ORIGINAL PAPER

Production of Bacteriocin E50-52 by Small Ubiquitin-Related Modifier Fusion in *Escherichia coli*

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Abstract

Bacteriocin E50-52, a class IIa bacteriocin with a wide antibacterial spectrum, and has a huge potential to be a substitute for conventional antibiotics. In this research, the bacteriocin E50-52 gene was cloned into the expression vector pET SUMO (small ubiquitin-related modifier) and introduced into *Escherichia coli* BL21 (DE3). The recombinant fusion protein SUMO-bacteriocin E50-52 expressed in a soluble form was purified to a purity of more than 90% by Ni-NTA sepharose column and 117 mg fusion protein was obtained per liter of fermentation culture. The fusion protein was cleaved with SUMO protease and re-applied to a Ni-NTA Sepharose column. Finally, about 16 mg recombinant bacteriocin E50-52 (rbE50-52) was obtained from a 1-liter fermentation culture with no less than 95% purity. The rbE50-52 had similar antimicrobial properties and molecular weight as the native bacteriocin E50-52 and showed very low hemolytic activity.

K e y   w o r d s: *Escherichia coli*, bacteriocin E50-52, purification, recombinant expression, small ubiquitin-related modifier

Introduction

Bacteriocins are a family of ribosomally synthesized antimicrobial peptides produced by bacteria (Klaenhammer, 1993; Cleveland *et al.*, 2001). Studies suggest that bacteriocins can inhibit bacteria by forming pores in the membrane and have effective activity against antibiotic-resistant pathogens (Jenssen *et al.*, 2006; Svetoch and Stern, 2010). There is no report showing that bacteriocins could be toxic for animals and accumulate in the treated subjects (Svetoch and Stern, 2010). Therefore, as promising new biological products, bacteriocins could be developed into novel alternatives to traditional antibiotics (Joerger, 2003). Bacteriocin E50-52 is a class IIa bacteriocin produced by *Enterococcus faecium* NRRL B-30746 and has a molecular weight of 3339.7 Da. Bacteriocin E50-52 showed potent activity against both Gram-positive and Gram-negative bacteria in vitro tests and therapeutic trials. Moreover, bacteriocin E50-52 is heat-stable and can inhibit drug-resistant nosocomial bacteria effectively (Svetoch *et al.*, 2008; 2009). Thus, bacteriocin E50-52 could be considered as a potential candidate for antibacterial therapy.

It is often cumbersome, time-consuming, and uneconomical to isolate bacteriocins from natural producer strains. Though chemical synthesis is a good way to obtain peptides of interest, it is limited by expensive reagents. Heterologous expression in *E. coli*, the first host used to produce a recombinant pharmaceutical peptides, is an adaptable method for the economical and rapid production of target proteins (Swartz, 2001; Li *et al.*, 2007; Jasniewski *et al.*, 2008). However, the significant antimicrobial activity of bacteriocins poses difficulties for direct expression in the host and high susceptibility to proteolytic degradation decreases the efficiency of purification (Li *et al.*, 2009). For these reasons, the potential fusion expression strategy is employed to protect *E. coli* and target peptide (Rao *et al.*, 2004; Wei *et al.*, 2005; Morin *et al.*, 2006; Shen *et al.*, 2010; Ma *et al.*, 2012).

SUMO is a member of the ubiquitin protein family that modulates the properties of intracellular proteins (Muller *et al.*, 2001; Johnson, 2004). When fused with proteins, SUMO can fold and protect them via its chaperoning functions (Li *et al.*, 2010). As a new fusion system, SUMO appears to have all the advantages of traditional systems and can be cleaved without extension in the N-terminus of the target proteins (Butt *et al.*, 2005). Therefore, in this study, SUMO fusion protein expression system was used to produce rbE50-52.

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Experimental

Materials and Methods

Bacterial strains and growth conditions. *E. coli* bacterial strain Mach1™-T1ᴿ and strain BL21 (DE3) (Invitrogen, USA) were used as subcloning and expression host, respectively. Both *E. coli* Mach1™-T1ᴿ cells and BL21 (DE3) cells were cultured and selected in LB broth or agar (1.8%) supplemented with kanamycin (50 μg/ml) at 37°C. *Staphylococcus aureu* ATCC 25923 was purchased from the China General Microbiological Culture Collection Center (Beijing, China), *E. coli* K88 and *Listeria monocytogenes* IVDCC 53005 were purchased from the China Veterinary Culture Collection Center (Beijing, China).

Plasmid and gene sequence. pET SUMO (Invitrogen) was used as expression plasmid. The GenBank accession number for the bacteriocin E50-52 used in this study is P85148.

Construction of expression vectors. The bacteriocin E50-52 gene was synthesized (Invitrogen) and amplified using primer pairs (Primer E50-52 F: 5'-ACCAC-CAAAAACTATGGCAAC-3', Primer R: 5'-TCACTA-TTACGCCAGTTTGCACAG-3'). The resulting PCR product was separated by 1.8% gel electrophoresis, purified using a DNA gel extraction kit (Tiangen), and ligated into the linearized pET SUMO plasmid by TA cloning. The ligation mixture was transformed into *E. coli* Mach1™-T1ᴿ cells for propagation of the recombinant plasmid. Another PCR was performed using primer pairs (Primer SUMO F: 5'-AGATTCTT-GTACGCAGGTATTAG-3'; Primer T7 R: 5'-TAGT-TATTGCTCAGCGGTGG-3'), and the product containing inserted fragment was sequenced to confirm insert orientation and sequence fidelity.

Expression of rbE50-52 fusion protein. The constructed pET SUMO-bacteriocin E50-52 plasmid was transformed into *E. coli* BL21 (DE3) strain. The recombinant expression strain was cultured in LB broth containing kanamycin at 37°C with shaking (180 rpm) to an optical density (OD$_{600}$) of 0.7–0.8. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was then added to a final concentration of 1.5 mM to induce the expression of the recombinant protein at 37°C for 5 h. To obtain the recombinant protein, the cells were treated according to the manufacturer's instruction. The disrupted cells were centrifuged at 13,000 g for 2 min to separate the soluble supernatant and the insoluble pellet fraction. Both supernatant and precipitate were analyzed by Tricine-SDS-PAGE. The non-induced cells were used as negative control.

Purification of fusion protein. The solubilized SUMO-bacteriocin E50-52 fusion protein was applied to a pre-equilibrated Ni-NTA (GE, USA) Sepharose column (5 ml), and the bound protein was eluted by elution buffer (50 mM Na$_3$PO$_4$ and 500 mM NaCl, 10–400 mM imidazole, pH 7.4) at 2 ml/min. The eluted samples were analyzed by Tricine-SDS-PAGE, and dialyzed against 150 mM NaCl overnight at 4°C.

Cleavage and purification of rbE50-52. The 500 μg fusion protein was treated with 250 U SUMO protease (Invitrogen) in 10×SUMO protease buffer (2% Igepal (NP-40), 1.5 M NaCl, 10 mM DTT, 500 mM Tris-HCl, pH 8.0) 500 μl at 4°C for 15 h. After the cleavage, proteins samples were subjected to a Ni-NTA Sepharose column to remove 6×His-tagged carrier and undigested fusion proteins. The purified rbE50-52 was analyzed by Tricine-SDS-PAGE.

Determination of the molecular weight by MS. MS analysis was carried out on AB4700 MALDI-TOF/TOF MS (AB Company, USA). Lyophilized rbE50-52 was dissolved with solvent A (70% acetonitrile, 0.1% trifluoroacetic acid in water [v/v/v]), and 0.5 μl samples mixed directly on the target with 1.5 μl of matrix (20 μg of α-cyno-β-hydroxy-cinnamic acid/μl in solvent A). The collected data were analyzed using Data Explorer V4.5 software (Applied Biosystems, USA).

Antimicrobial activity assay. The antimicrobial activity of rbE50-52 was tested by the agar diffusion method (cylinder plate method) using *S. aureus* ATCC 25923 as indicator strain. Cylinders were placed on the surface of agar inoculated with the test organism (about 1×10^7 CFU/ml) and filled with 200 μl of purified rbE50-52 (16 μg/ml). The same volume of sodium phosphate buffer (PBS) and SUMO-bacteriocin E50-52 fusion protein were used as negative control. After incubation overnight at 37°C, zones of growth inhibition surrounding the cylinders were measured. The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were determined by a liquid growth inhibition test using *S. aureus* ATCC 25923, *E. coli* K88 and *L. monocytogenes* IVDC C53005 as indicator strains as described by Karthikeyan (2006).

Hemolytic assay. The hemolytic property of rbE50-52 was evaluated by examining hemoglobin release at 540 nm in suspensions of rabbit erythrocytes. Bacteriocin at different concentrations (5, 10, 25, 50, 100 μg/ml) was used in this experiment. The fresh rabbit blood was treated according to the method of Tian (2009). The absorption was recorded at 540 nm using Imark™ Microplate Reader (Bio-Rad, US). The values for 0% and 100% hemolysis were determined in 10 mM PBS and 0.1% Triton X-100, respectively.

Results

Construction of expression vectors. The schematic representation of the bacteriocin E50-52 expression vector, containing a His-tag for affinity purification, is
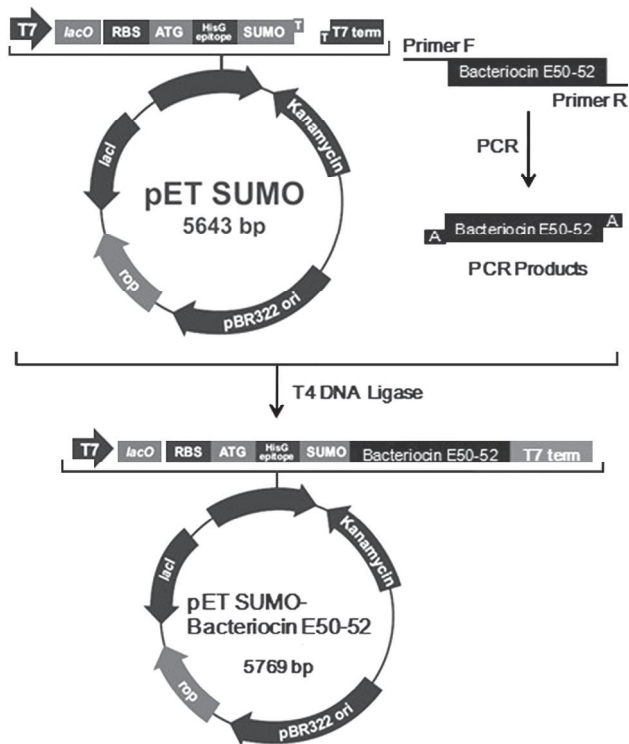
Fig. 1. Schematic representation of construction of the pET
SUMO-bacteriocin E50-52.

After amplification, bacteriocin E50-52 gene was inserted into pET
SUMO plasmid by TA cloning method. The plasmid with correct insert
orientation was selected by sequencing.

Fig. 3. Tricine-SDS-PAGE analysis of the purification
of SUMO-bacteriocin E50-52 fusion protein.

The eluted fusion protein showed about 90% purity by electrophoretic
analysis with Tricine-SDS-PAGE using the Bandscan software (BioMa-
rin Pharmaceutical Inc., UK). Lane M, protein markers; lane 1, elution;
lane 2, wash; lane 3, precipitation of cell lysate; lane 4, supernatant of cell
lysate. The arrow indicates the purified fusion protein.

presented in Fig. 1. The correct orientation of the insert
was confirmed by PCR amplification and sequencing
(data not shown).

Expression of the fusion protein. *E. coli* BL21
(DE3) was transformed with pET SUMO-bacteriocin
E50-52 plasmid and induced as described above. After
disruption by sonication, supernatant and precipitate
were subject to Tricine-SDS-PAGE. As shown in Fig. 2,

there was an obvious protein band after IPTG induction
compared with non-induced cells. The result indicated
that the fusion protein was efficiently produced in a sol-
uble form in the host induced by 1.5 mM IPTG at 37°C.

Purification of fusion protein. The fusion protein
contains polyhistidine (6 × His) tag at N-terminus and
can be separated efficiently using a Ni-NTA Sepharose
column. Most of the proteins without His-tag were
washed with washing buffer (the concentration of
imidazole < 300 mM), and the 6 × His-tagged SUMO-
bacteriocin E50-52 was eluted by the elution buffer
(50 mM Na_3PO_4, 500 mM NaCl, 300 mM Imidazole,
pH 7.4) with more than 90% purity (Fig. 3). About
117 mg fusion protein can be obtained from 1-liter of
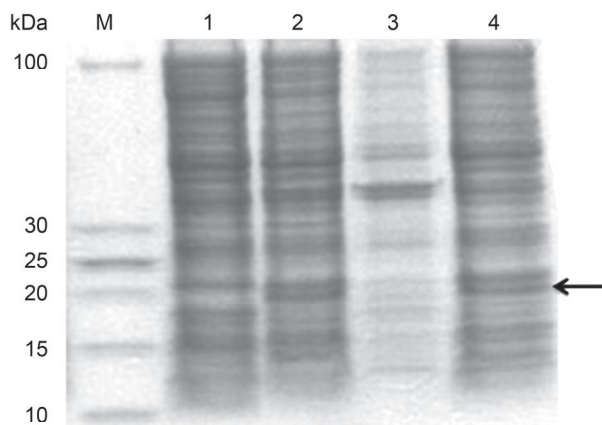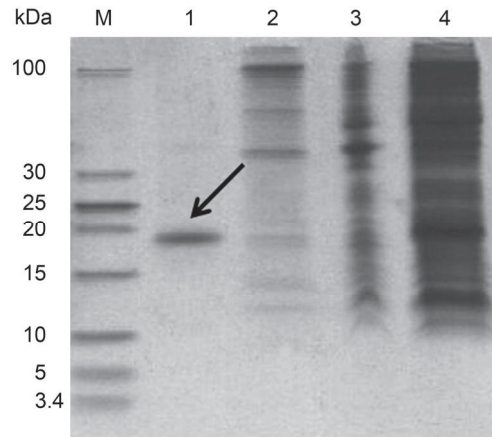fermentation culture.

Fig. 2. Tricine-SDS-PAGE analysis of expression fusion protein.

Lane M, protein markers; lane 1, negative control, lane 2
and 4 are soluble proteins when cultured at 1.5 mM IPTG;
lane 3 is the corresponding insoluble proteins.
The arrow indicates the location of the fusion protein.
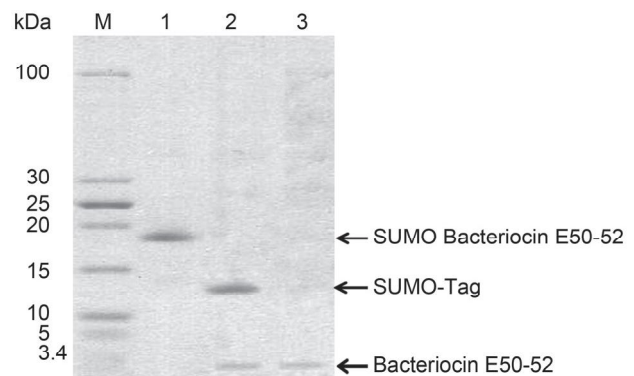

Fig. 4. Tricine-SDS-PAGE analysis of SUMO-bacteriocin
E50-52 fusion protein cleaved by SUMO protease and rbE50-52
purification.

Lane M, protein markers; lane 1, purified SUMO-bacteriocin E50-52
fusion protein; lane 2, mixture of SUMO-bacteriocin E50-52 fusion
protein by SUMO protease cleavage; lane 3, purified recombinant
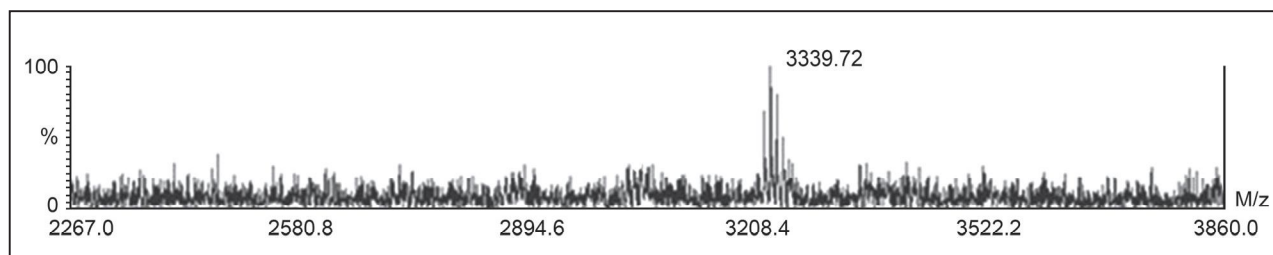bacteriocin E50-52 by Ni-NTA(GE).

Fig. 5. MALDI-TOF/TOF MS mass spectra of purified rpE50-52.

Cleavage and purification of rbE50-52. Purified SUMO-bacteriocin E50-52 fusion protein was cleaved using SUMO protease to release the rbE50-52, and then confirmed by Tricine-SDS-PAGE. Subsequently, the mixture was loaded to a Ni-NTA Sepharose column and rbE50-52 was washed with washing buffer. As shown in Fig. 4, the fusion protein was successfully cleaved (lane 2) and rbE50-52 was purified efficiently (lane 3). About 16 mg of purified mature rbE50-52 with more than 95% purity was obtained from 1-liter fermentation culture.

Determination of the molecular weight. The precise molecular weight of rbE50-52 determined by MS was 3339.72 Da, which is in good agreement with that of the native bacteriocin E50-52 (Fig. 5). The result suggested that the bacteriocin E50-52 was expressed correctly and the fusion protein was cleaved at expected position with no extra amino acid in the N-terminus of recombinant peptide.

Antimicrobial activities of rbE50-52. The rbE50-52 showed strong inhibitory activity against *S. aureus* ATCC 25923 (Fig. 6). The MIC and MBC against selected strains are presented in Table I. The results indicated that the rbE50-52 had similar antibacterial properties against indicator bacteria as the native bacteriocin E50-52 and was effective in killing bacteria.
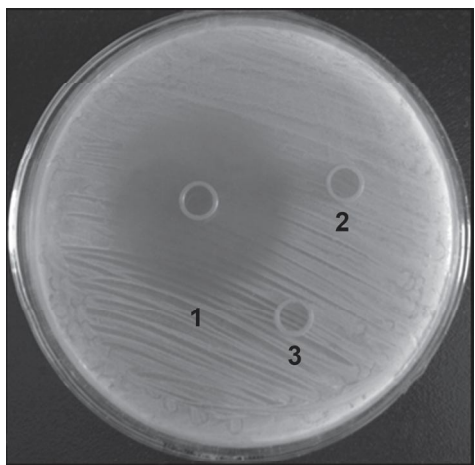

Fig. 6. The bioactivity of rbE50-52 against *S. aureus*.
(1) 16 μg rbE50-52 /ml; (2) the negative control, PBS;
(3) 117 μg SUMO-bacteriocin E50-52 fusion protein/ml.

Hemolytic activity. The release of hemoglobin was used to evaluate the hemolytic activity of rbE50-52. The hemolysis percentages of the peptide at concentration of 5, 10, 25, 50, 100 μg/ml were $0.76 \pm 0.65\%$, $0.85 \pm 0.57\%$, $1.98 \pm 0.67\%$, $2.11 \pm 0.49\%$, $2.87 \pm 0.76\%$, respectively.

Table I
The MIC/MBC of rbE50-52 and native bacteriocin E50-52 to selected microorganisms

Microorganisms	MIC/MBC (μg/ml)	
	rbE50-52	Native bacteriocin E50-52[a]
E. coli K88	0.9/1.7	<1.6/——[b]
S. aureus ATCC 25923	0.5/2	0.2–0.8/——
L. monocytogenes IVDCC 53005	14/32	2–32/——

[a] The MIC of the native bacteriocin E50-52 against *E. coli*, *S. aureus* and *L. monocytogenes* were determined using many different isolates.
[b] The MBC of native bacteriocin E50-52 against selected bacteria was not reported.

Discussion

Drug resistance among variety of microorganisms poses a potentially serious threat to public health and has caused substantial alarm and concern (Svetoch *et al.*, 2008; 2009). It becomes urgent to develop antimicrobial compounds with new mechanisms of action and potent activity against these microorganisms. Bacteriocins can inhibit bacterial growth by disruption of target cell membrane and have a low frequency of bacteriocin-resistant mutants (Sang and Blecha, 2008; Svetoch and Stern, 2010). Bacteriocin E50-52 displayed strong inhibitory property against both Gram-positive and Gram-negative bacteria and was also effective against antibiotic-resistant bacteria isolated from hospitals in vitro test (Svetoch *et al.*, 2008; 2009). *In vivo* test in broiler, oral treatment with bacteriocin E50-52 reduced both *Salmonella enteritidis* and *Campylobacter jejuni* by more than 100 000-fold in the ceca, and systemic *S. enteritidis* was reduced in the liver and spleen (Svetoch *et al.*, 2008). In this work, the MBC and hemolytic activity of rbE50-52 were also measured. The

peptide showed effective bactericidal properties against indicator strains and produced very low hemolysis against rabbit red blood cells. These characteristics of bacteriocin E50-52 make it potentially valuable as an agent for antimicrobial chemotherapy.

The low efficiency of bacteriocin purification from natural producer strains and high cost of chemical synthesis led to the exploration of heterologous expression of peptides with antimicrobial property against bacteria (Li *et al.*, 2010). As one of the most commonly used heterologous expression host, *E. coli* was utilized to produce rbE50-52 in this study. Due to their high toxicity to the expression host cells and sensitivity to proteases, direct expression systems of recombinant bacteriocins are difficult to be established. So far, the best available tools for expression of these proteins are fusion protein partners, which can also increase expression level, enhance protein solubility, and simplify purification and detection of recombinant proteins (Butt *et al.*, 2005; Esposito and Chatterjee, 2006; Li, 2009; Sun *et al.*, 2008). Today, a number of fusion protein partners are used in the expression of proteins, such as MBP (maltose-binding protein), GST (glutathione S-transferase), TRX (thioredoxin), Nus A, Ub (ubiquitin) and SUMO. Comparative study showed that SUMO was superior to commonly used fusion partners (Marblestone *et al.*, 2006). SUMO proteases are able to cleave SUMO tag accurately and release the recombinant protein with native N-terminus, which is prerequisite for maintaining the activity of bacteriocins (Wu and Hancock, 1999; Butt *et al.*, 2005). In the current study, the fusion protein was expressed and purified efficiently with the aid of SUMO fusion protein partners. After cleavage, the rbE50-52 with desired N-terminus was released. The recombinant peptides had similar antimicrobial properties (MIC) and molecular weight as the native bacteriocin E50-52. About 16 mg rbE50-52 was obtained from one liter of fermentation culture, the yield of native E50-52 was not reported. Compared with isolating from natural producer stains, producing of rbE50-52 needed cheaper medium, shorter fermentation time and simpler purification procedures (Svetoch *et al.*, 2009).

In summary, we constructed an efficient SUMO fusion system to express and purify E50-52 in *E. coli*. The system can produce biologically active bacteriocin E50-52 with acceptable yield, and the purification is achieved in two steps. This expression system can potentially be a powerful tool for obtaining sufficient quantities of bacteriocin E50-52 for further studies or possible application.

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