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Expression and Purification of Lacticin Q by Small Ubiquitin-Related Modifier Fusion in *Escherichia coli*

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Lacticin Q is a broad-spectrum class II bacteriocin with potential as an alternative to conventional antibiotics. The objective of this study was to produce recombinant lacticin Q using a small ubiquitin-related modifier (SUMO) fusion protein expression system. The 168-bp lacticin Q gene was cloned into the expression vector pET SUMO and transformed into *Escherichia coli* BL21(DE3). The soluble fusion protein was recovered with a Ni-NTA Sepharose column (95% purity); 130 mg protein was obtained per liter of fermentation culture. The SUMO tag was then proteolytically cleaved from the protein, which was re-applied to the column. Finally, about 32 mg lacticin Q (≥96% purity) was obtained. The recombinant protein exhibited antimicrobial properties similar to that of the native protein, demonstrating that lacticin Q had been successfully expressed by the SUMO fusion system.

Keywords: bacteriocin, *Escherichia coli*, recombinant expression, lacticin Q, small ubiquitin-related modifier

Introduction

Bacteriocins are ribosomally synthesized antibacterial peptides or proteins produced by bacteria (Riley and Wertz, 2002; Torkar and Bogovi, 2003). These biological products show considerable potential as substitutes for antibiotics because of their antibacterial activity and low toxicity (Gillor and Ghazaryan, 2007; Xie *et al.*, 2009). Lactic acid bacteria produce a wide variety of bacteriocins (Beaulieu *et al.*, 2005). Lacticin Q is a bacteriocin produced by *Lactococcus lactis* QU 5 that exhibits the same spectrum of activity as nisin A. Moreover, lacticin Q is resistant to heat treatment and pH changes, showing stability at high pH values that inactivate nisin A (Fujita *et al.*, 2007). Therefore, lacticin Q shows potential as an ecologically friendly alternative to conventional antibiotics.

Only low levels of bacteriocin can be obtained through growth of the natural producer strains, and these compounds are typically difficult to purify. Heterologous expression of bacteriocin has been widely studied in recent years in an attempt to increase yields. *Escherichia coli* is commonly used because of its high level of expression, rapid growth rate, and low cost (Jasniewski *et al.*, 2008; Li *et al.*, 2009). However, the broad antibacterial spectrum and high susceptibility to proteolytic degradation of bacteriocins pose difficulties for their direct expression in *E. coli* (Li, 2009). For this reason, affinity tags are fused to target proteins to neutralize the toxicity of antibacterial peptides to the host cells (Čipáková *et al.*, 2004; Rao *et al.*, 2004; Wei *et al.*, 2005; Morin *et al.*, 2006) and for purification by chromatographic separation (Shen *et al.*, 2010).

Small ubiquitin-related modifier (SUMO) is a member of a ubiquitin-like protein family that regulates cellular processes such as apoptosis, nuclear transport, and cell cycle progression (Muller *et al.*, 2001). When fused at the N-terminus of proteins, SUMO facilitates folding and protects the protein through its chaperoning properties (Li *et al.*, 2010). In addition, fusing a heterologous protein to SUMO can increase expression, enhance solubility, and simplify purification and detection of the recombinant protein (Sun *et al.*, 2008). Therefore, the objective of the current study was to produce recombinant lacticin Q using a SUMO fusion protein expression system.

Materials and Methods

Bacterial strains and growth conditions

E. coli bacterial strain Mach1^{TM}-T1^{R} (Invitrogen, USA) was used for subcloning and plasmid amplification. *E. coli* bacterial strain BL21 (DE3) (Invitrogen) was used as the expression host. Both *E. coli* Mach1^{TM}-T1^{R} cells and BL21 (DE3) cells were grown and selected in LB broth or agar (2.0%) containing 50 μg kanamycin/ml at 37°C. *Enterococcus hirae* (ATCC 10541) was obtained from the China General Microbiological Culture Collection Center (Beijing, China), and *Staphylococcus aureus* (ATCC 12600) was obtained from the Agricultural Culture Collection of China (Beijing, China). *Listeria innocua* (ATCC 33090) was conserved by our laboratory.

Plasmid and gene sequence

The plasmid pET SUMO (Invitrogen) is a commercial fusion protein expression vector. A codon usage table for *E. coli* is available (http://www.kazusa.or.jp/codon). GenBank

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accession no. for the lacticin Q used in this study is **BAF57910** (Fujita *et al.*, 2007).

PCR amplification and construction of expression vectors

The lacticin Q gene was synthesized (Invitrogen) and cloned into pMD18-T (TaKaRa, Japan). Plasmid DNA was isolated using a TIAN prep Midi Plasmid Kit (Tiangen, China). The 168 bp gene of lacticin Q was amplified using Taq DNA polymerase (TaKaRa) and primer pairs (Primer F: 5′-ATG GCGGGCTTTCTGAAAGTG-3′, Primer R: 5′-TCACTAT TATTTAATGCCCAGAATCTGTT-3′). The PCR conditions were 30 cycles of 30 sec at 95°C, 30 sec at 55°C, 1 min at 72°C after denaturing for 5 min at 94°C. The PCR products were separated by 2% gel electrophoresis, purified with a DNA gel extraction kit (Tiangen), and inserted into the linearized pET SUMO plasmid (Invitrogen) by TA cloning using T4 DNA Ligase (TaKaRa). The ligation mixture was transformed into *E. coli* Mach1^{TM}-T1^{R} cells and the recombinant plasmid was verified by PCR amplification and sequencing.

Expression of SUMO-lacticin Q fusion protein

The pET SUMO-lacticin Q plasmid that had been constructed was transformed into competent *E. coli* BL21 (DE3). The recombinant expression strain was cultivated in LB broth containing 50 μg kanamycin/ml (1% glucose) at 37°C with shaking (200 rpm) to an optical density (OD 600) of 0.6–0.8. Isopropyl β-D-1-thiogalactopyranoside (IPTG) (1.5 mM) was then added to induce the expression of the recombinant protein at 37°C for 5 h. The cells were harvested by centrifuging at 13,000×g for 2 min, resuspended in lysis buffer (50 mM K_3PO_4, 400 mM NaCl, 100 mM KCl, 10% glycerol, 0.5% Triton X-100, 10 mM imidazole, pH 7.8), and disrupted on ice by sonication at 200 W for 45 cycles (2 sec working, 8 sec free). Following centrifugation at 13,000×g for 2 min, the supernatant was collected and subjected to purification. Both supernatant and precipitate were analyzed by Tricine-SDS-PAGE.

Purification of SUMO-lacticin Q fusion protein

SUMO-lacticin Q fusion peptide was purified using a Ni-NTA Sepharose (GE, USA) column (5 ml). The column was pre-equilibrated with binding buffer (50 mM Na_3PO_4, 500 mM NaCl, pH 7.4) and the bound protein was eluted by a linear gradient of 10–400 mM imidazole in buffer (50 mM Na_3PO_4 and 500 mM NaCl, pH 7.4) at 2 ml/min. The eluted fractions were analyzed by Tricine-SDS-PAGE, and dialyzed overnight at 4°C against 150 mM NaCl.

Cleavage of SUMO-lacticin Q fusion protein

The 500 μg SUMO-lacticin Q protein was reacted with 250 U SUMO protease (Invitrogen) in 10× SUMO protease buffer [500 mM Tris-HCl; pH 8.0, 2% Igepal ca-360 (NP-40), 1.5 M NaCl, 10 mM DTT] 500 μl at 30°C for 3 h. The cleaved mixture was analyzed by Tricine-SDS-PAGE.

Purification of recombinant lacticin Q

After SUMO protease cleavage, the SUMO-lacticin Q fusion protein sample was loaded onto another Ni-NTA Sepharose column (5 ml) to remove His tagged carrier and any undigested fusion proteins. The purified proteins were analyzed

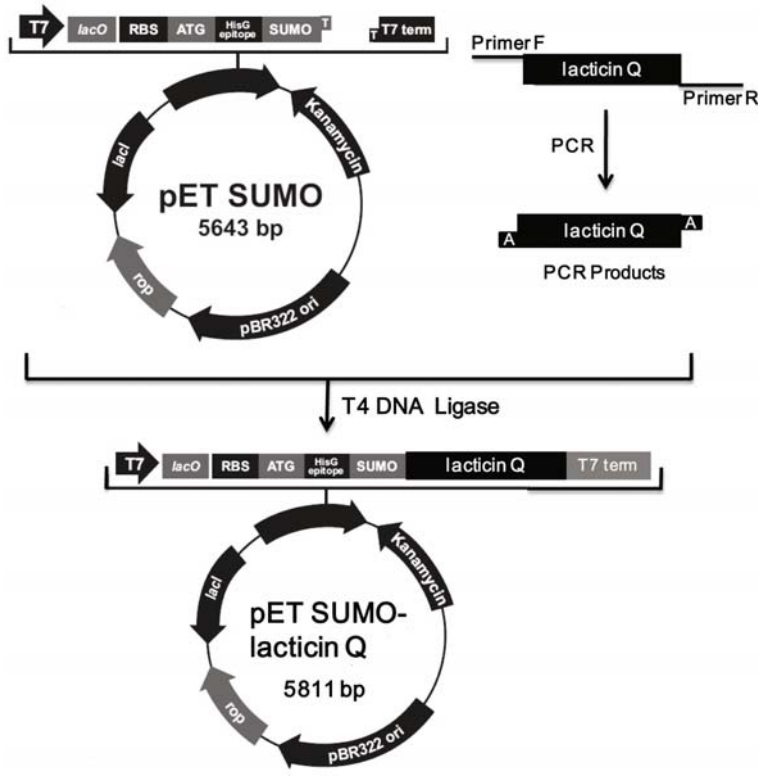

Fig. 1. **Construction of the recombinant plasmid pET SUMO-lacticin Q.**

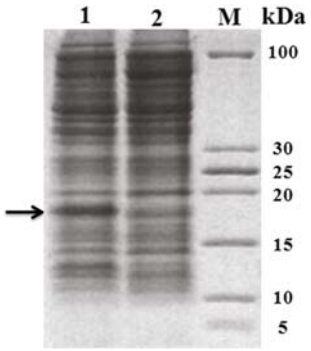

Fig. 2. Tricine-SDS-PAGE analysis of fusion protein expression. Lanes: M, protein markers; 1, supernatant of 1.5 mM IPTG-induced bacterial lysate; 2, supernatant of non-induced bacterial lysate. Arrow indicates the location of the fusion protein.

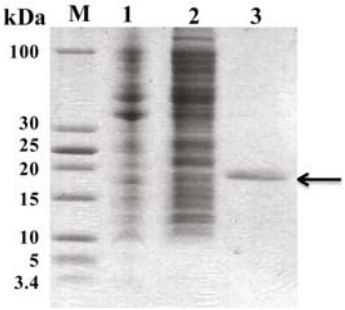

Fig. 3. Tricine-SDS-PAGE analysis for the purification of SUMO-lacticin Q fusion protein. The eluted fusion protein showed about 95% or more purity by electrophoretic analysis with Tricine-SDS-PAGE as analyzed by Bandscan software (BioMarin Pharmaceutical Inc.). Lanes: M, protein markers; 1, precipitate of cell lysate; 2, supernatant of cell lysate; 3, elution. Arrow indicates the purified fusion protein.

by Tricine-SDS-PAGE and the samples were stored at -20°C for molecular weight determination and activity assay.

Tricine-SDS-PAGE

Tricine-SDS-PAGE (4% and 16% acrylamide for the stacking and separating gel, respectively) was performed according to the procedure of Schagger (Schagger, 2006) to separate the proteins and determine the molecular weight of the recombinant protein. Protein markers (Fermentas, Canada) including 100 kDa, 30 kDa, 25 kDa, 20 kDa, 15 kDa, 10 kDa, 5 kDa, and 3.4 kDa were subjected to Tricine-SDS-PAGE. The initial working voltage was at 70 V. When the protein marker reached the edge of the separating gel, the voltage was changed to 120 V until the end of electrophoresis. All gels were stained overnight in solution containing Coomassie brilliant blue G-250. The background blue color was destained with a solution made up of glacial acetic acid (7%, v/v) and methanol (50%, v/v) to visualize the proteins. The gels were analyzed by Bandscan software (BioMarin Pharmaceutical Inc., UK).

Determination of molecular weight by MS

AB4700 MALDI-TOF/TOF MS (AB Company, USA) was used to determine the molecular weight of lacticin Q, with a 337 nm nitrogen laser for desorption and ionization. The total acceleration voltage was 20 kV, and the delay time was 330 ns. Lyophilized peptide fractions were dissolved in solvent A (70% acetonitrile, 0.1% trifluoroacetic acid in water [v/v/v]), and part of the samples (0.5 µl) mixed directly on the target with 1.5 µl of matrix (20 µg of α-cycno-β-hydroxy-cinnamic acid/µl in solvent A). The data were collected and analyzed using Data Explorer V4.5 software (Applied Biosystems, USA).

Recombinant lacticin Q activity assay

Recombinant lacticin Q activity was assayed by the agar diffusion method, with *Staphylococcus aureus* used as target strain. A dilution of the strain was spread on nutrient broth (NB) plates. Cylinders were placed on the agar surface, and 100 µl of purified lacticin Q (100 µg/ml) was added to each well. The same volume of sodium phosphate buffer (PBS) was used as negative control and 100 µl PBS containing 150 µg ampicillin/ml was used as positive control. Plates were incubated overnight at 37°C, and subsequently areas of growth inhibition (antibacterial activity) surrounding the cylinders were detected. The minimal inhibitory concentration (MIC) was determined by a liquid growth inhibition assay using *Enterococcus hirae*, *Staphylococcus aureus*, and *Listeria innocua* as indicator strains (Lee *et al.*, 2002). The indicator organisms were cultured at 37°C until the optical density reached the logarithmic stage, and distributed into a 96-well plate with 10^5 cells per well (90 µl). The cells were then treated with 10 µl of purified lacticin Q at a series of concentrations. Each assay was repeated three times. After 12 h incubation at 37°C, the plate was assessed

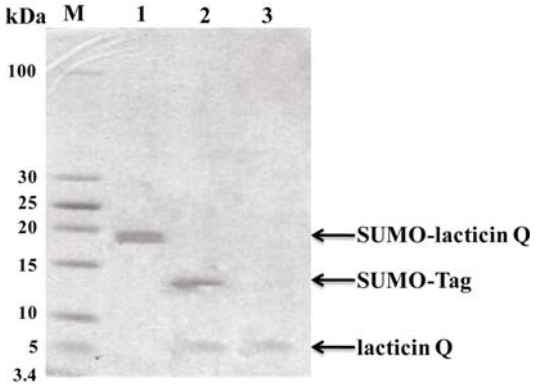

Fig. 4. Tricine-SDS-PAGE analysis of SUMO-lacticin Q fusion protein cleaved by SUMO protease and recombinant lacticin Q purification. Lanes: M, protein markers; 1, purified SUMO-lacticin Q fusion protein; 2, mixture of SUMO-tag and lacticin Q recombinant protein after SUMO protease cleavage; 3, purified recombinant lacticin Q by Ni-NTA.

Table 1. Isolation of recombinant lacticin Q from SUMO-lacticin Q fusion protein

Purification steps	Fusion protein (mg)	Recombinant lacticin Q (mg)	Purity (%)[c]
Ni-NTA Sepharose column	130[a]	40[b]	>95
Ni-NTA Sepharose column	Not applicable	32[a]	>96

[a] Based on 1 L of bacterial culture (about 60 g wet weight). Protein concentration was determined by Bradford protein assay
[b] The amount of recombinant lacticin Q was calculated as a fraction of SUMO-lacticin Q fusion protein
[c] Purity of protein or peptide was estimated by SDS gel stained by Coomassie blue

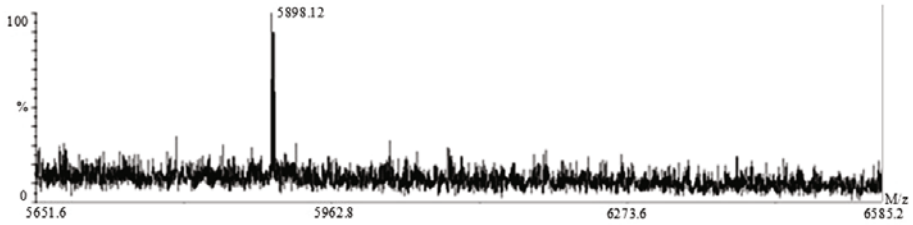

Fig. 5. MALDI-TOF/TOF MS mass spectrum of purified lacticin Q.

by measuring the optical density at 600 nm. The MIC was defined as the lowest lacticin Q concentration at which there was no change in optical density.

Results

Construction of expression vectors

The schematic for construction of the lacticin Q expression vector, containing a His-tag for affinity purification, is depicted in Fig. 1. The correct orientation of the insert was confirmed by PCR and DNA sequencing (data not shown).

Expression of SUMO-lacticin Q fusion protein

The correct recombinant plasmid was transformed into competent expression host *E. coli* BL21 (DE3) cells, and subjected to a pilot expression test. After induction with 1.5 mM IPTG at 37°C, there was an obvious protein band on the gel (Fig. 2), showing that the fusion protein was efficiently produced in a soluble form.

Purification of SUMO-lacticin Q fusion protein

The recombinant protein was loaded on a Ni-NTA Sepharose column pre-equilibrated with the binding buffer at a flow rate of 2 ml/min. Most of the proteins without 6×His tags were washed out with wash buffer (the concentration of imidazole <300 mM), and the 6×His-tagged SUMO-lacticin Q was eluted by the elution buffer (50 mM Na_3PO_4, 500 mM NaCl, 300 mM Imidazole, pH 7.4) with more than 95% purity (Fig. 3). About 130 mg fusion protein was obtained from 1-L of fermentation culture.

Cleavage and purification of recombinant lacticin Q

The lacticin Q was released from the fusion protein by cleavage with the SUMO protease, and confirmed by checking the proteins by Tricine-SDS-PAGE (Fig. 4). After cleaving, the sample was re-applied to a Ni-NTA Sepharose column, and the purified lacticin Q (more than 96% purity) was obtained (Fig. 4). The purity of each purification step can be seen in Table 1. About 32 mg purified lacticin Q was obtained from the 1-L fermentation culture.

Determination of molecular weight

The precise molecular weight of lacticin Q is 5926.50 Da (Fujita *et al.*, 2007). MS analysis revealed that the molecular weight of purified recombinant lacticin Q was 5898.12 Da (Fig. 5), which is 28.38 Da less than that of native lacticin Q.

Antimicrobial activities of recombinant lacticin Q

The recombinant lacticin Q inhibited the growth of *S. aureus* in the agar diffusion assays (Fig. 6). Determination of its MICs against selected microorganisms indicated that the recombinant lacticin Q had antimicrobial properties similar to the native lacticin Q (Table 2).

Discussion

Lacticin Q is a bacteriocin produced by *L. lactis* QU 5 that exhibits the same antibacterial spectrum as nisin A. The MICs of lacticin Q are lower than those of nisin A against the following indicator strains: *Bacillus cereus* JCM 2152^T, *Bacillus circulans* JCM 2504^T, *Bacillus coagulans* JCM 2257^T, *Enterococcus faecalis* JCM 5803^T, *Enterococcus mundtii* QU 2, *Lactococcus lactis* ATCC 19435^T, and *Lactobacillus acidophilus* JCM 1132^T. In addition, lacticin Q is highly stable under neutral and alkaline pH conditions in which nisin A is inactivated (Fujita *et al.*, 2007), indicating its potential as an ecologically friendly alternative to conventional antibiotics.

Expression systems have been difficult to establish for the production of recombinant bacteriocins (Beaulieu *et al.*, 2005), perhaps due to their high toxicity to the host bacterial cells and sensitivity to intracellular proteases. The use of fusion protein partners is one strategy used to address these problems (Yu *et al.*, 2010). In particular, SUMO is used to produce recombinant proteins because it can enhance protein folding and solubility.

The SUMO protease recognizes only the tertiary structure of the SUMO fusion protein, allowing the recombinant

Table 2. The MIC of recombinant lacticin Q and native lacticin Q to selected microorganisms

Microorganism[a]	MIC (μg/ml)	
	Recombinant lacticin Q	Native lacticin Q[b]
Enterococcus hirae ATCC 10541	1.4	1.35
Staphylococcus aureus ATCC 12600	10	10
Listeria innocua ATCC 33090	0.5	0.41

[a] Abbreviations: ATCC, American Type Culture Collection
[b] The MIC value of native lacticin Q is from the original data of Fujita *et al.* (2007)

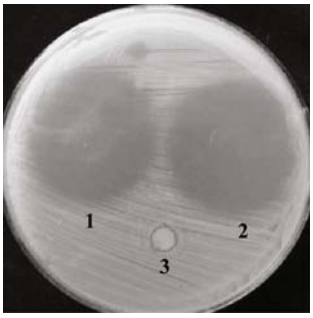

Fig. 6. The antimicrobial activity of recombinant lacticin Q against *S. aureus*. (1) 100 μg recombinant lacticin Q/ml; (2) The positive control, 150 μg ampicillin/ml; (3) the negative control, PBS.

protein to retain the native N-terminus after cleavage (Butt *et al*., 2005). In the present study, the SUMO-lacticin Q fusion protein was completely cleaved by SUMO protease, as shown in Fig. 4. However, the molecular weight of recombinant lacticin Q was 28.38 Da less than that of native lacticin Q (Fig. 5), perhaps because the N-terminal methionine of recombinant lacticin Q was not formylated (Fujita *et al*., 2007).

Several studies recently reported use of the SUMO fusion system to express antimicrobial peptides, including defense peptides (Bommarius *et al*., 2010; Li *et al*., 2010) and CM4 (Li *et al*., 2009). Antimicrobial activities were detected for these recombinant peptides. In the present study we describe the high-yield production of lacticin Q using a SUMO fusion protein partner. With this system, the fusion protein accounted for about 8% of the total protein. We obtained 32 mg pure lacticin Q from the 1-L fermentation culture, and the recombinant protein exhibited antimicrobial properties (i.e., MIC) similar to that of the native protein. In contrast, wild-type strain fermentation produces only 0.4 mg/L lacticin Q (Fujita *et al*., 2007), demonstrating that SUMO fusion technology significantly increases yields.

In summary, we developed a SUMO fusion system for the expression and purification of lacticin Q in *E. coli*, which markedly improved the efficiency of lacticin Q production. SUMO fusion technology can potentially be adapted to produce other unmodified bacteriocins. *E. coli* does not glycosylate or phosphorylate proteins, nor does this host strain recognize proteolytic processing signals from eukaryotes; therefore, this system maybe not suitable for proteins that require post-translational modification.

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