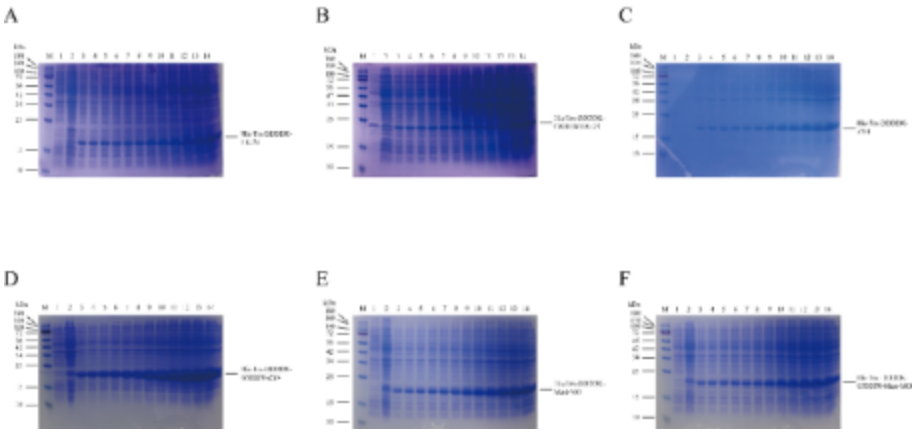


Figure 3

SDS-PAGE analysis of recombinant protein expression. Expression was assessed on 15% separating gels. Cells harbouring pET32a-LK-24 (A), pET32a-GSHHW-LK-24 (B), pET32a-ZY4 (C), pET32a-GSHHW-ZY4 (D), pET32a-Mast-MO (E) and pET32a-GSHHW-Mast-MO (F) were cultured in LB medium with 100 μg/mL ampicillin sodium at 37 °C and 220 rpm until OD600 reached ~0.6. IPTG was added to 1 mM and cells were harvested at 0-6 h post-induction (lanes 2-8). For auto-induction, cells were cultured in ZYM-5052 medium with 100 μg/mL ampicillin sodium at 37 °C and 220 rpm until OD600 reached ~0.6 and then shifted to 25 °C; cells were harvested at 16, 18, 20, 22, 24 and 26 h (lanes 9-14). M, protein marker; lane 1, pET32a empty vector control.

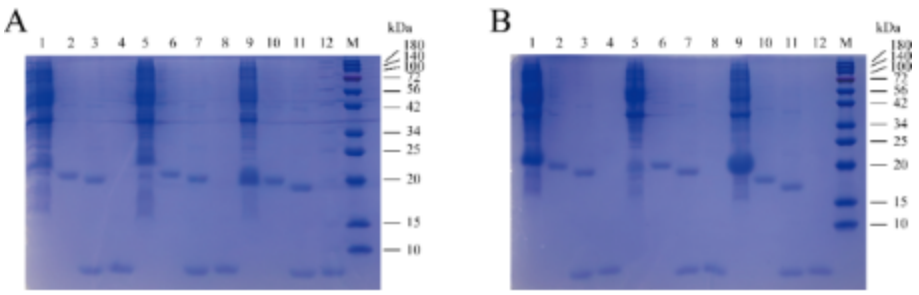


Figure 4

SDS-PAGE analysis of the purified AMPs. Fusion proteins expressed by auto-induction for 24 h were cleaved by enterokinase digestion or SNAC chemical cleavage, followed by 10 kDa MWCO ultrafiltration. (A) Lanes 1, 5 and 9, total protein of pET32a-LK-24, pET32a-GSHHW-LK-24 and pET32a-ZY4; lanes 2, 6 and 10, Ni-NTA-purified fusion proteins; lanes 3 and 11, enterokinase cleavage products of His-Trx-DDDDK-LK-24 and His-Trx-DDDDK-ZY4; lane 7, SNAC cleavage product of His-Trx-DDDDK-GSHHW-LK-24; lanes 4, 8 and 12, ultrafiltration-concentrated LK-24, SHHW-LK-24 and ZY4. (B) Lanes 1, 5 and 9, total protein of pET32a-GSHHW-ZY4, pET32a-Mast-MO and pET32a-GSHHW-Mast-MO; lanes 2, 6 and 10, Ni-NTA-purified fusion proteins; lanes 3 and 11, SNAC cleavage products of His-Trx-DDDDK-GSHHW-ZY4 and His-Trx-DDDDK-GSHHW-Mast-MO; lane 7, enterokinase cleavage product of His-Trx-DDDDK-Mast-MO; lanes 4, 8 and 12, ultrafiltration-concentrated SHHW-ZY4, Mast-MO and SHHW-Mast-MO.

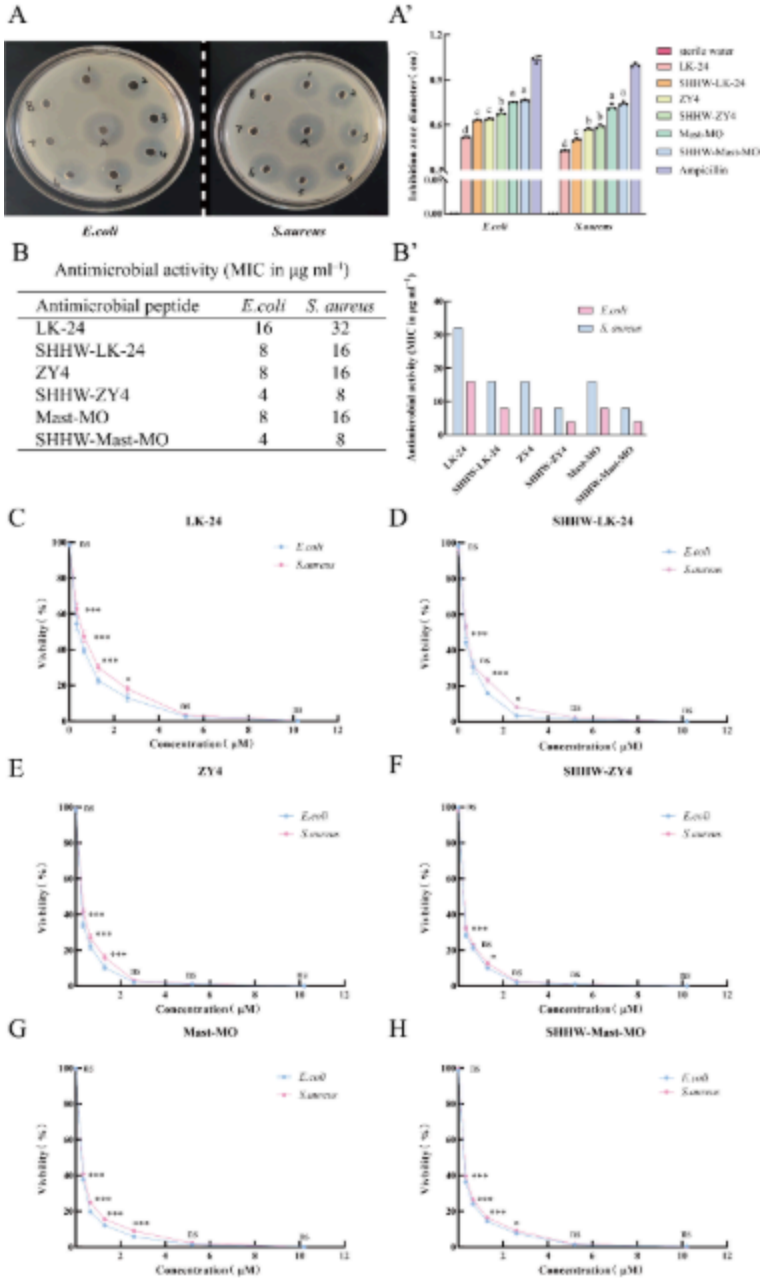


Figure 5

Zone-of-inhibition and MIC assays of the six AMPs. Wells were punched into LB agar plates seeded with *E. coli* or *S. aureus* and 50 μL (1 μg/μL) of each AMP was added per well (A). Wells: 1, LK-24; 2, SHHW-LK-24; 3, ZY4; 4, SHHW-ZY4; 5, Mast-MO; 6, SHHW-Mast-MO; 7 and 8, sterile water (negative control); A, ampicillin sodium (positive control). Inhibition zone diameters were measured and analysed statistically (A'). MIC values were determined (B, B') and dose-dependent antibacterial activity against *E. coli* and *S. aureus*was evaluated (C-H).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1TableS1.xlsx