

Expression of plectasin in *Bacillus subtilis* using SUMO technology by a maltose-inducible vector

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Abstract Plectasin, the first fungus defensin, is especially efficient against Gram-positive bacteria. To explore an effective approach for expressing plectasin in *Bacillus subtilis*, the sequence encoding plectasin fused with the small ubiquitin-like modifier (SUMO) gene, the 6 × His gene and the signal peptide of SacB were cloned into an *E. coli*–*B. subtilis* shuttle vector pGJ148 in which the maltose utilization operon promoter Pglv directed the expression. The fusion protein successfully secreted in culture and approximately, 41 mg of the recombinant fusion protein SUMO-plectasin was purified per liter of culture supernatant. After purification by Ni-NTA resin column and digestion by SUMO protease, 5.5 mg of plectasin with a purity of 94 % was obtained from 1 L fermentation culture. Recombinant plectasin was found inhibition activity against *S. pneumoniae*, *S. aureus* and *S. epidermidis*. These results indicate that the maltose-induced expression system may be a safe and efficient way for the large-scale production of soluble peptides in *B. subtilis*.

Keywords Antimicrobial peptide · Plectasin · *Bacillus subtilis* · Maltose-inducible promoter · SUMO

Introduction

In this ‘antibiotic-resistance era’, antimicrobial peptides (AMPs), have been acknowledged as a promising approach

for treating infections owing to their unique mechanisms of action and no drug resistance [6]. AMPs are generally small (12–50 amino acids), and contain both cationic and hydrophobic amino acids [5]. Ubiquitous in nature, AMPs exhibit activity against bacteria, fungi, parasite, virus, and even cancer cells [4]. Defensin, a major family of AMPs, has three pairs of disulfide bonds connected by six conserved Cys residues [1]. Plectasin, isolated from *Pseudoplectanina nigrella*, is the first fungus-derived defensin peptide [15]. This peptide, which comprises 40 amino acids, contains an α - β - β structure and is stabilized by three disulfide bonds (Cys4–Cys30, Cys15–Cys37 and Cys19–Cys39) [15]. This peptide is especially active against Gram-positive bacteria, particularly *Streptococcus pneumoniae*, including clinical isolated drug-resistant strains [15]. Plectasin was neither cytotoxic to murine fibroblasts, human A549 cells, normal human bronchial epithelial cells or lung fibroblasts, nor hemolytic to human erythrocytes. In addition, plectasin did not induce IL-8 in A549 cells [7]. These findings indicated that plectasin would be an attractive future candidate for clinical use as an anti-infective agent; therefore, large amounts of peptides are needed for future research and clinical.

However, isolation of plectasin from natural sources and chemical synthesis are costly and complex [11]. Recombinant DNA technology appears to offer an economic effective means to produce plectasin in greater quantity. To avoid the lethality of AMPs to the host strain and enhance the stability of the AMPs, AMPs are often fused to a carrier protein [16]. An example carrier protein is small ubiquitin-like modifier (SUMO), which consists of approximately 100 amino acids. As an N-terminal fusion partner, SUMO not only significantly enhances expression, but also improves the stability and solubility of the target proteins. Moreover, a unique advantage of a SUMO tag is a specific

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SUMO protease that recognizes SUMO's tertiary structure rather than short degenerate sequences [14]. The SUMO protease is efficient under a wide range of buffer conditions, pH values and temperatures [25].

Although *Escherichia coli* is the most widely used host strain for heterogenous protein engineering, matters of inclusion bodies and lipopolysaccharides (LPS) limit the development of *E. coli* expression system, and make the downstream purification difficult [16]. The Gram-positive bacterium *Bacillus subtilis* is regarded as a potential host for the production of recombinant proteins [18]. It is non-pathogenic and has a naturally high secretory capacity. In contrast to *Escherichia coli*, *Bacillus subtilis* does not contain LPS and can secrete proteins directly into the cultivation medium, which makes recombinant protein purification simpler.

Nowadays, isopropyl- β -D-thiogalactopyranoside (IPTG) induction promoter *Pspac* has been widely used in the expression of AMPs in *B. subtilis* [8, 13]. However, IPTG is expensive and toxicity, which determines that *Pspac* is not an ideal choice for an expression system. Recently, another strong inducible promoter, maltose utilization operon promoter *Pglv* was developed in *B. subtilis* [21]. Maltose, the inducer of *Pglv*, is much cheap than IPTG and is not toxic to host strains. Therefore, *Pglv* is superior to *Pspac* in cost and safety, and more suitable in industrial application.

In our study, SUMO-plectasin was successfully expressed using a maltose-inducible promoter in *B. subtilis*. An efficient environmental method for producing recombinant plectasin using SUMO technology was established in a *B. subtilis* expression system.

Materials and methods

Strains, vectors and reagents

Escherichia coli DH5 α was used for subcloning and plasmid amplification. *B. subtilis* WB800 N and the shuttle plasmid pGJ148 were used for protein expression and secretion (kindly provided by Professor Yizhen Wang, Key Laboratory of Animal Nutrition and Feed Science, Zhejiang University, China). *S. aureus* ATCC 29213, *S. epidermidis* ATCC 12228, *E. coli* ATCC 25922 and *E. coli* UB 1005 (maintained in our lab) were for antimicrobial activity detection. *S. pneumoniae* CVCC 2350 was kindly provided by Prof. Jianhua Wang (Feed Research Institute, Chinese Academy of Agricultural Science, China). Restriction enzymes, T4 DNA ligase and protein molecular weight markers were purchased from TaKaRa Biotechnology (Shiga, Japan). Ni-NTA affinity chromatography columns (5 ml) were purchased from Sangon Biotech Co., Ltd (Shanghai, China). Other chemical reagents were of analytical grade.

Construction of recombinant plasmid pGJ148-SUMO-plectasin

The gene encoding mature plectasin (Genbank, DD265332.1), with the previously reported sequence GFGCNGPWEDDMQCHNHCKSIKGYKGGYCAKGGFVCKCYO, was synthesized as a full-length oligonucleotide with the SUMO gene and signal peptide of SacB using standard solid-phase methods at Sangon Biotechnology Company (Shanghai, China) (Fig. 1a). In addition, a 6 \times His-Tag was between the SP_{sacB} and SUMO coding sequences, which was used to separate the fusion protein by affinity chromatography. After digestion with *EcoR* I and *BamH* I, the fragment was subcloned into *E. coli/B. subtilis* shuttle vector pGJ148 (Fig. 1b). The recombinant plasmid pGJ148-SUMO-plectasin was transformed into competent *E. coli* DH5 α cells. Positive colonies were identified by restriction analysis and DNA sequencing (Table 1).

Transformation and expression

The confirmed recombinant plasmid was transformed into the *B. subtilis* WB800 N according to the method of Ilk [9]. Transformants were selected based on neomycin and chloramphenicol resistance. A positive transformant was inoculated into 10 ml of Luria-Bertani (LB) broth containing 10 μ g/ml neomycin and chloramphenicol, respectively, and grown overnight at 37 $^{\circ}$ C. The culture was then inoculated into fresh LB broth at a ratio of 1:100 and incubated with shaking at 37 $^{\circ}$ C for 3 h. The culture was induced with maltose and incubated at 37 $^{\circ}$ C, with a rotation speed of 220 rpm. The fermentation liquid was collected 0, 6, 12, 24, 36, 48, 60 and 72 h post-induction to quantify the wet cell weight. The supernatant was then collected by centrifugation at 14,000g for 10 min at 4 $^{\circ}$ C, and then cell pellets were incubated with lysozyme (final concentration 1 mg/ml) for 30 min at 37 $^{\circ}$ C to lyse the cells. Total protein concentration was quantified by Bradford.

Purification of fusion protein SUMO-plectasin

SUMO-plectasin was purified by an Ni-NTA resin column. The column was pre-equilibrated with 4 column volumes of binding buffer (20 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole; pH 8.0). The culture supernatant was mixed with 5 \times binding buffer (0.1 M Tris-HCl, 2.5 M NaCl, 0.1 M imidazole; pH 8.0). The mixture was passed through a 0.2- μ m filter and then applied to the column. SUMO-plectasin was eluted at a flow rate of 1 ml/min with 5 column volumes of elution buffer containing 20, 50, 80, 200, 300 and 500 mM imidazole, respectively. The fractions were analyzed by 15 % SDS-PAGE.

Fig. 1 a SP_{SacB} $-6 \times His$ -SUMO-plectasin nucleotide sequence and its amino acid sequence. **b** Schematic representation of the recombinant expression plasmid

a
 GAATTC ATG AAC ATC AAA AAATTCGCT AAA CAA GCT ACA GTT CTT ACA TTC ACA ACAGCT CTT CTT GCT
EcoRI M N I K K F A K Q A T V L T F T T A L L A
 GGC GGC GCT ACA CAA GCT TTC GCT CAT CAT CAT CAT CAT ATG TCT GAT TCT GAA GTT AAC CAA GAA
 G G A T Q A F A H H H H H M S D S E V N Q E
 GCT AAA CCT GAA GTT AAA CCT GAA GTT AAA CCT GAA ACACAT ATC AAC CTT AAA GTT TCT GAT GGC TCT
 A K P E V K P E V K P E T H I N L K V S D G S
 TCT GAA ATC TTC TTC AAA ATC AAAAAACAACACCT CTT CGT CGT CTT ATG GAA GCT TTC GCT AAA CGT
 S E I F F K I K K T T P L R R L M E A F A K R
 CAA GGC AAA GAA ATG GAT TCT CTT CGT TTC CTT TAC GAT GGC ATC CGT ATC CAA GCT GAT CAA ACACCT
 Q G K E M D S L R F L Y D G I R I Q A D Q T P
 GAA GAT CTT GAT ATG GAA GAT AAC GAT ATC ATC GAA GCT CAT CGT GAA CAA ATC GGC GGC GCT ACA TAC
 E D L D M E D N D I I E A H R E Q I G G A T Y
 GGC TTC GGC TGC AAC GGC CCT TGG GAT GAA GAT GAT ATG CAA TGC CAT AAC CAT TGC AAA TCT ATC AAA
 G F G C N G P W D E D D M Q C H N H C K S I K
 GGC TAC AAA GGC GGC TAC TGC GCT AAA GGC GGC TTC GTT TGC AAA TGC TAC TAA GGATCC
 G Y K G G Y C A K G G F V C K C Y * *BamHI*

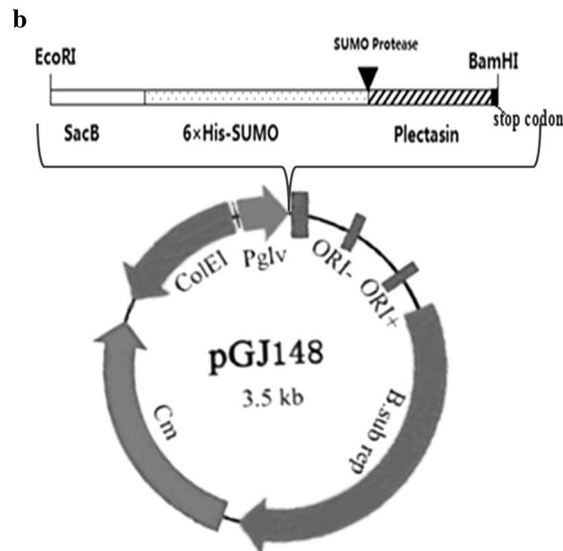


Table 1 Plasmid and strains used in this study

Plasmid and strains	Source	Character
Plasmid		
PGJ148	Zhejiang University	Cm ^r
Strain		
<i>E. coli</i> DH5α	Laboratory stock	No antibiotic resistance
<i>B. subtilis</i> WB800 N	Zhejiang University	Ne ^r , protease-deficient strain
<i>E. coli</i> UB 1005	Laboratory stock	–
<i>E. coli</i> ATCC 25922	Laboratory stock	–
<i>S. epidermidis</i> ATCC 12228	Laboratory stock	–
<i>S. aureus</i> ATCC 29213	Laboratory stock	–
<i>S. pneumoniae</i> CVCC 2350	Chinese Academy of Agricultural Science	–

Cm^r Chloramphenicol resistant Ne^r Neomycin resistance

Immunoblotting

The samples were separated by 15 % SDS-PAGE and then transferred to a nitrocellulose membrane (Millipore, Billerica, MA, USA). Anti-SUMO tag rabbit polyclonal antibody (Sagon, Shanghai, China) and HRP-conjugated affinipure goat anti-rabbit IgG (H + L; Sagon, Shanghai, China) were used to detect the recombinant fusion protein. The immunoreactive protein on the membrane was visualized using enhanced chemiluminescence and was exposed to an X-ray film (Bio-Rad, Hercules, CA).

Cleavage of SUMO-plectasin and purification of plectasin

The fraction containing the SUMO-plectasin was dialyzed overnight at 4 °C against SUMO protease buffer (50 mM Tris-HCl, 0.2 % NP-40, 150 mM NaCl, and 10 mM DTT; pH 8.0). The SUMO-plectasin and His-tagged SUMO protease (GeneCopoeia, Rockville, MD, USA) were mixed at a ratio of 1.0 unit per 5 µg of fusion protein. After incubation at 4 °C for 16 h, the mixture was dialyzed overnight against binding buffer at 4 °C and loaded onto an equilibrated Ni-NTA column. The flow-through fraction was collected and analyzed with by 16.5 % Tricine-SDS-PAGE. The purified peptide was dialyzed against deionized water by 1000 Da MWCO dialysis tubing, lyophilized and stored at -20 °C until needed. The peptide yield was determined as described by Bradford, and the purity of recombinant plectasin was evaluated with Quantity One Software (Bio-Rad, USA). The molecular weight was determined by MALDI-TOF.

Antimicrobial and hemolytic assays

The antimicrobial activity of recombinant proteins was tested against several bacteria. Minimal inhibitory concentrations (MICs) were measured by a modified version of Clinical Laboratory Standards Institute (CLSI) manual broth microdilution method as described previously [5]. In brief, bacteria were grown overnight at 37 °C to mid-log phase and then diluted to a final concentration ranging from 2×10^5 to 7×10^5 CFU/ml with Mueller-Hinton broth (MHB). The MHB was supplemented with 5 % defibrinated sheep blood for *S. pneumoniae*. The purified plectasin was dissolved and diluted in 0.01 % acetic acid and 0.2 % bovine serum albumin (BSA). Bacterial aliquots of 100 µl were incubated for 18–24 h at 37 °C with 100 µl of plectasin in MHB. Cultures containing no plectasin were positive controls. Uninoculated MHB was used as the negative control. The lowest concentration of peptide at which no visible turbidity occurred was designated as the MIC. Each experiment was repeated three times.

The hemolytic activities of plectasin were determined using fresh and healthy human red blood cells (hRBCs) by a previously described method [5]. HRBCs were collected and then centrifuged at 1000g for 5 min. The collected erythrocytes were washed three times and resuspended in phosphate-buffered saline (PBS) buffer (pH 7.2). Plectasin was added and the suspension was incubated for 1 h at 37 °C. After centrifugation at 1000g for 5 min at 4 °C, the supernatant was transferred to a 96-well microtitre plate. The absorbance of the supernatant was measured at 570 nm. HRBCs in PBS and 0.1 % Triton X-100, respectively, are served as the negative and positive controls, respectively.

Results

Construction of recombinant plasmid pGJ148-SUMO-plectasin

The recombinant plasmid pGJ148-SUMO-plectasin was constructed by inserting a DNA fragment of the sacB signal peptide, 6 × His-tag and SUMO-plectasin into pGJ148 downstream of the maltose-inducible promoter Pglv. The recombinant plasmid pGJ148-SUMO-plectasin was transformed into *B. subtilis* WB800 N and confirmed by sequencing. A transformation of pGJ148 was employed as the negative control.

Expression and purification of SUMO-plectasin

To examine the appropriate supplement of maltose on the expression system, we added maltose at 1, 3, 5, 7 and 9 % concentrations into LB medium. Supernatants were collected 24 h post-induction and subjected to Western blotting. A protein band was detected when the supplementation of maltose was at 1, 3, 5, 7, 9 %, and the highest expression level appeared at 7 % (Fig. 2a). As shown in Fig. 3, total protein level increased over the course of the induction. To determine the optimal induction time for expression, the supernatants were collected 0, 6, 12, 24, 36, 48, 60 and 72 h post-induction and analyzed by Western blotting. As shown in Fig. 2b, a band of approximately 25 kDa was observed after 12 h of induction, and the yield of the fusion protein increased as the induction time increased. The amount of SUMO-plectasin in the supernatant and in whole cells at 36 h was examined by Western blotting, and the majority of the produced SUMO-plectasin was secreted into the medium (Fig. 2c).

The supernatants were collected 48 h post-induction, and then, SUMO-plectasin was purified using Ni affinity

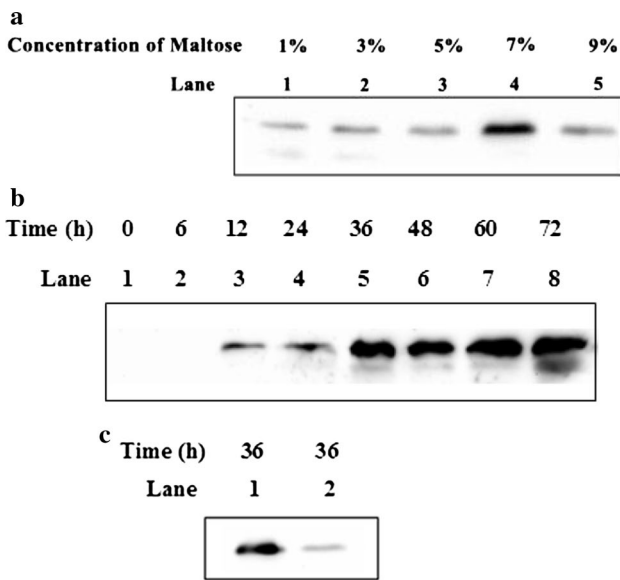


Fig. 2 Expression of fusion protein SUMO-Plectasin from *B. subtilis* WB800 N/pGJ148/His-SUMO-plectasin. **a** Western blot analysis of the supernatant sample at 24 h post-induction by different maltose induction concentrations. Lane 1–5 fermentation supernatants taken under 1, 3, 5, 7, 9 % maltose concentrations. **b** Western blot analysis of the supernatant sample by 7 % maltose induction. Lane 1–9 fermentation supernatants taken at 0, 6, 12, 24, 36, 48, 60, 72 h after induction, respectively. **c** Western blot analysis of intra- and extracellular expression of SUMO-Plectasin in *B. subtilis* WB800 N

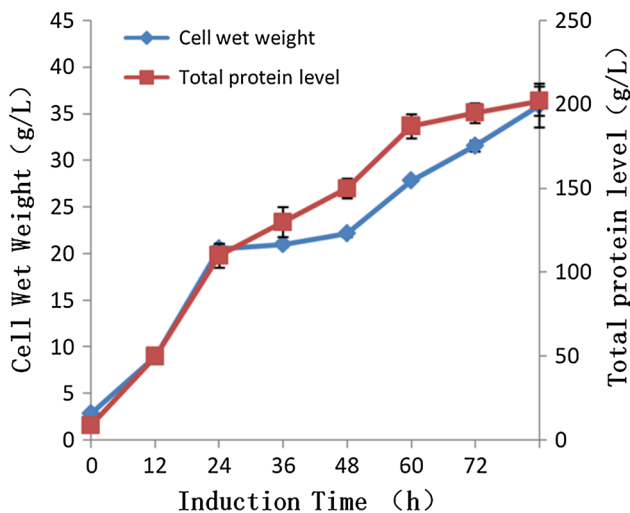


Fig. 3 A time curve of the total protein levels and cell wet weight during induction

chromatography. After analyzed by SDS-PAGE, the results showed that SUMO-plectasin could be effectively eluted by elution buffer containing 300 mM imidazole with more than 95 % purity, yielding 41 mg of fusion protein per liter of fermentation culture.

Purification of recombinant plectasin

After SUMO protease cleavage, plectasin was released from SUMO-plectasin. The cleavage reaction mixture was loaded on the Ni-NTA resin column to remove the 6 × His-SUMO tag, His-tagged SUMO protease and undigested SUMO-plectasin. The purity of the recombinant plectasin was >94 %. Finally, a yield of 5.5 mg recombinant plectasin was obtained from 1 L culture medium (Fig. 4a). MALDI-TOF MS analysis showed that the main peak of recombinant plectasin had a molecular mass of 4405.39 Da, which is consistent with the calculated value of 4404.82 Da (http://web.expasy.org/cgi-bin/compute_pi/pi_tool) (Fig. 4b).

Antimicrobial and hemolytic activities of recombinant plectasin

An antibacterial activity assay was performed to determine the function of the recombinant plectasin. As shown in Table 2, the peptide exhibited obvious activity against Gram-positive bacteria (*S. aureus* ATCC 29213, *S. epidermidis* ATCC 12228, and *S. pneumoniae* CVCC 2350), with an MIC of 1 ug/mL for *S. aureus* ATCC 29213, 8 ug/mL for *S. epidermidis* ATCC 12228, and 2 ug/mL for *S. pneumoniae* CVCC 2350. However, the peptide appeared to have no activity against Gram-negative bacteria, such as *E. coli* ATCC 25922 and *E. coli* UB 1005. These results indicated that recombinant plectasin had similar antimicrobial properties as observed previously.

In the hemolysis assays, plectasin did not cause hemolysis against human red blood cells for the concentrations of the peptide up to 256 ug/mL, indicating that plectasin displays no hemolytic activity to hRBC. These results showed that plectasin has potential for development as a therapeutic agent.

Discussion

In recent years, the continuous use and misuse of antibiotics have brought about some problems, such as, the appearance of drug-resistant and multidrug-resistant bacteria and the risk of antibiotic residues in the environment, which cause an increasingly serious worldwide public health problem [20]. Plectasin, the first fungus defensin, exhibits strong antibacterial property against Gram-positive bacteria and prevalent resistant bacterium, and has potential to be a novel antimicrobial agent against *Streptococcus* and *Staphylococcus* [26]. Recombinant DNA technology has provided an opportunity for large-scale plectasin production. Thus, an effective, safe and economical expression system needs to be established.

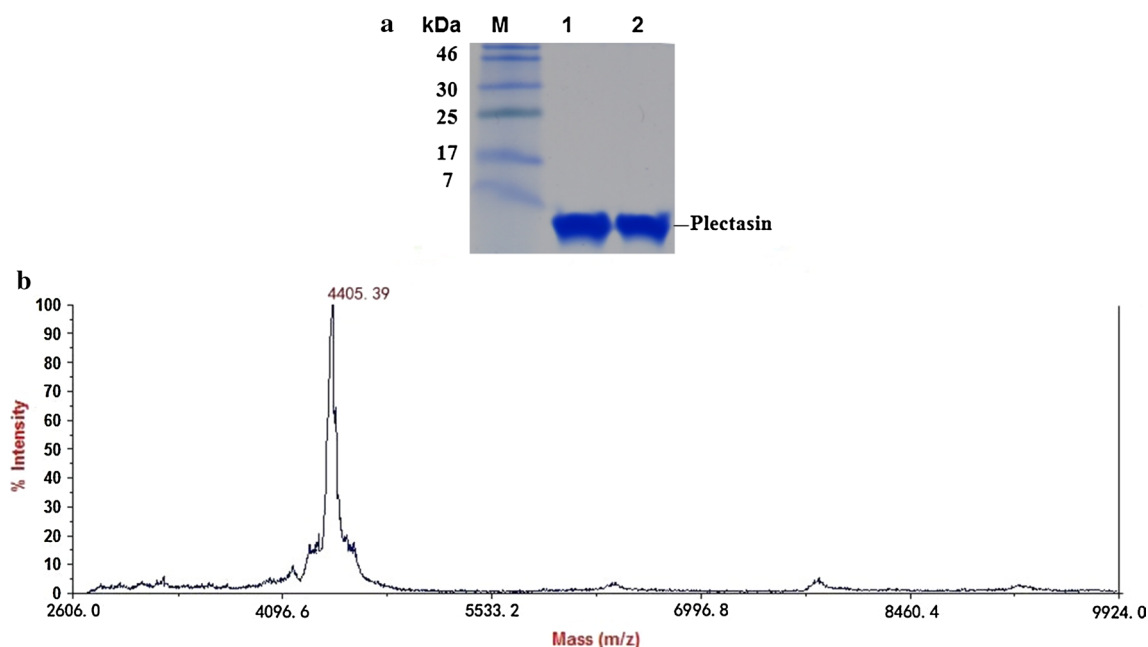


Fig. 4 **a** 16 % Tricine-SDS-PAGE analysis of recombinant of plectasin. **b** MALDI-TOF mass spectrum of purified recombinant plectasin

Table 2 Antimicrobial activity of recombinant Plectasin

Microorganism	MIC ($\mu\text{g/ml}$)
Gram-positive bacteria	
<i>S. aureus</i> ATCC 29213	1
<i>S. pneumoniae</i> CVCC 2350	2
<i>S. epidermidis</i> ATCC 12228	8
Gram-negative bacteria	
<i>E. coli</i> ATCC 25922	>128
<i>E. coli</i> UB 1005	>128

Bacillus subtilis is one of the most widely used hosts to produce heterologous proteins. Compared to *E. coli*, *B. subtilis* is generally recognized as safe and non-pathogenic. Another advantage is that the protein is secreted extracellularly with bioactivity, which simplifies protein purification. There are several additional reasons for *B. subtilis* being an attractive expression host: desirable secretion capacity, no significant bias in codon usage, well-described mechanisms for gene transcription and translation, and capability of large-scale fermentation [19, 22]. In this study, we chose *B. subtilis* strain WB800 N, which is a strain deficient in eight extracellular proteases to avoid protein degradation by extracellular *B. subtilis* proteases.

Promoter is a significant regulatory element in a genome, and regulates genes expression and expression strength; therefore, strong promoter is essential for an

effective *B. subtilis* expression system [22]. The *E. coli*-*B. subtilis* shuttle vector pGJ148 was used in the current study to secrete the fusion protein SUMO-plectasin, which was directed by the *B. subtilis* maltose utilization operon promoter *Pglv*. *Pglv* is much stronger than a constitutive promoter, and maltose is a cheaper and safer inducer than IPTG or xylose, commonly used inducers of *Pspac* and *PxyIA* [23]. In addition, *Pglv* has been used successfully for the expression of the xylanase gene. Thus, the promoter *Pglv* is a potential promoter in industrial application. This study is the first to use the promoter *Pglv* in the expression of AMPs.

We used the SUMO protein as a fusion partner, which not only overcomes the toxicity of plectasin to the host strain but also increases the expression yield. In addition, SUMO has been shown to improve folding, promote solubility and simplify protein detection [3]. Usually, an N-terminal $6 \times$ His-tag is fused to SUMO for Ni-NTA affinity purification to obtain suitable purity of the target fusion protein. Now, the SUMO fusion technology has been proved to be an effective expression tool of AMPs [3, 11, 13]. In our work, a $6 \times$ His-tag and yeast SUMO (*Saccharomyces cerevisiae* Smt3) were fused to the N-terminal of plectasin. Fusion protein SUMO-plectasin was highly expressed following induction by 7 % maltose at 37 °C. Under the control of the *Pglv* promoter, SUMO-plectasin began to accumulate 12 h post-induction. The SUMO-plectasin fusion protein increased up to 41 mg/L after 60 h

of induction following Ni-NTA affinity purification. The results indicate that the shuttle vector pGJ148 works well for the secretion of the fusion protein SUMO-plectasin, and the promoter Pglv plays a key role in high expression level of SUMO-plectasin.

The SUMO protease effectively recognizes tertiary structure of SUMO. Therefore, the SUMO protease never cleaves within the protein of interest and generates a target protein with a native N-terminus [12]. Furthermore, the SUMO protease is cheaper and more stable than enterokinase, factor Xa and thrombin. The SUMO protease 1 has been successfully produced in *E. coli* and *B. subtilis*, and demonstrated a high level of activity, which promotes the application of the SUMO fusion technology in the industrial production of AMPs [17]. After SUMO protease cleavage, we chose Ni-NTA affinity chromatography to purify recombinant plectasin. Because the SUMO protease, cleaved SUMO tag and fusion SUMO-plectasin all have His tags, after loading the cleavage mixtures onto the Ni-NTA column, the recombinant plectasin appeared in the flow through. Recombinant plectasin released from SUMO-plectasin only accounted for 13.4 % of the fusion protein. We supposed that a portion of recombinant plectasin was lost during column chromatography, as is often the case.

Plectasin has been successfully expressed in *E. coli* and *Pichia pastoris* [10, 24, 26]. In previous study, Chen et al. expressed plectasin in *E. coli* using SUMO technology and obtained 35.8 mg/L fusion protein, which is lower than the fusion protein we obtained [2]. Zhang et al. and Yang et al. have obtained 3.5 mg and 1.75 mg of recombinant plectasin polypeptide, respectively, from 1 L of *E. coli* fermentation culture medium, which is lower than the 5.5 mg/L of recombinant plectasin polypeptide expressed by *B. subtilis* in this study [10, 24]. The molecular weight of recombinant plectasin is 57 Da more than that of native plectasin, probably because the three disulfide bonds were not oxidized completely. Recombinant plectasin showed similar bioactivity as previously observed and had no hemolytic activity against human red blood cells.

In conclusion, the secretory expression of plectasin fused with SUMO under a maltose utilization operon promoter Pglv has been established. To our knowledge, this is the first report on the expression and purification of recombinant plectasin in the *B. subtilis*. The expression system not only may promote plectasin application in clinical use but also could be applied to produce other antimicrobial peptides in the future.

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