J. Microbiol. Biotechnol. (2015), 25(7), 1007–1014
http://dx.doi.org/10.4014/jmb.1501.01056

jmb

The Stability, and Efficacy Against Penicillin-Resistant *Enterococcus faecium*, of the Plectasin Peptide Efficiently Produced by *Escherichia coli*

Xin Chen[1*†], Yaoan Wen[2†], Ling Li[3†], Jiawei Shi[2†], Zhe Zhu[1], Yuwen Luo[1], Yun Li[1], and Rui Chen[4*]

[1]*Department of Respiratory Medicine, Zhujiang Hospital, Southern Medical University, Guangzhou 510282, P.R. China*
[2]*Nanfang Hospital, First Clinical Medical College, Southern Medical University, Guangzhou 510515, P.R. China*
[3]*Biosafety Level-3 Laboratory, School of Public Health and Tropical Medicine, Southern Medical University, Guangzhou 510515, P.R. China*
[4]*Department of Respiratory Diseases, Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University, Guangzhou 510120, P.R. China*

Received: January 20, 2015
Revised: March 7, 2015
Accepted: March 7, 2015

First published online
March 20, 2015

*Corresponding authors
X.C.
Phone: +86-20-62782296;
Fax: +86-20-61643010;
E-mail: chen_xin1020@hotmail.com
R.C.
Phone: +86-20-81332441;
Fax: +86-20-81332960;
E-mail: gzchenrui@163.com

†These authors contributed equally to this work.

pISSN 1017-7825, eISSN 1738-8872

Plectasin, the first defensin extracted from a fungus (the saprophytic ascomycete *Pseudoplectania nigrella*), is attractive as a prospective antimicrobial agent. The purpose of this study was to establish a bacterium-based production system and evaluate the antimicrobial activity of the resulting plectasin. A gene encoding plectasin, with the codon preference of *Escherichia coli*, was optimized based on its amino acid sequence, synthesized using gene-splicing with overlap extension PCR, and inserted into the expression vector pGEX-4T-1. The fusion protein was expressed in the soluble fraction of *E. coli* and purified using glutathione *S*-transferase affinity chromatography. Plectasin was cleaved from the fusion protein with thrombin and purified by ultrafiltration. The purified plectasin showed strong, concentration-dependent antimicrobial activity against gram-positive bacteria, including antibiotic-resistant bacteria, especially penicillin-resistant *Enterococcus faecium*. This antimicrobial activity was equal to chemically synthesized plectasin and was maintained over a wide range of pH and temperatures. This soluble recombinant expression system in *E. coli* is effective for producing plectasin at a relatively lower cost, and higher purity and efficiency than prior systems, and might provide a foundation for developing a large-scale production system. Overall, plectasin shows potential as a novel, high-performance, and safe antibiotic for the treatment of refractory diseases caused by drug-resistant bacterial strains.

Keywords: Antimicrobial peptide, antimicrobial activity, fusion protein, plectasin, prokaryotic expression, penicillin-resistant bacteria

Introduction

With the long-term overuse of antimicrobials in both clinical treatment and agriculture, bacterial drug resistance is raising increasing concerns regarding the treatment of many infectious diseases and bringing a substantial economic burden for patients. Therefore, there is a need to develop novel, high-performance, low-risk antimicrobial agents.

Plectasin is the first defensin extracted from a fungus, the saprophytic ascomycete *Pseudoplectania nigrella* [10]. Unlike many animal defensins that require very low ionic strength to exert their antimicrobial activities, plectasin exhibits antimicrobial actions at physiological ionic levels [14]. *In vitro*, plectasin is especially active against some species of gram-positive bacteria. Further research in murine models indicates that plectasin shows clinical effects against *Streptococcus pneumonia* comparable to vancomycin in peritonitis and penicillin in pneumonia [2, 10]. Other research results reveal that the effectiveness of plectasin is not reduced by anti-plectasin antibodies found in animal plasma [1]. Thus, plectasin may be an attractive candidate antimicrobial.

Current preparations of antimicrobial peptides are obtained by isolation from organisms, chemical synthesis, and genetic engineering. Because of the low levels of natural plectasin in organisms, and the presence of a

disulfide bond that can affect its antimicrobial activity, [11] isolation from organisms or chemical synthesis can be complex and costly with no guarantee of yield. Recently, several studies reported that plectasin could be produced using prokaryotic or eukaryotic host cells [3, 6, 9, 13, 17]. However, these methods usually required higher costs but yielded lower purity material. Moreover, the molecular chaperones used to avoid toxicity to the host cells were low molecular weight, which may affect the ultimate purity or antimicrobial activity of the product. Overall, highly purified plectasin has not yet been produced, and its antimicrobial activity against drug-resistant bacteria remains unclear.

In the current study, a bacterium-based plectasin-producing system was established. The plectasin-encoding gene was optimized according to its amino acid sequence with the synonymous codon bias of *Escherichia coli*, cloned into the pGEX-4T-1 vector, and subsequently expressed as a glutathione S-transferase (GST) fusion protein. The fusion protein was then induced by isopropyl β-D-1-thiogalactopyranoside (IPTG), purified by affinity chromatography, and excised by thrombin. The antimicrobial activity of plectasin was measured at different concentrations by the Kirby-Bauer assay. The stability of plectasin at different pH and temperatures was also assessed in terms of its antimicrobial activity.

Materials and Methods

Strains, Vectors, Reagents, and Enzymes

Mueller-Hinton broth was purchased from Hopebiol (Qingdao, China). Restriction enzymes, T4 DNA ligase, and DNA molecular weight markers were purchased from TaKaRa (Dalian, China). A pre-stained protein molecular weight marker was purchased from Dingguo Changsheng Biotech Co. (Beijing, China). GST Sepharose was purchased from Galaxy Bio (Jinan, China). Primers were synthesized by Genewindows (Guangzhou, China). *E. coli* DH5α, *E. coli* BL21 (DE3), and pGEX-4T-1 were preserved in a biosafety level-3 laboratory (Southern Medical University, Guangzhou, China). Penicillin-resistant *Enterococcus faecium* (PREF) and other bacteria were provided by Nanfang Hospital (Southern Medical University). Chemically synthesized plectasin was prepared by Sangon Biotech (Shanghai, China). Thrombin was purchased from Sigma-Aldrich (St. Louis, MO, USA). Amicon Ultra 10K centrifugal filter units were purchased from Millipore (Darmstadt, Germany).

Creation and Amplification of the Target Gene

The amino acid sequence of plectasin (RGFGCNGPWDEDDMQ CHNHCKSIKGYKGGYCAKGGFVCKCY) was retrieved from the NCBI GenBank. Gene splicing by overlap extension PCR (SOE-PCR) was utilized to obtain the target gene. Based on the amino acid sequence of plectasin, and the synonymous codon bias of *E. coli* [7], the overlap primers were designed as P1 (5′-CGCGGCTTTGGCTGCAACGGCCCGTGGGATGAAGATGATAT GCAGTGCCATAACCATTGCAAAAGCATTAAAGGC-3′) and P2 (5′-ATAGCATTTGC ACACAAAGCCGCCTTTCGCGCAAAGCCG CCTTTATAGCCTTTAATGCTTTTGCAAT-3′). A PCR technique in which the primers used one another as templates was adopted to obtain the target gene. Two additional primers were designed as P3 (5′-GGAATTC (*Eco*RI) CGCGGCTTTGGCT-3′) and P4: (5′-CCTCGAG (*Xho*I) TCA (termination codon) ATAGCATTTGCACACA-3′) to insert the target gene into the pGEX-4T-1 vector.

Construction, Transformation, and Identification of the Recombinant Plasmid

The target gene and pGEX-4T-1 were digested by the restriction enzymes *EcoR*I and *Xho*I at 37°C for 3 h, separated using 1% agarose gel electrophoresis, recovered with a DNA gel extraction kit, and reconnected by incubating with T4 DNA ligase at 16°C overnight. The recombinant plasmid was transformed into *E. coli* (DH5α) and amplified on Luria broth (LB) agar plates containing ampicillin. The positive recombinant plasmid was called pGEX-4T-1-PS. The conceptual diagram for the construction of pGEX-4T-1-PS is shown in Fig. 1.

Positive colonies were selected and inoculated into 5 ml of LB liquid culture medium by shaking (225 rpm) at 37°C overnight. The recombinant plasmid was identified by PCR using primers P3 and P4. Agarose gel electrophoresis was used to detect the product. The target gene and vector pGEX-4T-1 were used as the positive and negative control groups, respectively. The positive clone was sequenced by Genewindows (Guangzhou, China).

Expression of the Fusion Protein

The correct plasmid was transformed into competent BL21 cells to induce the target protein. The expression bacterium BL21 was

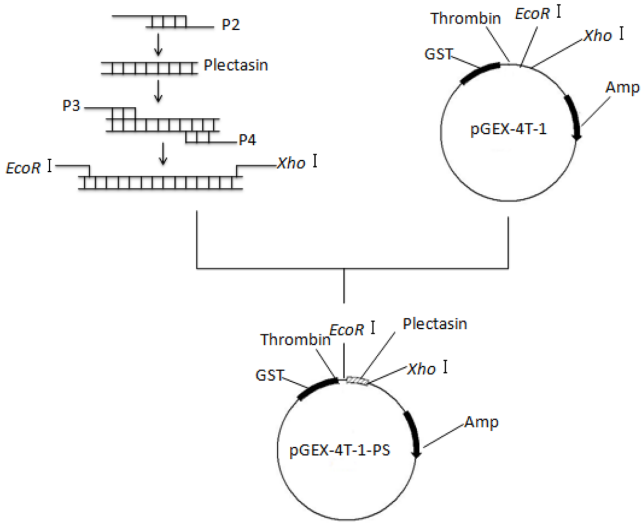

Fig. 1. Conceptual diagram of the construction of pGEX-4T-1-PS.

inoculated into 500 ml of LB culture medium. When the cultures reached an optical density of 0.6 at 600 nm, 1 mM IPTG was added at 37°C for 4 h to induce the recombinant protein. Bacterial cells were then harvested by centrifugation, resuspended in phosphate-buffered saline (PBS) containing 5% β-mercaptoethanol, and lysed by sonication (Ultrasonic Cell Crusher) at 300 W for 50 cycles (5 sec on, 5 sec off) in an ice-water bath. The whole-cell lysate was then centrifuged at 20,000 ×*g* for 30 min at 4°C to separate the soluble and insoluble fractions. Proteins in both the soluble and insoluble fractions were separately subjected to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Purification and Cleavage of the Fusion Protein

GST affinity chromatography was utilized to purify the fusion protein. The supernatant of the bacterial lysate was applied to an affinity column packed with beads. The column was washed with PBS (10 times of resin volume) and then with PBS containing 10 mM reduced glutathione (4 times of resin volume) to elute the fusion protein. The total binding capacity was ≥5 mg GST/ml chromatography medium. The eluent was assayed by SDS-PAGE. For cleavage of plectasin from the GST fusion protein, the purified fusion protein was incubated with thrombin for 16 h at 20°C. The mixture was placed into an Amicon Ultra10K centrifugal filter unit and centrifuged at 7,500 ×*g* for 30 min at 4°C. The concentrated solute was recovered with a pipettor and the mature plectasin was subjected to 18% SDS-PAGE. The purified target peptide was confirmed by ESI mass spectrometry at Sangon Biotech (Shanghai, China). Determination of the target peptide concentration was done using the BCA method.

Antimicrobial Activity Assay of Plectasin

The antimicrobial activity of purified plectasin was determined using the Kirby-Bauer assay. Pure cultures of PREF and other bacteria were prepared separately on nonselective medium until the culture reached an optical density of 0.6 at 600 nm (18–24 h). A lawn of bacteria was streaked onto Mueller-Hinton agar using a sterile cotton swab. The lid was left off the agar for 3–5 min to allow the plate to dry. Plectasin was applied drop-wise on the bacterial lawn. Ampicillin and sterile deionized water were used as positive and negative controls, respectively. The plates were incubated for 16–18 h at 37°C and the zones of inhibition were measured as an index of antibacterial activity. The minimal inhibitory concentration (MIC) of purified plectasin was determined by the microtiter broth dilution method [16]. PREF were grown to 0.5 OD_{600} at 37°C in LB medium. The target bacterial culture was diluted to 1×10^5 CFU/ml with the same medium. A total of 90 µl of cell suspenstion and 10 µl of peptide were added into each well. The activity of plectasin was tested over a concentration range of 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, and 0.125 µM. Plates were incubated for 16–18 h at 37°C, and the absorption of the cell culture was recorded at 590 nm. Ampicillin and sterile deionized water were used as positive and negative controls, respectively. In this study, the MIC was defined as the lowest concentration of peptide that reduced growth by 50% compared with the positive control.

Influence of Peptide Concentration,pH, and Temperature on the Antibacterial Activity of Plectasin

The concentration dependence of the antibacterial activity of plectasin was determined using 1, 2, and 3 mg/ml in PBS. The influence of pH on the antibacterial activity of purified plectasin was determined after pre-incubation of plectasin at 37°C for 16 h in buffer at pH 2.0, 4.0, 6.0, 8.0, or 10.0. The thermostability of purified plectasin in PBS was determined by pre-incubating at 35°C, 55°C, 85°C, or 100°C for 1 h followed by rapid cooling to room temperature. Sterile deionized water was used as a negative control. The influences of peptide concentration, pH, and temperature on the activity of plectasin against PREF were measured by the Kirby-Bauer assay as described above.

Results

Construction of the Recombinant Plasmid

After the first step of SOE-PCR, the target DNA fragments of plectasin (126 bp) were successfully amplified by PCR (Fig. 2A). After the second step of SOE-PCR, the target DNA fragments of plectasin with restriction enzyme cutting sites (140 bp) were successfully amplified (Fig. 2B). The target DNA fragments of plectasin were then inserted into the vector pGEX-4T-1. The recombinant vectors extracted from positive colonies were identified by PCR using primers P3 and P4. Agarose gel analysis showed that the target fragments were successfully inserted into the vector (Fig. 2C, lane 2). Subsequent DNA sequencing validated that the recombinant plasmid pGEX-4T-1-PS (plectasin) was constructed successfully (data not shown).

Expression of the Fusion Protein

The recombinant plasmid pGEX-4T-1-PS was transformed into *E. coli*. Compared with the sample without IPTG induction, a protein band with an expected molecular mass (~30 kDa) was visible in the total cell lysate of induced *E. coli* BL21 bearing the recombinant plasmid pGEX-4T-1-PS (Fig. 3, lane 2). The target protein was visible in the supernatant fraction (Fig. 3, lane 3) but not in the precipitant (Fig. 3, lane 4). This suggested that the GST-PS fusion protein was expressed in the cytosol in *E. coli*.

Purification and Cleavage of the Fusion Protein

Because the recombinant fusion protein contained GST, affinity chromatography was used for purification. The

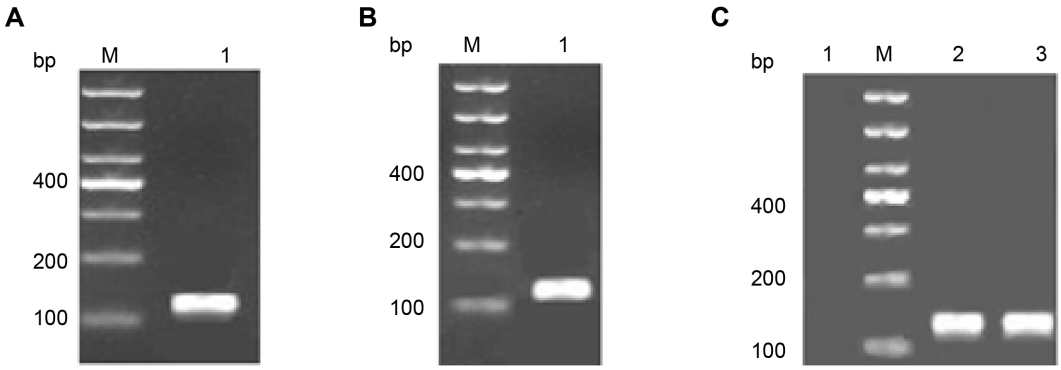

Fig. 2. Construction of the recombinant expression plasmid pGEX-4T-1-PS.
(**A**) Agarose gel analysis of the first-step product of SOE-PCR. (**B**) Agarose gel analysis of the second-step product of SOE-PCR. (**C**) Agarose gel analysis of PCR products of the plasmid pGEX-4T-1 (lane 1) as a negative control, recombinant plasmid pGEX-4T-1-PS (lane 2), and second-step product of SOE-PCR (lane 3) as a positive control.

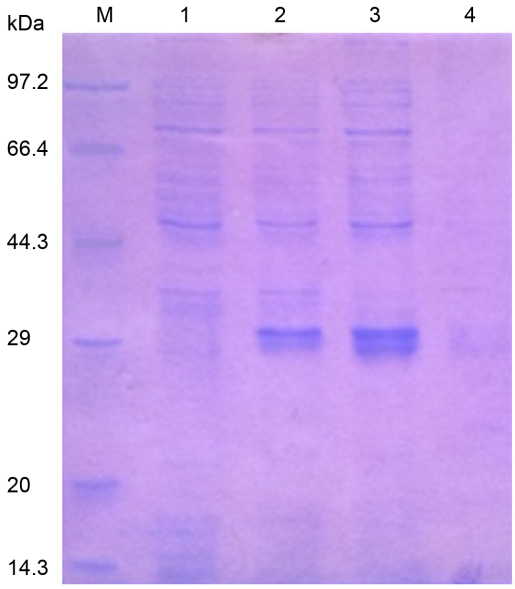

Fig. 3. SDS-PAGE analysis of the fusion protein expressed in *E. coli* BL21 (DE3).
Lane 1: Expression products without IPTG induction were used as the negative control. Lane 2: Expression products with IPTG induction in the whole-cell lysate. Lane 3: Expression products with IPTG induction in the soluble fraction. Lane 4: Expression products with IPTG induction in the insoluble fraction.

total supernatant of the bacterial lysate was applied to the affinity column. After elution with buffer containing reduced glutathione, the GST-PS fusion proteins could be eluted from the column (Fig. 4A). From one-step affinity chromatography, about 35 mg of fusion protein was obtained from 1 liter of culture medium. The fusion protein was stored at −20°C.

Table 1. Overall yield and purity at each main step in the purification procedure.

Purification step	Yield in LB (mg)	Purity
Captured fusion protein	35	96%
Cleavage removal of GST	0.6	98%

The fusion protein contained a specific thrombin recognition site and was digested with thrombin to release plectasin. Complete cleavage of the fusion protein was achieved (Fig. 4B). After filtration, the residue was dissolved and analyzed. GST (~26 kDa) and thrombin mainly existed in the liquid fraction (Fig. 4B, lane 1). Compared with chemically synthesized plectasin (4,408 Da, Fig. 4B, lane 3), the target polypeptide band (Fig. 4B, lane 2) could be observed. About 1.6 mg of plectasin was purified from 1 liter of culture medium (Table 1) and was stored at −20°C. The results of ESI mass spectrometry analysis showed one prominent peak (calculated molecular mass: 4,406.37 Da) from the purified plectasin. This was consistent with its theoretical value of 4,408 Da.

Antimicrobial Activity of Plectasin

Purified plectasin induced a large zone of clearance, whereas sterile deionized water had no inhibitory effect on the growth of PREF (Fig. 5A, spot 1 *vs*. 3). Purified plectasin (spot 3) showed antibacterial activity against PREF, similar to that of ampicillin (spot 2) and chemically synthesized plectasin (spot 4) (Fig. 5A). Except for ampicillin (spot 2), none of the tested products generated an inhibition zone with *E coli*, suggesting that this strain was resistant to plectasin (Fig. 5B). Using the same method, *S. aureus*, *S. suis*, and *S. pneumonia* appeared to be sensitive, whereas

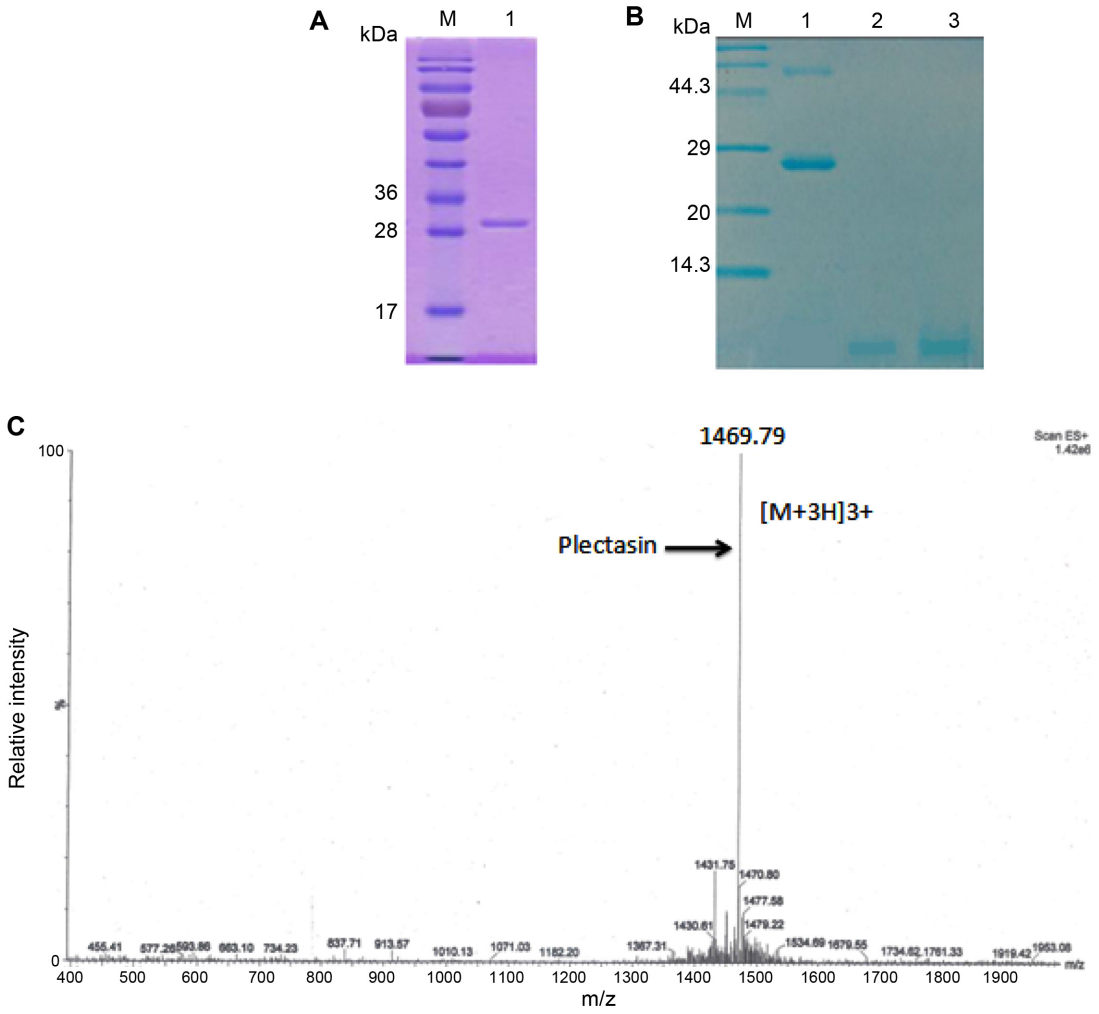

Fig. 4. SDS-PAGE analysis of the purified and cleaved GST-PS fusion proteins.
(**A**) Purified GST-PS fusion protein. After elution with buffer, the eluate (lane 1) was subjected to SDS-PAGE. (**B**) Cleaved GST-PS fusion protein. After cleavage, the residue in the redissolved liquid (lane 1) and filtrate (lane 2) were subjected to SDS-PAGE. Chemically synthesized plectasin (lane 3) was used as a positive control. (**C**) ESI mass spectrometry analysis of purified plectasin.

P. aeruginosa and *P. nigrella* appeared to be resistant, to plectasin (Table 2). The concentration-response curves showed that purified plectasin had equal antibacterial properties to chemically synthesized plectasin (Fig. 5C). The MIC of plectasin against PREF was 16 μM.

Table 2. Antimicrobial activity of plectasin.

Microorganism	Inhibition zone (cm)[a]
S. aureus	2.3
S. suis	2.2
S. pneumonia	2.0
P. aeruginosa	0
P. nigrella	0

[a]The amount of plectasin was 15 μg.

Influence of Peptide Concentration, pH, and Temperature on Plectasin Antibacterial Activity

The diameter of the inhibition zone produced by plectasin increased along with the peptide concentration (Fig. 6A). This indicated that the antimicrobial activity of plectasin was concentration-dependent. The pH from 2.0 to 10.0 had little effect on the antibacterial activity of plectasin (Fig. 6B). Furthermore, the influence of temperature on plectasin was very slight, especially from 35°C to 85°C. However, plectasin lost nearly half its antimicrobial activity at 100°C (Fig. 6C).

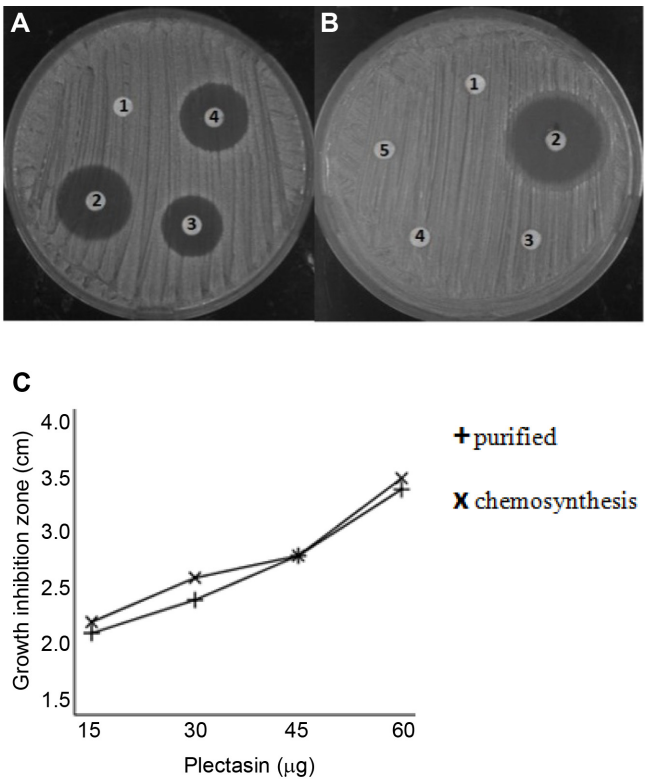

Fig. 5. Antimicrobial activity assay using gram-positive PREF and gram-negative *E. coli*.
(**A**) PREF treated with (1) 10 μl of sterile deionized water as a negative control, (2) 5 μg ampicillin as a positive control, (3) 15 μg purified plectasin, and (4) 15 μg chemically synthesized plectasin. (**B**) *E. coli* treated with (1) 10 μl of sterile deionized water as a negative control, (2) 5 μg ampicillin as a positive control, (3) pGEX-PS fusion protein as a negative control, (4) 15 μg purified plectasin, and (5) 15 μg chemically synthesized plectasin. (**C**) Concentration-response curves comparing purified plectasin to chemically synthesized plectasin. The horizontal axis represents the amount of plectasin. The vertical axis represents the size of the inhibition zone.

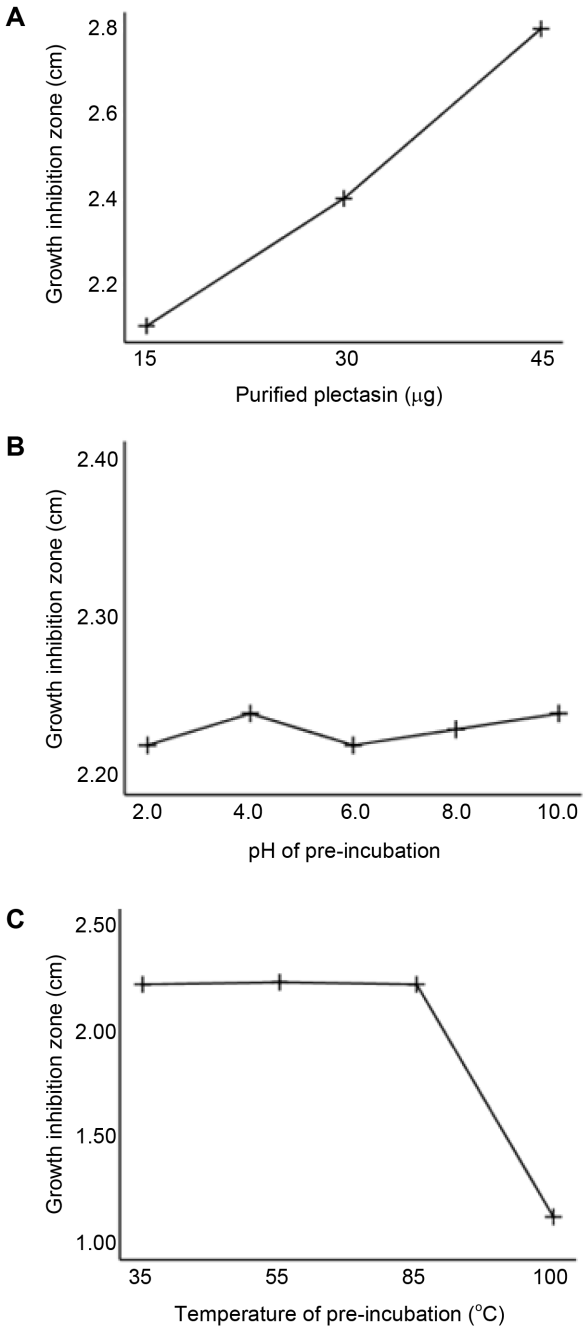

Fig. 6. Influence of concentration, pH, and temperature on the activity of plectasin against PREF.
(**A**) PREF were treated with 15, 30, or 45 μg purified plectasin. (**B**) The 15 μg purified plectasin was pre-incubated at 37°C for 10 h in buffers at pH 2.0, 4.0, 6.0, 8.0, or 10.0. (**C**) The 15 μg purified plectasin was treated at 35°C, 55°C, 85°C, or 100°C before assessing its antibacterial activity.

Discussion

Using microorganisms to express antimicrobial peptides can be advantageous because of their strong expressive orientation, biosafety, unlimited sources of raw materials, and low cost. We chose *E. coli* as our expression system because of the ease of culturing, fast growth, and availability of effective prevention against bacterial contamination [4].

Because plectasin will inhibit the growth of *E. coli* and hinder prokaryotic expression of this peptide [8], a fusion partner has proven to be a successful strategy for microbial synthesis of recombinant antimicrobial peptides [5, 18]. pGEX-4T-1 is an efficient prokaryotic expression vector with an IPTG-inducible promoter. The N-terminal of the expression protein is connected with a GST tag containing

Table 3. Differences in our experiment strategy compared with previous reports.

	Our study	Previous reports
Creation of target gene	SOE-PCR	PCR, SOE-PCR
Expression system	*E. coli*	*E. coli* and *Pichia pastoris*
Molecular chaperone	GST	TrxA, SUMO, Intein, and GST
Purified with plectasin	GST affinity chromatography	Affinity chromatography
Identified with plectasin	ESI mass spectrometry	MALDI-TOF mass spectrometry
Purity	⩾ 96%	----
Strain sensitive to plectasin	PREF, *S. aureus*, *S. suis*, and *S. pneumonia*	MRSR, gram-positive bacteria
Influence of concentration on antibacterial activity	Concentration-dependent	----
Influence of pH on antibacterial activity	Little effect	Little effect
Influence of temperature on antibacterial activity	Slight from 35°C to 85°C, lost half at 100°C	Slight from 35°C to 85°C, lost half at 100°C

thrombin cleavage sites. Thus, the fusion protein can be cleaved by thrombin and then rapidly purified by GST affinity chromatography [15].

The results of our study using a GST fusion protein expression system showed that the fusion protein had no antimicrobial activity against *E. coli*. As the fusion protein was present in the soluble portion of *E. coli* BL21 (DE3) lysates, there was no need to consider renaturation of the inclusion body. The results of ESI mass spectrometry analysis showed that the construction of the purified plectasin was correct. The yield of fusion protein reached 35 mg/l. The fusion protein could be specifically adsorbed on the chromatography column and, after digestion with thrombin, the released plectasin could be purified to over 98%. The amount of the purified target polypeptide (1.6 mg per liter of culture medium) was lower compared with published data. However, compared with previous reports [3, 6, 9, 17], this procedure provided a method of preparing the plectasin peptide at a relatively lower cost and higher purity. The most probable reason for the lower quantity was that some plectasin might adhere to the ultrafiltration tubes. In addition, several expression conditions could have affected the production of plectasin, such as the induction time, concentration of inducer, and inducing temperature.

Antibiotics tend to lose their efficacy over time owing to the emergence of strains with resistance to multiple antibiotic classes, such as *Enterococcus* spp. and *Staphylococcus aureus* [12]. To solve this problem, developing new antibiotics is an urgent need. Although Mao *et al*. [9] found that agplectasin showed remarkable antibacterial activity against methicillin-resistant *Staphylococcus*, many gram-positive bacteria appeared to be resistant. In the current study, we found that purified plectasin exhibited remarkable antibacterial activity against PREF and other gram-positive bacteria, which was equal to that of chemically synthesized plectasin. This suggests that plectasin may have universal applicability.

Concentration, pH, and temperature may influence the activity of antibacterial peptides. Plectasin appeared to be highly stable between 35°C and 85°C, although a reduction of up to 40% in its antimicrobial activity was observed at 100°C. Plectasin also retained its antimicrobial activity against PREF at pH between 2.0 and 10.0. Our results regarding the influence of pH and temperature on the antibacterial activity of plectasin were similar to a previous report [17]. In addition, our results showed that plectasin inhibited the growth of PREF in a concentration-dependent manner. This may be related to its unique mechanism of antimicrobial activity as well as its low molecular weight, simple structure, and stable conformation because of a high content of cysteine.

In conclusion, we propose an improved approach that expresses plectasin in *E. coli*. By fusing plectasin with GST, it can be efficiently produced in the cytoplasm of *E. coli* and purified to 98%. Additionally, plectasin showed significant concentration-dependent antimicrobial activity against gram-positive and antibiotic-resistant bacteria, especially PREF. The stability of bacterially produced plectasin was similar to chemically synthesized plectasin over a wide range of pH and temperatures. The differences in our experiment strategy compared with previous reports [3, 6, 9, 17] are shown in Table 3. Therefore, our purified plectasin shows potential as a high-performance and safe antibiotic for the treatment of refractory diseases caused by drug-resistant bacterial strains.

Acknowledgments

This work was supported by the Combinational Project of Guangdong Province and the Education Ministry for Manufacturing, Education & Research (2010B090400540), the National College Students' Innovation and Entrepreneurship Training Program (201212121080), the Guangdong Provincial Science and Technology Project (2011B080701062), and the Guangdong Province College Students' Innovative Experiment Project (1212111003). The authors confirm that there are no conflicts of interest regarding the content of this article. We are most grateful to Guangqiao Zeng MD, State Key Laboratory of Respiratory Disease, Guangzhou Medical University, Guangzhou, China, for his assistance in medical writing.

References

1. Brinch KS, Frimodt-Moller N, Hoiby N, Kristensen HH. 2009. Influence of antidrug antibodies on plectasin efficacy and pharmacokinetics. *Antimicrob. Agents Chemother.* **53:** 4794-4800.
2. Brinch KS, Sandberg A, Baudoux P, Van Bambeke F, Tulkens PM, Frimodt-Moller N, *et al.* 2009. Plectasin shows intracellular activity against *Staphylococcus aureus* in human THP-1 monocytes and in a mouse peritonitis model. *Antimicrob. Agents Chemother.* **53:** 4801-4808.
3. Chen X, Shi J, Chen R, Wen Y, Shi Y, Zhu Z, *et al.* 2014. Molecular chaperones (TrxA, SUMO, Intein, and GST) mediating expression, purification, and antimicrobial activity assays of plectasin in *Escherichia coli*. *Biotechnol. Appl. Biochem.* DOI: 10.1002/bab.1303.
4. Huang L, Leong SS, Jiang R. 2009. Soluble fusion expression and characterization of bioactive human beta-defensin 26 and 27. *Appl. Microbiol. Biotechnol.* **84:** 301-308.
5. Ingham AB, Moore RJ. 2007. Recombinant production of antimicrobial peptides in heterologous microbial systems. *Biotechnol. Appl. Biochem.* **47:** 1-9.
6. Jing XL, Luo XG, Tian WJ, Lv LH, Jiang Y, Wang N, Zhang TC. 2010. High-level expression of the antimicrobial peptide plectasin in *Escherichia coli*. *Curr. Microbiol.* **61:** 197-202.
7. Kane JF. 1995. Effects of rare codon clusters on high-level expression of heterologous proteins in *Escherichia coli*. *Curr. Opin. Biotechnol.* **6:** 494-500.
8. Kim JM, Jang SA, Yu BJ, Sung BH, Cho JH, Kim SC. 2008. High-level expression of an antimicrobial peptide histonin as a natural form by multimerization and furin-mediated cleavage. *Appl. Microbiol. Biotechnol.* **78:** 123-130.
9. Mao R, Teng D, Wang X, Xi D, Zhang Y, Hu X, *et al.* 2013. Design, expression, and characterization of a novel targeted plectasin against methicillin-resistant *Staphylococcus aureus*. *Appl. Microbiol. Biotechnol.* **97:** 3991-4002.
10. Mygind PH, Fischer RL, Schnorr KM, Hansen MT, Sonksen CP, Ludvigsen S, *et al.* 2005. Plectasin is a peptide antibiotic with therapeutic potential from a saprophytic fungus. *Nature* **437:** 975-980.
11. Padovan L, Crovella S, Tossi A, Segat L. 2010. Techniques for plant defensin production. *Curr. Protein Pept. Sci.* **11:** 231-235.
12. Rossolini GM, Arena F, Pecile P, Pollini S. 2014. Update on the antibiotic resistance crisis. *Curr. Opin. Pharmacol.* **18c:** 56-60.
13. Rothan HA, Mohamed Z, Suhaeb AM, Rahman NA, Yusof R. 2013. Antiviral cationic peptides as a strategy for innovation in global health therapeutics for dengue virus: high yield production of the biologically active recombinant plectasin peptide. *Omics* **17:** 560-567.
14. Schneider T, Kruse T, Wimmer R, Wiedemann I, Sass V, Pag U, *et al.* 2010. Plectasin, a fungal defensin, targets the bacterial cell wall precursor lipid II. *Science* **328:** 1168-1172.
15. Sharrocks AD. 1994. A T7 expression vector for producing N- and C-terminal fusion proteins with glutathione S-transferase. *Gene* **138:** 105-108.
16. Tian ZG, Dong TT, Yang YL, Teng D, Wang JH. 2009. Expression of antimicrobial peptide LH multimers in *Escherichia coli* C43(DE3). *Appl. Microbiol. Biotechnol.* **83:** 143-149.
17. Zhang J, Yang Y, Teng D, Tian Z, Wang S, Wang J. 2011. Expression of plectasin in *Pichia pastoris* and its characterization as a new antimicrobial peptide against *Staphyloccocus* and *Streptococcus*. *Protein Expr. Purif.* **78:** 189-196.
18. Zorko M, Jerala R. 2010. Production of recombinant antimicrobial peptides in bacteria. *Methods Mol. Biol.* **618:** 61-76.