Sani Marc-Antoine (Orcid ID: 0000-0003-3284-2176)

Expression and purification of the native C-amidated antimicrobial peptide maculatin 1.1

Shiying Zhu[1], Daniel K Weber[2], Frances Separovic[1], Marc-Antoine Sani[1,*]

1 School of Chemistry, Bio21 Institute, University of Melbourne, Melbourne, VIC 3010, Australia

2 Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, Minneapolis, MN 55455, USA

*** Corresponding author:**
Dr Marc-Antoine Sani
msani@unimelb.edu.au

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Abstract

Maculatin 1.1 (Mac1) is an antimicrobial peptide (AMP) from an Australian tree frog and exhibits low micromolar activity against Gram-positive bacteria. The antimicrobial properties of Mac1 are linked to its disruption of bacterial lipid membranes, which has been studied extensively by *in vitro* nuclear magnetic resonance (NMR) spectroscopy and biophysical approaches. While *in vivo* NMR has recently proven effective in probing peptide-lipid interplay in live bacterial cells, direct structural characterisation of AMPs has been prohibited by low sensitivity and overwhelming background noise. To overcome this issue, we report a recombinant expression protocol to produce isotopically enriched Mac1. We utilized a double-fusion construct to alleviate toxicity against the *E. coli* host and generate the native N-free and C-amidated termini Mac1 peptide. The SUMO and intein tags allowed native N-terminus and C-terminal amidation, respectively, to be achieved in a one-pot reaction. The protocol yielded 0.1 mg/L of native, uniformly

^{15}N-labelled, Mac1, which possessed identical structure and activity to peptide obtained by solid-phase peptide synthesis.

1 INTRODUCTION

Antimicrobial peptides (AMPs) are seen as promising alternatives to antibiotics in order to restrain the alarming rise in antibiotic resistance [1-3]. Since bacteria are less likely to evolve resistance against AMPs due to their targeting of and damage to the cell membrane, AMPs have potential for clinical therapy and pharmaceutical development [4-7]. Maculatin 1.1 (Mac1, GLFGVLAKVAAHVVPAIAEHF-NH_2) is a cationic AMP isolated from the skin glands of the Australian tree frog *Litoria genimaculata.* This peptide is in the class of AMPs that disrupt bacterial cell membranes via a pore-formation mechanism and is effective in killing a broad spectrum of Gram-positive bacteria, in particular *S. aureus* [4, 8]. The mode of action of Mac1 has been extensively studied in model systems, such as liposomal membranes and detergents, yet there is still little known of its structure and self-assembly under *in vivo* conditions. [9-11]. Nuclear magnetic resonance (NMR) is a cutting-edge technology that provides key structural information to better understand the complex interaction between AMPs and bilayer membranes with the ability to do so in a native membrane environment [12, 13]. A key limitation of NMR is the inherent low sensitivity and strong background signals in complex mixtures, which is commonly overcome by specific isotopic labelling [14, 15]. Isotope labelling is typically achieved through chemical and biological synthesis [16, 17] but latter post-translational, structural modifications, small size, proteolytic degradation and toxicity have been difficult to overcome when expressing AMPs from bacteria. In particular, the C-amidation is a structural feature found in most cationic AMPs that is important for maintaining complete biological activity, stability and proper structural functionality [9, 18-20]. Recently developed fusion partners, such as the small ubiquitin-like modifier (SUMO), thioredoxin (Trx), Glutathione-S-transferase (GST) and *Mxe* GyrA intein or Calmodulin (CaM) [21-24], have presented an opportunity to overcome these limitations. Fusion tags can simplify the lengthy purification process, increase the protein solubility, allow quantification and generate native sequences. For instance, the SUMO fusion strategy is widely used for the soluble expression of target proteins and preserves the native N-terminal amino acid sequence (*i.e.*, without left over residues) during stereo-specific cleavage by ubiquitin-like-specific protease 1 (Ulp1) [25, 26, 27]. Similarly, the self-cleavable *Mxe* GyrA intein tag has been effectively used to produce C-amidated AMPs by exploiting the reactive thioester intermediate in the presence of dithiothreitol (DTT) reducing agent in saturated ammonia [21, 28].

In this study, a cost-effective biological expression method was designed to generate uniformly ^{15}N-labelled Mac1 with its native sequence and C-terminal amidation. The SUMO tag was placed at the N-terminus and *Mxe* GyrA intein was placed at the C-terminus by fusion using the commercial pTXB1 vector (Figure 1). The purified recombinant Mac1 was then compared against its synthesized form using circular dichroism (CD), solution NMR, electrospray ionization mass spectrometry (ESI-MS) and several antimicrobial assays.

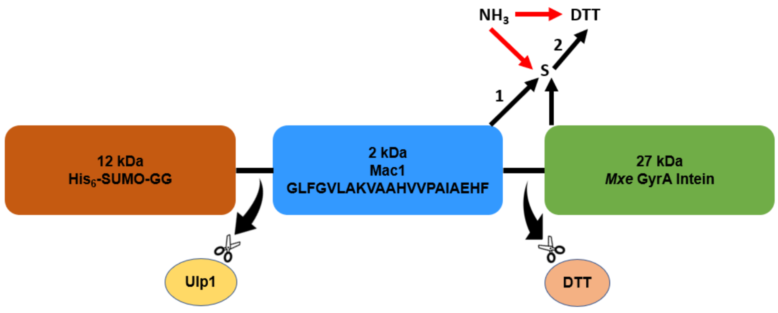

FIGURE 1 Design of the fusion construct to generate native Mac1 sequence and chemical reaction to release amidated C-terminus: **1**, Acyl-thioester formation with the intein thiol and **2** possible transthioesterification to exogenous DTT. Ammonia can cleave

either the intein thioester or the DTT thioester, and S represents sulphur in thioester. Ulp1 cleaves at -GG site of SUMO.

2 MATERIALS AND METHODS

2.1 Design and cloning of the SUMO-Mac1-*Mxe* GyrA fusion construct

Codon-optimised Mac1 DNA was ligated into a pE-SUMOpro (ampicillin-resistant; LifeSensors Inc., Malvern, USA) expression plasmid using BsaI and XbaI restriction sites. Initial attempts to express SUMO-Mac1 fusion protein were unsuccessful due to high toxicity of the construct against *Escherichia coli*. Subsequently, the SUMO-Mac1 DNA sequence was amplified from the plasmid by polymerase chain reaction (PCR) and ligated into a pTXB1 vector (ampicillin-resistant; New England Biolabs, Ipswich, USA) using NdeI (5') and SapI (3') restriction sites to encode the *E. coli*-friendly double fusion construct SUMO-Mac1-*Mxe* GyrA. The SUMO partner contained an N-terminal His$_6$-tag, allowing purification by nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography and removal upon cleavage by Ulp1 (i.e., SUMO protease). The chitin-binding domain included with the C-terminal *Mxe* GyrA intein was not utilised in this work. Both the cleavage of SUMO and *Mxe* GyrA are completely traceless of extraneous amino acids and leave only the native sequence of Mac1. The amino-acid sequence of the SUMO-Mac1-*Mxe* GyrA double fusion construct is:

MGHHHHHHGS *LQDSEVNQEA KPEVKPEVKP ETHINLKVSD GSSEIFFKIK KTTPLRRLME 60*

*AFAKRQGKEM DSLRFLYDGI RIQADQAPED LDMEDNDIIE AHREQIGG***GL FGVLAKVAAH** 120

VVPAIAEHF*C ITGDALVALP EGESVRIADI VPGARPNSDN AIDLKVLDRH GNPVLADRLF 180*

HSGEHPVYTV RTVEGLRVTG TANHPLLCLV DVAGVPTLLW KLIDEIKPGD YAVIQRSAFS 240

VDCAGFARGK PEFAPTTYTV GVPGLVRFLE AHHRDPDAQA IADELTDGRF YYAKVASVTD 300

*AGVQPVYSLR VDTADHAFIT NGFVSHAT*GL TGLNSGLTTN PGVSAWQVNT AYTAGQLVTY 360

NGKTYKCLQP HTSLAGWEPS NVPALWQLQ 389

where the Mac1 sequence is underlined and bolded; the His_6 and chitin-binding domains are shaded; and SUMO and *Mxe* GyrA sequences are displayed in italics.

2.2 Expression, purification, and C-terminal amidation of Mac1

A single colony of transformed *E. coli* one shot BL21(DE3) expression cells was used to inoculate overnight a 20 mL starter culture containing 100 μg/mL of ampicillin. For producing unlabeled SUMO-Mac1-*Mxe* GyrA, 500 mL of LB-Miller (containing 100 μg/mL ampicillin) was inoculated from starter culture and grown until an OD_{600} of 0.6 (250 rpm, 3 hours, 37°C). Cells were induced with 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and the growth was continued for 4 hours. For expression of uniformly labeled ^{15}N fusion protein, cells were initially grown in 100 ml LB medium overnight and harvested by centrifugation (8000g, 20 min, 4 °C). Pellets were resuspended in fresh ^{15}N-enriched Neidhardt's minimal media[16, 29] and induced with 0.2 mM IPTG for 4 hours once the OD_{600} reached 0.6. SDS-PAGE revealed that SUMO-Mac1-*Mxe* GyrA was found to accumulate in inclusion bodies. Cells were thus harvested by centrifugation (8000 g, 20 mins, 4°C), resuspended in denaturing buffer (6 M urea, 100 mM NaH_2PO_4, 10 mM Tris, 300 mM NaCl, pH 8.0), then lysed by mechanical homogenization. The insoluble debris from the cell lysate was removed by centrifugation (15000 g, 20 min, 4°C) prior to loading onto Ni-NTA resin. The resin was washed with denaturing buffer (pH 7.0) to remove impurities. The fusion protein was released using low-pH (pH 4.0) denaturing buffer. A yield of 60 mg of SUMO-Mac1-*Mxe* GyrA (natural abundance) per L of culture was determined according to UV-vis absorbance at 280 nm and theoretical extinction coefficient of 35560 $M^{-1}cm^{-1}$. The fusion protein was refolded from denaturing buffer into concentrated ammonium buffer (20 mM PBS, 100 mM NaCl, 3.5 M $(NH_4)_2CO_3$) using a PD-10 column with the eluent dripped directly into cleaving buffer (100 mM DTT and Ulp1 at a 1:500 molar ratio to fusion protein) and incubated for 3 days at room temperature. The cleaved Mac1 formed an insoluble precipitate that was collected by centrifugation then dissolved into CH_3CN/H_2O (1:9, v/v) and purified by RP-HPLC using Phenomenex C18 analytical column (4.6 x 250 mm, pore size 300 Å, particle size 5µm). Elution was carried out with a linear gradient from 0% to 100% of solvent B (0.1% TFA/CH_3CN) in solvent A (0.1% TFA/H_2O) for 45 mins at 1 mL/min. Purified peptide was lyophilized and stored at -20°C (Figure 2).

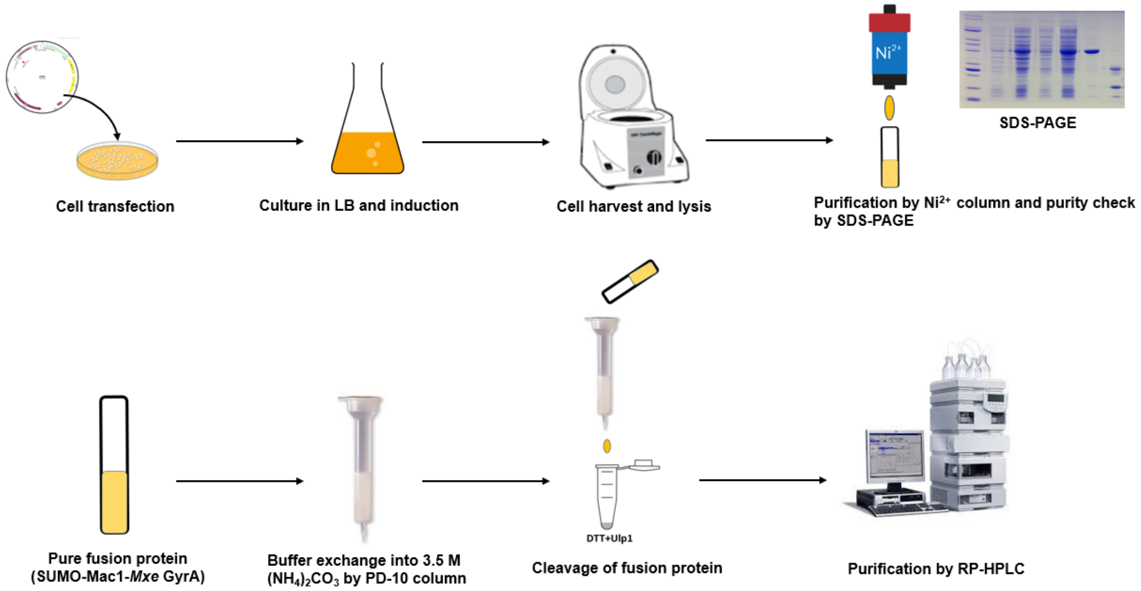

FIGURE 2 Summary of protein expression, purification and extraction to produce recombinant native Mac1.

2.3 NMR spectroscopy

Solution NMR samples were prepared by dissolving 184 μM Mac1 in phosphate buffer (50 mM NaCl, 20 mM phosphate buffer pH 4.74, 0.1 mM NaN_3, 0.03 mM DSS, 10% D_2O) containing 46 mM deuterated sodium dodecyl sulfate (d_{25}-SDS) micelles.

NMR spectra were recorded on a Bruker Avance II 800 MHz spectrometer (Bruker Biospin, Rheinstetten, Germany) and a Bruker AVIII 600 MHz spectrometer at 298 K. All spectra were referenced internally to 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS). 2D ^{1}H-^{15}N heteronuclear single quantum correlation (HSQC) spectrum was acquired with time domains of 2048 and 256 complex points for ^{1}H (F2) and ^{15}N (F1), respectively, a total of 8 scans, and a recycle delay of 1.6 s. A 3D ^{15}N edited-TOCSY-HSQC experiment was acquired with 60 ms mixing time, 50% non-uniform sampling (NUS) and 2048 complex points in the direct (HN) dimension, 128 and 64 in the first (H_{NOE}) and second (^{15}N) indirect dimensions, respectively, with a recycle delay of 1.2 s. A 3D ^{15}N edited-NOESY-HSQC experiment was performed with 120 ms mixing time, and 4096 complex points in the direct (HN) dimension, 512 and 64 in the first (H_{NOE}) and second (^{15}N) indirect dimensions, respectively, and recycle delay of 1.6 s. All spectra were processed with NMRPipe, reconstructed with MDDnmr and analysed through TopSpin 4.0.5 and CCPNMR2.4 [30, 31]. Full backbone and side chains assignments were made using ^{1}H-^{15}N HSQC and ^{15}N edited-NOESY-HSQC and TOCSY-HSQC spectra.

2.4 Circular dichroism

Circular dichroism (CD) measurements were recorded at room temperature using a Chirascan-Plus (Applied Photophysics, Leatherhead, UK) spectrometer. The sample was scanned from 190 to 260 nm in triplicate with 1 nm step size, 1 nm bandwidth, 1 s time-per-point and 1 mm pathlength cuvettes. Spectra were zeroed at 260 nm and normalized to give units of mean-residue ellipticity (MRE) according to the following equation:

$$\mathrm{MRE} = \frac{\theta}{C \times L \times N}$$

where θ is the recorded ellipticity in milli-degrees, C is the peptide concentration in dmol/L, L is the cell path-length in cm, and N is the number of residues per peptide.

The CD samples were made by dissolving synthesized Mac1 to 155 μM and expressed Mac1 to 140 μM either in aqueous buffer (10 mM phosphate, pH 7.4, 1 mM NaCl) or in 25 mM SDS (Sigma Aldrich, Castle Hill, Australia) micelles (10 mM phosphate, pH 7.4, 1 mM NaCl).

2.5 Antimicrobial assay

Staphylococcus aureus (ATCC 29213) was cultured in 10 mL LB medium until OD_{600} 0.3. 1 mL cell culture was diluted 10^6 times and then incubated with peptides at 37°C for 90 minutes. After incubation, the diluted cell culture was sprayed onto four LB agar plates for statistical analysis. Plates without peptide incubation were used as positive controls. Colonies were counted after 15 hours incubation at 37°C. All counted bacterial colonies were processed in Excel, expressed as mean ± standard deviation (SD), and paired t-test with two tailed ρ value was used to calculate the level of probability (ρ) for the differences in activity observed between synthetic and recombinant Mac1.

2.6 Solid Phase Peptide Synthesis of maculatin 1.1.

Mac1 (GLFGVLAKVAAHVVPAIAEHF-NH2) was synthesised by Fmoc/tBu solid-phase peptide synthesis on a CEM Liberty microwave-assisted synthesiser using a DIC/Oxyma system starting with 0.1 mmol Rink Amide resin. The peptide was cleaved from the resin support using a cleavage cocktail made of TFA/H_2O/TIPS (38:1:1) for two hours at room temperature. The filtrates were then concentrated under a stream of nitrogen, and the crude peptide was obtained by precipitation, washed three times with cold diethyl ether and followed by lyophilisation overnight.

The crude synthetic Mac1 was purified on a Phenomenex C18 column (22 x 250 mm, pore size 300, particle size 10 µm) with a gradient of 25% to 70% acetonitrile (0.1% trifluoracetic acid, TFA). Mac1 was characterised by infusion orbitrap MS (2145.24 Da observed, 2145.56 Da calculated), RP-HPLC (purity > 98%) and TFA salt was removed by 3 rounds of lyophilization in 5 mM HCl [32].

3 RESULTS AND DISCUSSION

3.1 Expression and purification of fusion protein SUMO-Mac1-*Mxe* GyrA

SUMO-Mac1-*Mxe* GyrA was expressed from *E. coli* BL21(DE3) cells under control of the T7/lac promoter system using either LB media for natural isotopic abundance, or Neidhardt's minimal media for uniform ^{15}N labelling. After culture reached an OD_{600} of 0.6, *E. coli* cells containing recombinant proteins were induced by the addition of 0.2 mM IPTG (optimized concentration). After 4 hrs, a prominent band with an apparent molecular mass of ~40 kDa was observed by SDS-PAGE (Figure 3). The fusion protein was expressed in higher yield in the LB media (60 mg/ml) compared to minimal media (30 mg/ml).

Prior studies have shown that SUMO fusion proteins accumulate primarily into inclusion bodies[16], which was also observed in this expression method. Thus, lysis and initial purification steps were done using denaturing urea buffer (6 M). The fusion protein was isolated by Ni-NTA affinity chromatography taking advantage of the N-terminal 6xHis tag, whereby the use of chilled buffers was sufficient for averting risks of carbamylation modifications from urea degradation [33] and self-splicing. The fusion protein from column elution displayed a 42.2 kDa (42.3 kDa calculated) mass as revealed by ESI-TOF-MS, also corresponding to the molecular weight and band intensity observed on SDS-PAGE (Figure 3).

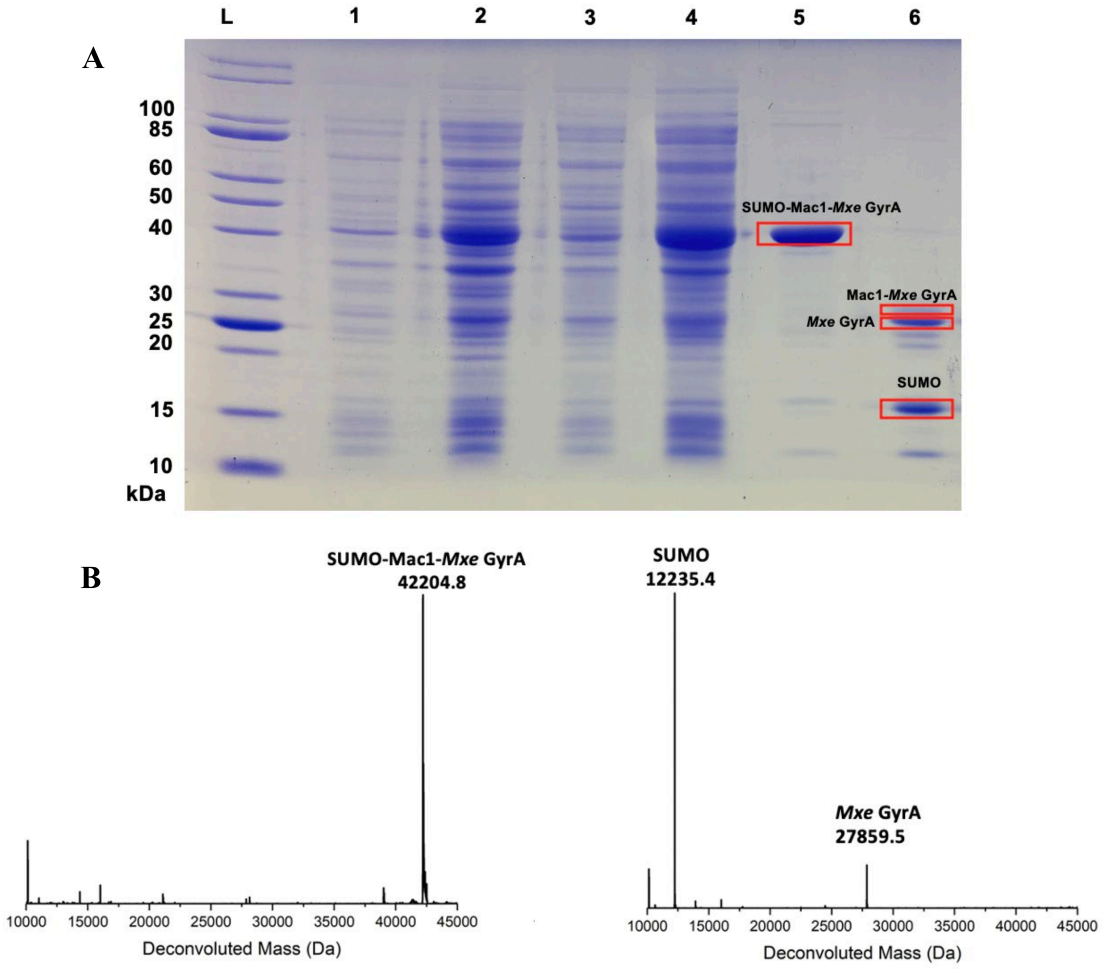

FIGURE 3 A) SDS-PAGE of protein expression and purification: Lanes 1 and 2 are culture pellets before and after induction; Lanes 3 and 4 are cell lysate supernatant obtained from PBS buffer and urea buffer, respectively; Lanes 5 and 6 are fusion protein pre- and post-cleavage. B) ESI-TOF mass spectra of pre- (left) and post-cleaved (right) SUMO-Mac1-*Mxe* GyrA.

3.2 Cleavage and purification of recombinant Mac1

The fusion protein was refolded into high concentration of ammonium bicarbonate by buffer exchange on a PD10 column and eluted into the cleaving buffer containing the SUMO stereospecific Ulp1 protease and exogenous DTT (Figure 2) [21]. Rapid on-column refolding (~2 min) followed immediately by cleavage in DTT essentially minimized the intermittent time when the yield of the thioester intermediate can be reduced by the spontaneous hydrolysis of the intein. Upon removal of both fusion tags,the C-terminal thioester reacted immediately with ammonia to produce Mac1 in its amidated form. SDS-PAGE revealed that 90% of fusion tags were successfully cleaved to release *Mxe* GyrA with mass 27.8 kDa (27.8 kDa calculated) and SUMO with mass 12.2 kDa (12.4 kDa calculated) along with a trace (10%) of noncleaved Mac1-*Mxe GyrA* seen by ESI-MS-TOF (Figure 3 & Figure S3). The extra faint bands were trace impurities which were not detected by mass spectrometry (Figure 3). The HPLC trace at 214 nm revealed that 75% C-terminus amidation of Mac1 was obtained and 25% remaining in acidic form, with impurities trace from SUMO and *Mxe* GyrA (Figure 4) also visible. Amidation of Mac1 (Mac1-NH_2) was confirmed based on its differing retention time by HPLC compared to non-amidated species and expected mass difference of ~ -1 Da determined by ESI-MS (Figures 4, S1 and S2). The final yield was 0.1-0.3 mg/L for unlabelled and 0.1 mg/L for uniformly ^{15}N labelled Mac1. These yields correspond to a recovery rate below 10% of the expected peptide mass from the 60 mg/L and 30 mg/L of expressed fusion protein (Table1). ESI-MS confirmed the mass for unlabelled Mac1 to be 2145.24 Da (2145.21 Da calculated) and uniformly ^{15}N labelled Mac1 to be 2171.16 Da (2171.36 Da calculated). The purity of expressed peptides was above 95% as indicated by HPLC spectra (Figure S1 & S2).

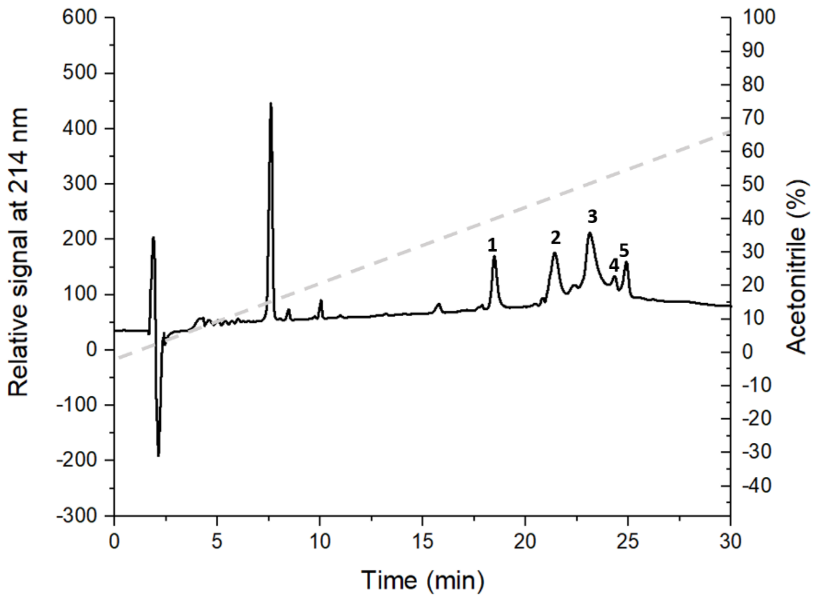

FIGURE 4 HPLC trace at 214 nm of peptide mixture after cleavage. Labels indicate SUMO (1), SUMO+*Mxe* GyrA (2-3), Mac1-OH (4), and Mac1-NH_2 (5) as determined by ESI-MS-TOF.

TABLE 1 Summary of yields of fusion proteins and labelled peptide

Protein and peptides	**Yield (mg/L of culture)**	**Recovery (%) of peptide from fusion protein**
SUMO-Mac1-*Mxe* GyrA	60.0	––
^{15}N SUMO-Mac1-*Mxe* GyrA	30.0	––
Mac1	0.1-0.3	3.2%-9.8%
^{15}N Mac1	0.1	6.7%

3.3 Characterization by CD and NMR

The secondary structures of synthesized and expressed Mac1 were analyzed by CD spectroscopy in phosphate buffer or in the presence of SDS micelles. Both peptides gave a negative peak around 200 nm, which is indicative of a random coil structure. In the presence of SDS micelles, the expressed Mac1 adopted the same α-helical structure as synthetic Mac1, as determined by the similar amplitudes of ellipticity at 208 nm and 222 nm (Figure 5A).

High resolution structures were investigated by solution NMR in SDS micelles. Well resolved $^{1}H^{15}N$ HSQC spectra of recombinant ^{15}N labelled Mac1 displayed good dispersion of cross peaks, corresponding to 19 residues in peptide backbone excluding glycine-1 and proline-15 (Figure 5B). The peaks were subsequently assigned based on 3D ^{15}N HSQC-NOESY and 3D ^{15}N edited-TOCSY-HSQC (Figure 5C). The slightly greater H^{N} chemical shift dispersion (1.6 ppm) and negative Hα and ^{15}N secondary chemical shift of recombinant Mac1 indicated a predominantly helical conformation (Figure S4A&B). These results suggest that the expressed Mac1 adopts the same α-helical structure as synthetic Mac1 in the presence of SDS micelles, which concurs with the similar amplitude of ellipticity in CD experiment.

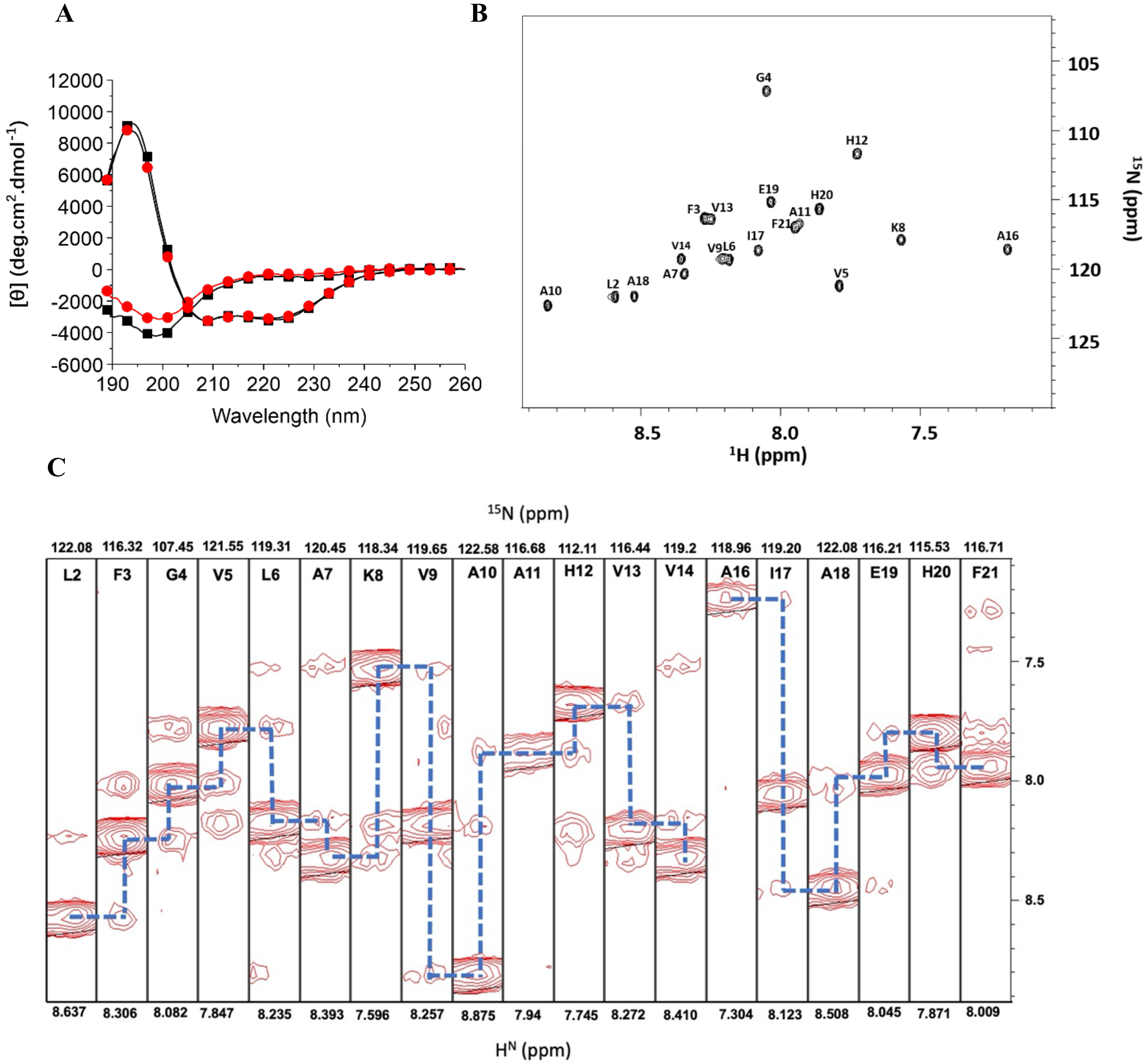

FIGURE 5 A) CD spectra of 155 µM synthesized Mac1 (black squares) and 140 µM expressed Mac1 (red circle) in PBS buffer and 25 mM SDS at 37°C; B) ^{1}H-^{15}N HSQC,

and C) 3D HSQC-NOESY of uniformly ^{15}N-labeled Mac1 in SDS micelles at 25°C. Cross peaks are labelled based on amino acid types and sequence.

3.4 Bioactivity test

The minimum inhibitory concentration against *S. aureus* (ATCC 29213) for the synthetic Mac1 has been previously obtained, showing low micromolar activity (8 µM) [4]. The activity between the recombinant and the synthetic Mac1 was thus compared with a simple colony count at *ca.* 4-fold lower MIC. Both synthetic Mac1 and expressed Mac1 were effective against *S. aureus,* as revealed by the lower bacterial colony count compared to the controls after overnight incubation. There was no statistically significant difference between the means of the two groups (equal variance), indicating a similar bioactivity for both peptides (Figure 6 & Table S1).

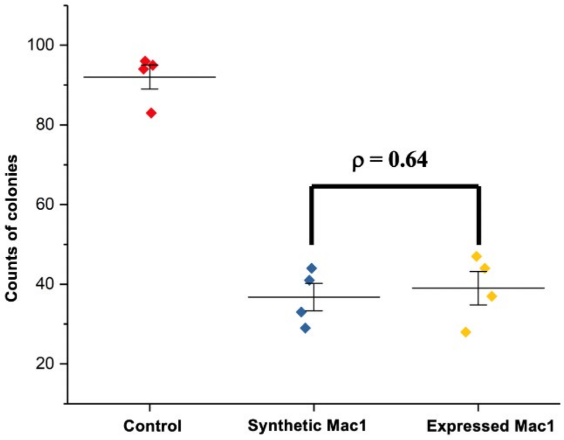

FIGURE 6 Box-and whisker plot of *S. aureus* growth in the presence of 1.7 µM Mac1. Error bar are the standard deviations from biological replicates.

4 DISCUSSION

Mac1 is a potential antibiotic alternative for the treatment of bacterial infections, with low micromolar inhibitory concentrations against Gram-positive bacteria. The molecular mechanism by which Mac1 inhibits bacteria growth is still not fully known although its propensity to interact with bacterial membrane to form helical structures is key [1, 11]. NMR and in particular solid-state NMR, are powerful techniques to understand the self-assembly mechanism of Mac1 into bacterial membranes [9, 34]. Isotopically labelled peptides are usually needed to increase sensitivity and alleviate interference from background signals for in-cell NMR experiment [35]. An *E. coli*-based cell expression system was selected because of its fast growth kinetics to achieve high cell density cultures, low cost and technical simplicity [36, 37]. However, the direct expression of cationic AMPs in *E. coli* is difficult due to their toxicity to host cells and sensitivity to the host intracellular proteolytic degradation [38]. An example is the initial failed expression of SUMO-Mac1 fusion protein due to high toxicity of the construct against *E. coli* (data not shown). Here, an *E. coli*-based expression system was successfully established to produce SUMO-Mac1-*Mxe* GyrA fusion protein without toxicity of the construct against the host cell. The intein fusion system with inducible self-cleavage activity and tryptophan residues (absent in SUMO and Mac1) provided additional benefits for UV-vis detection of the fusion construct [39]. SUMO acts as a nucleation site for the proper folding of the fusion protein with increased solubility and expression level due to its highly hydrophilic surface and hydrophobic core [25]. The fusion partner -*Mxe* GyrA cleavage efficiency (~90%) was consistent with previous reports (> 50%) having phenylalanine as last amino acid with DTT cleavage in concentrated ammonium solution [21].

The fusion strategy allowed the production of 0.1 mg/L of ^{15}N labelled Mac1, with a C-terminal amidated and the level of C-amidation (~75%) was consistent with previous studies[21]. Theoretically, ~1.56 mg (100% recovery) ^{15}N labelled Mac1 should be obtained from 30 mg fusion protein, but the necessary buffer exchange and HPLC steps drastically reduced the final recovery percentage of labelled peptide (6.7%, Table 1). Other reasons for the loss are probably inclined to the spontaneous hydrolysis upon fusion cleavage resulting in ~25% non-amidated Mac1 (Mac1-OH) and minor non-precipitated Mac1 in cleavage buffer. While AMPs have been produced without using fusion proteins [40, 41], several difficulties such as C-amidation or non-native sequence could led to detrimental structural modifications, particularly for such short peptide sequence [42-44]. Here, the expressed Mac1 maintained the same α-helix structure as its synthesized form as indicated by CD and ^{1}H-^{15}N HSQC spectra. The results correlated well with activity tests, with the same antibacterial ability against *S. aureus*. Therefore, the expression system produced functional labelled peptides, suitable for future in-cell NMR studies, albeit such studies required high amounts of labelled peptides. Although the expression yield was high (60 mg/L), Mac1 represents only *ca.* 5% of the total mass. Thus, greater yield of C-amidated Mac1 peptide could eventually be obtained by optimising the size of the fusion tags. For example, human β-defensin-2 AMP (~4 kDa) was fused to a self-cleaving tag ΔI-CM mini intein (~17 kDa) for the expression and resulted with yield of ~1mg/L of recombinant AMP [45]. High yield expression (~58 mg/L) of cecropin B2 AMP (~4 kDa) also derived from using small intein Ssp DnaB intein (~18 kDa) [46]. Overall, the expression of Mac1 with native N and C termini was achieved which could provide a mean to use different labelling schemes, such as partial labelling – a requisite for performing high-resolution in-cell solid-state NMR studies.

CONFLICTS OF INTEREST

The author declares no conflicts of interest.

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REFERENCES

1. Zhu S, Sani MA, Separovic F. Interaction of cationic antimicrobial peptides from Australian frogs with lipid membranes. *Pept Sci.* 2018;110(3):e24061.
2. Bahar AA, Ren D. Antimicrobial peptides. *Pharmaceuticals (Basel).* 2013;6(12):1543-1575.
3. Kumar P, Kizhakkedathu JN, Straus SK. Antimicrobial Peptides: Diversity, Mechanism of Action and Strategies to Improve the Activity and Biocompatibility In Vivo. *Biomolecules.* 2018;8(1):
4. Sani M-A, Whitwell T, Gehman J, Robins-Browne R, Pantarat N, Attard T, Reynolds E, O'Brien-Simpson N, Separovic F. Maculatin 1.1 disrupts Staphylococcus aureus lipid membranes via a pore mechanism. *Antimicrob Agents Chemother.* 2013;57(8):3593-3600.
5. Sani M-A, Henriques ST, Weber D, Separovic F. Bacteria may cope differently from similar membrane damage caused by the Australian tree frog antimicrobial peptide maculatin 1.1. *J Biol Chem.* 2015;290(32):19853-19862.
6. Hashemi MM, Rovig J, Weber S, Hilton B, Forouzan MM, Savage PB. Susceptibility of colistin-resistant, Gram-negative bacteria to antimicrobial peptides and ceragenins. *Antimicrob Agents Chemother.* 2017;61(8):
7. Mahlapuu M, Hakansson J, Ringstad L, Bjorn C. Antimicrobial Peptides: An Emerging Category of Therapeutic Agents. *Front Cell Infect Microbiol.* 2016;6(194.
8. Chia BC, Carver JA, Mulhern TD, Bowie JH. Maculatin 1.1, an anti-microbial peptide from the Australian tree frog, Litoria genimaculata: Solution structure and biological activity. *Eur J Biochem.* 2000;267(7):1894-1908.
9. Sani M-A, Le Brun AP, Separovic F. The antimicrobial peptide maculatin self assembles in parallel to form a pore in phospholipid bilayers. *Biochimica et Biophysica Acta (BBA)-Biomembranes.* 2020;1862(5):183204.
10. Fernandez DI, Lee T-H, Sani M-A, Aguilar M-I, Separovic F. Proline facilitates membrane insertion of the antimicrobial peptide maculatin 1.1 via surface indentation and subsequent lipid disordering. *Biophys J.* 2013;104(7):1495-1507.
11. Chia CS, Torres J, Cooper MA, Arkin IT, Bowie JH. The orientation of the antibiotic peptide maculatin 1.1 in DMPG and DMPC lipid bilayers. Support for a pore-forming mechanism. *FEBS Lett.* 2002;512(1-3):47-51.

12. Renaud J-P, Chari A, Ciferri C, Liu W-t, Remigy H-W, Stark H, Wiesmann C. Cryo-EM in drug discovery: achievements, limitations and prospects. *Nat Rev Drug Discov.* 2018;17(7):471-492.
13. Sani MA, Separovic F. Progression of NMR studies of membrane-active peptides from lipid bilayers to live cells. *J Magn Reson.* 2015;253(138-142.
14. Lee JH, Okuno Y, Cavagnero S. Sensitivity enhancement in solution NMR: Emerging ideas and new frontiers. *J Magn Reson.* 2014;241(18-31.
15. Smernik RJ, Oades JM. Background signal in solid state 13C NMR spectra of soil organic matter (SOM)—quantification and minimization. *Solid State Nucl Magn Reson.* 2001;20(1-2):74-84.
16. Weber DK, Sani M-A, Gehman JD. A routine method for cloning, expressing and purifying Aβ (1–42) for structural NMR studies. *Amino Acids.* 2014;46(10):2415-2426.
17. Li Y. Recombinant production of antimicrobial peptides in Escherichia coli: a review. *Protein Expr Purif.* 2011;80(2):260-267.
18. Mura M, Wang J, Zhou Y, Pinna M, Zvelindovsky AV, Dennison SR, Phoenix DA. The effect of amidation on the behaviour of antimicrobial peptides. *Eur Biophys J.* 2016;45(3):195-207.
19. Shahmiri M, Mechler A. The role of C-terminal amidation in the mechanism of action of the antimicrobial peptide aurein 1.2. *The EuroBiotech Journal.* 2020;4(1):25-31.
20. Crusca Jr E, Rezende AA, Marchetto R, Mendes-Giannini MJ, Fontes W, Castro MS, Cilli EM. Influence of N-terminus modifications on the biological activity, membrane interaction, and secondary structure of the antimicrobial peptide hylin-a1. *Pept Sci.* 2011;96(1):41-48.
21. Cottingham IR, Millar A, Emslie E, Colman A, Schnieke AE, McKee C. A method for the amidation of recombinant peptides expressed as intein fusion proteins in Escherichia coli. *Nat Biotechnol.* 2001;19(10):974-977.
22. Daniel V. Glutathione S-transferases: gene structure and regulation of expression. *Crit Rev Biochem Mol Biol.* 1993;28(3):173-207.
23. Kim SJ, Miyoshi Y, Taguchi T, Tamaki Y, Nakamura H, Yodoi J, Kato K, Noguchi S. High thioredoxin expression is associated with resistance to docetaxel in primary breast cancer. *Clin Cancer Res.* 2005;11(23):8425-8430.
24. Ishida H, Nguyen LT, Gopal R, Aizawa T, Vogel HJ. Overexpression of antimicrobial, anticancer, and transmembrane peptides in Escherichia coli through a calmodulin-peptide fusion system. *J Am Chem Soc.* 2016;138(35):11318-11326.
25. Kuo D, Nie M, Courey AJ. (2014) SUMO as a solubility tag and in vivo cleavage of SUMO fusion proteins with Ulp1, In *Protein Affinity Tags*, pp 71-80, Springer.
26. Xie Y-G, Luan C, Zhang H-W, Han F-F, Feng J, Choi Y-J, Groleau D, Wang Y-Z. Effects of thioredoxin: SUMO and intein on soluble fusion expression of an

antimicrobial peptide OG2 in Escherichia coli. *Protein Peptide Lett.* 2013;20(1):54-60.

27. Esposito D, Chatterjee DK. Enhancement of soluble protein expression through the use of fusion tags. *Curr Opin Biotechnol.* 2006;17(4):353-358.
28. Király O, Guan L, Sahin-Tóth M. (2011) Expression of recombinant proteins with uniform N-termini, In *Heterologous Gene Expression in E. coli*, pp 175-194, Springer.
29. Neidhardt FC, Bloch PL, Smith DF. Culture medium for enterobacteria. *J Bacteriol.* 1974;119(3):736-747.
30. Vranken WF, Boucher W, Stevens TJ, Fogh RH, Pajon A, Llinas M, Ulrich EL, Markley JL, Ionides J, Laue ED. The CCPN data model for NMR spectroscopy: development of a software pipeline. *Proteins: structure, function, and bioinformatics.* 2005;59(4):687-696.
31. Bostock M, Nietlispach D. Compressed sensing: Reconstruction of non-uniformly sampled multidimensional NMR data. *Concepts in Magnetic Resonance Part A.* 2017;46(2):e21438.
32. Sani MA, Loudet C, Gröbner G, Dufourc EJ. Pro-apoptotic bax-α1 synthesis and evidence for β-sheet to α-helix conformational change as triggered by negatively charged lipid membranes. *Journal of peptide science: an official publication of the European Peptide Society.* 2007;13(2):100-106.
33. Kollipara L, Zahedi RP. Protein carbamylation: in vivo modification or in vitro artefact? *Proteomics.* 2013;13(6):941-944.
34. Lee T-H, Sani M-A, Overall S, Separovic F, Aguilar M-I. Effect of phosphatidylcholine bilayer thickness and molecular order on the binding of the antimicrobial peptide maculatin 1.1. *Biochimica et Biophysica Acta (BBA)-Biomembranes.* 2018;1860(2):300-309.
35. Burz DS, Breindel L, Shekhtman A. (2019) Improved sensitivity and resolution of in-cell NMR spectra, In *Methods Enzymol*, pp 305-328, Elsevier.
36. Michel E, Wüthrich K. High-yield Escherichia coli-based cell-free expression of human proteins. *J Biomol NMR.* 2012;53(1):43-51.
37. Schwarz D, Junge F, Durst F, Frölich N, Schneider B, Reckel S, Sobhanifar S, Dötsch V, Bernhard F. Preparative scale expression of membrane proteins in Escherichia coli-based continuous exchange cell-free systems. *Nat Protoc.* 2007;2(11):2945.
38. Abou Aleinein R, Hamoud R, Schäfer H, Wink M. Molecular cloning and expression of ranalexin, a bioactive antimicrobial peptide from Rana catesbeiana in Escherichia coli and assessments of its biological activities. *Appl Microbiol Biotechnol.* 2013;97(8):3535-3543.

39. Sydor JR, Mariano M, Sideris S, Nock S. Establishment of intein-mediated protein ligation under denaturing conditions: C-terminal labeling of a single-chain antibody for biochip screening. *Bioconjugate Chem.* 2002;13(4):707-712.
40. Raran-Kurussi S, Waugh DS. The ability to enhance the solubility of its fusion partners is an intrinsic property of maltose-binding protein but their folding is either spontaneous or chaperone-mediated. *PLoS One.* 2012;7(11):
41. Fox JD, Kapust RB, Waugh DS. Single amino acid substitutions on the surface of Escherichia coli maltose-binding protein can have a profound impact on the solubility of fusion proteins. *Protein Sci.* 2001;10(3):622-630.
42. Moreno-Gonzalez I, Soto C. (2011) Misfolded protein aggregates: mechanisms, structures and potential for disease transmission, In *Semin Cell Dev Biol*, pp 482-487, Elsevier.
43. Valore EV, Ganz T. (1997) Laboratory production of antimicrobial peptides in native conformation, In *Antibacterial peptide protocols*, pp 115-131, Springer.
44. Wei Q, Kim YS, Seo JH, Jang WS, Lee IH, Cha HJ. Facilitation of expression and purification of an antimicrobial peptide by fusion with baculoviral polyhedrin in Escherichia coli. *Appl Environ Microbiol.* 2005;71(9):5038-5043.
45. Luan C, Xie YG, Pu YT, Zhang HW, Han FF, Feng J, Wang YZ. Recombinant expression of antimicrobial peptides using a novel self-cleaving aggregation tag in Escherichia coli. *Can J Microbiol.* 2014;60(3):113-120.
46. Fang Y-T, Li S-Y, Hu N-J, Yang J, Liu J-H, Liu Y-C. Study on Cecropin B2 Production via Construct Bearing Intein Oligopeptide Cleavage Variants. *Molecules.* 2020;25(4):1005.