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Expression of Recombinant Antibacterial Lactoferricin-Related Peptides from *Pichia pastoris* Expression System

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Four recombinant antimicrobial peptide (rAMP) cDNAs, constructed from two goat lactoferricin-related peptide cDNAs (GLFcin and GLFcin II) with/without $(His)_6$-Tag, were cloned into pPICZαC and transformed into *Pichia pastoris* SMD1168H. After methanol induction, these rAMPs were expressed and secreted into broth. They were purified after CM-Sepharose (without His-tg), HisTrap (with His-tg) and Sephadex G-25 chromatographies. The yield of purified rAMP was 0.15 mg/mL of broth. These 4 rAMPs were thermal-stable and with high antibacterial activity against *Escherichia coli* BCRC 11549, *Pseudomonas aeruginosa* BCRC 12450, *Bacillus cereus* BCRC 10603, *Staphylococcus aureus* BCRC 25923, *Propioni bacterium acnes* BCRC 10723, and *Listera monocytogenes* BCRC 14845. The minimum inhibitory concentration (MIC) of rAMPs against these indicators ranged from 4.07 to 16.00 mg/mL.

KEYWORDS: Goat lactoferricin; antimicrobial peptides; *Pichia pastoris*

INTRODUCTION

The menace of antibiotic-resistant bacteria to both animal and human health has long been a concern of many scientists and governments worldwide, which consequently led to development of effective as well as human and environmentally compatible antibiotic alternatives for feed and medical industries (*1*). Scientists have paid much attention to the development of antimicrobial peptides (AMPs) derived from animals and plants. This might be due to the advantages of easier possession, more effective activities and exhibition of a unique mechanism of killing bacteria compared with current antibiotics (*2*−*9*). During the past few years, natural cationic AMPs have been proven to possess broad-spectrum activities against many microbes, including bacteria, fungi and virus, and ability to kill cancer cells (*10*−*12*). In addition to natural peptides, AMPs could also be obtained by digestion of proteins such as bactericidal/permeability-increasing proteins (*13*), tenecin (*14*) or non-histone chromosomal protein H6 (*15*).

The mechanism of antimicrobial activity is still an unsolved puzzle. One of the most acceptable explanations is that cationic AMPs interact electrostatically with the negatively charged phospholipids in bilayer membranes, which consequently causes barrel-stave or toroidal pores (*2*) or destroys the membrane via a "carpet" mechanism (*16*, *17*). These phenomena usually result in membrane-bilayer permeability and eventually cause cells to die off. This special mode of action is considered not to form the resistance to AMPs, suggesting that AMP may be a safe and effective therapeutic alternative against pathogenic microbes that are resistant to conventional antibiotics.

Lactoferricin (LFcin) is a strong cationic AMP against a wide range of microorganisms including Gram-positive and Gram-negative bacteria as well as fungi (*18*−*24*). It is released from the N-terminal of lactoferrin by pepsin digestion (*25*, *26*) and found to be rich in arginine, lysine and tryptophan (*20*, *27*). Even though they are at the same molar concentration, the bactericidal activity of LFcin is much higher than that of lactoferrin (*25*, *26*). Furthermore, these LFcin peptides were also found to have antiviral (*28*), antitumor (*29*), anti-inflammatory (*22*), immune response stimulation (*22*) and angiotensin I-converting enzyme inhibition (ACEI) (*30*) activities.

This study aimed to overexpress the antimicrobial lactoferricin-related peptides, GLFcin, GLFcin-$(His)_6$-Tag, GLFcin II and GLFcin II-$(His)_6$-Tag, from *Pichia pastoris* expression system and further to determine the antibacterial properties of these 4 recombinant peptides. These data will shed light on evaluating the application potentials in food and cosmetic industries of these rAMPs, either as an additive or control of bacterial contamination during processing.

MATERIALS AND METHODS

The pGEM-T Easy (Promega, Madison, WI) was used for T/A cloning of PCR products. *Escherichia coli* Top 10 F′ (Invitrogen) was used for subcloning and expression vector propagation. The pPICZαC (Invitrogen, Carlsbad, CA) vector was used for extracellular expression of rAMPs. *Pichia pastoris* SMD1168H (Invitrogen) was the host for pPICZαC. All restriction enzymes, T4 DNA ligase, and *pfu* DNA polymerase were purchased from Invitrogen. Peptone and yeast extract were purchased from Difco Co. Ltd. (Detroit, MI). Isopropyl-β-D-thio-galactopyranoside

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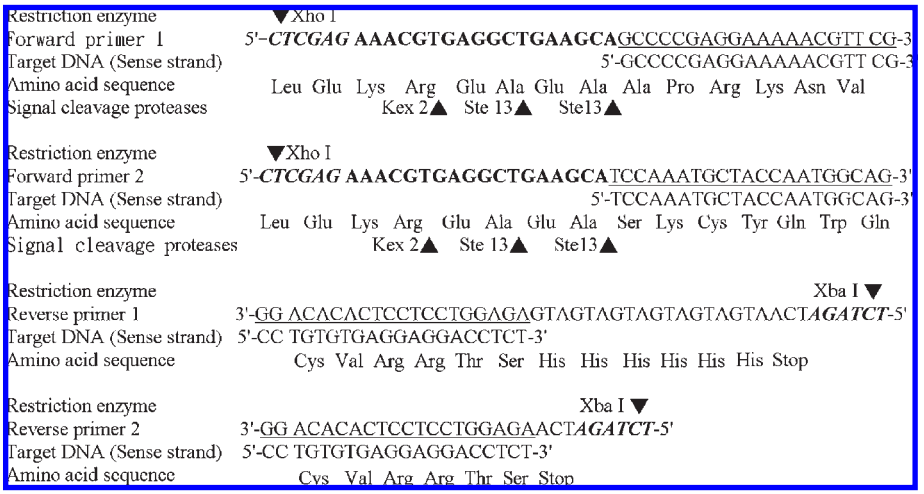

Figure 1. Primers design for amplification of rAMPs by PCR and for ligation the PCR products into pPICZαC expression vector through the 5′-extended restriction enzyme (*Xho*I and *Xba*I) cutting sites. Forward primer 1 and 2, used as forward primers for PCR reactions of GLFcin and GLFcin II, respectively, were collocated with reverse primer 1 and 2 for PCR products with [GLFcin $(His)_6$-Tag or GLFcin II $(His)_6$-Tag] and without [GLFcin or GLFcin II] $(His)_6$-Tag, respectively.

(IPTG), ampicillin, kanamycin, chloramphenicol, dNTPs and X-Gal were the products of MDBio (MDBio, Inc. (Taipei, Taiwan). All other chemical reagents were analytical grade and purchased from Sigma (St. Louis, MO). Goat milk gland cell was purchased from Kong-Shan Slaughterhouse (Kaohsiung, Taiwan). All primers used in PCR were synthesized by Misihin Biotech Co. Ltd. (Taipei, Taiwan). Indicator strains of *Escherichia coli* BCRC 11549, *Pseudomonas aeruginosa* BCRC 12450, *Bacillus cereus* BCRC 10603, *Staphylococcus aureus* BCRC 25923, *Propioni bacterium acnes* BCRC 10723 and *Listera monocytogenes* BCRC 14845 were obtained from the Culture Collection at Bioresource Collection and Research Center (Hsinchu, Taiwan) and maintained in nutrient agar, cystine trypticase agar, tryptic soy agar, nutrient agar, reinforced clostridial agar, and tryptic soy agar, respectively, at 4 °C. They were subcultured monthly and used for antibiotic assays.

Screening and Amplification of GLFcin cDNA from Goat Milk Gland mRNA. Total RNA was extracted from goat milk gland using Trizol RNA extraction kit (Gibco BRL products). The single strain cDNA, produced from RT-PCR, was used as a template. The oligonucleotides with sequences 5′-***CTCGAG*** **AAA CGT GAG GCT GAA GCA** GCCCCGAGGAAAAACGTTCG-3′ and 5′-***CTCGAG*** **AAA CGT GAG GCT GAA GCA** TCCAAATGCTACCAATGGCAG-3′, based on the nucleotide residues 58−77 and 106−126 of goat lactoferricin (underlined), were used as forward primers for PCR reactions of GLFcin and GLFcin II, respectively. The oligonucleotides with sequences of 5′-***TCTAGA*** TCA ATG ATG ATG ATG ATG ATG AGAGGTCCTCCTCACACAGG-3′ and 5′-***TCTAGA*** TCA AGA GGT CCT CCT CAC ACA GG-3′, based on the antisense nucleotide residues 161−180 of goat lactoferricin (underlined), were used as reverse primers for PCR reactions of rAMP fused with/without $(His)_6$-Tag, respectively. Restriction sites at the 5′ ends of the primers for *Xho*I and *Xba*I (boldfaced italic) were incorporated to facilitate subcloning of the PCR product. A boldfaced sequence (as shown in **Figure 1**) designed between the *Xho*I site and specific sequence of the forward primer was coded for amino acid sequence of Kex2 and Ste 13 cutting sites to facilitate cleavage of the signal sequence without any additional amino acid survived on the N-terminus of mature rAMPs. Amplification was performed using proofreading polymerase (Invitrogen Inc., Carlsbad, CA) by PCR reaction for 30 cycles: denaturation, 95 °C for 30 s; annealing, 56 °C for 30 s; and extension, 68 °C for 50 s, in a thermal cycler (Perkin-Elmer, GeneAmp PCR system 2400).

Construction of rAMP-pPICZαC Expression Vector. The standard techniques of molecular cloning were performed essentially according to Sambrook and others (*30*). The PCR product was cloned into pGEM-T Easy vector (Promega) and then transformed into *E. coli* Top 10. After blue/white selection and midi-preparation, the insert was released with *Xho*I/*Xba*I digestion and subcloned into *Xho*I/*Xba*I digested pPICZαC vector to generate the construct of rAMP-pPICZαC vector.

Transformation of rAMP-pPICZαC into *Pichia pastoris* SMD1168H by Electroporation. The rAMP-pPICZαC vector was digested with *Sac*I restriction enzyme in the *AOX1* promoter region to linearize the vector prior to *P. pastoris* transformation and generated a fragment capable of integrating into the chromosomal *AOX1* promoter locus by homologous recombination. For electroporation, 0.5 mL of the overnight culture *P. pastoris* SMD1168H was inoculated into 500 mL of YPD medium (1% yeast extract, 2% peptone, 2% dextrose) in a 2 L shake flask and grown at 30 °C to an OD_{600} of 1.3−1.5. The cells were then washed twice and resuspended in 1 mL of ice-cold 1.0 M sorbitol. An aliquot of competent *P. pastoris* SMD1168H (80 μL) was mixed with 10 μg of linearized transforming DNA (in 10 μL of sterilized water) and then transferred to an ice-cold 0.2 cm electroporation cuvette. The cells were pulsed using an electroporator (MicroPulser, Bio-Rad) according to the manufacturer's instructions. The cells were incubated at 30 °C for 1 h and then spread on YPDS (YPD medium plus 1.0 M sorbitol) plates using Zeocin (100 μg/mL) as selective marker. The resulted cells were cultured at 30 °C for 2 to 3 days.

Screening and Isolation of Multicopy Recombinant Colonies. After 3-day incubation, the colonies were confirmed by PCR according to Paramasivam and others (*32*). Each colony on the plate was inoculated individually into 5 mL of YPD medium in a 30 mL sterilized glass tube with gas permeable cap and incubated at 30 °C in a shaking incubator (300 rpm). After 4-day incubation, 1 mL of culture cells of each colony was harvested by 10 min centrifugation at 10000*g*, 4 °C. The cell pellets were resuspended in 400 μL of yeast lysis buffer (*33*) and vortexed vigorously with acid-washed glass beads for 4 min. The lysed cell suspensions were centrifuged at 12000*g*, 4 °C for 10 min. The clear supernatant was subjected to phenol−chloroform extraction and then by ethanol precipitation. The chromosome DNA pellet obtained was washed twice with 70% ethanol, dried and redissolved in 40 μL of distilled water. From each colony, 2 μL of chromosome DNA solution was used as template for the PCR with specific rAMP primers (**Figure 1**). Then, 5 μL of each PCR amplified product was analyzed by 1% agarose horizontal electrophoresis, separated in DC 100 V for 15 min. The positive reactions of the PCR indicated that the corresponding *Pichia* colonies had been integrated with rAMP-pPICZαC vector into their chromosome. These colonies were selected for further multicopy recombinant screening.

To screen multicopy recombinant colonies, an enhanced Zeocin selection was performed by spreading the PCR selected colonies over YPD plates with 5−20-fold concentrations (500 to 2000 μg/mL) of Zeocin. *Pichia* transformants with a high Zeocin resistance were selected for rAMPs expression.

Growth Curve and Methanol Induction for the Expression of rAMPs. The high Zeocin resistant transformant was cultivated in 5 mL of YPD + Zeocin broth (peptone, 20 g/L; yeast extract, 10 g/L; dextrose, 20 g/L; Zeocin, 100 mg/L, pH 7.5 adjusted by NaOH) in a 50 mL flask at 30 °C using a shaking incubator (200 rpm) overnight. Ten milliliters of the resulting culture was inoculated into 1.0 L of fresh YPD broth (peptone, 20 g/L; yeast extract, 10 g/L; dextrose, 20 g/L). The inoculated broth was further divided into 4 parts. They were cultivated in 1.0 L flasks in a 30 °C shaking incubator (200 rpm). Except for the control group (without methanol), methanol (2%) was added at the 24th, 48th and 72nd hours during incubation to induce the production of rAMPs. Microbial growth was monitored during cultivation by measuring the colony forming units (CFU)/mL. After a series of dilutions, they were incubated at 30 °C on YPD agar plates for 48 h and the formed colonies were counted.

Purification of rAMP-$(His)_6$-Tag. After 4 days cultivation, the cells, harvested by 10 min centrifugation at 10000*g*, 4 °C, were filtrated using 0.45 μm membranes to eliminate the microorganisms. The filtrated supernatants were collected, heated at 100 °C for 3 min and then centrifuged again at 10000g for 10 min to completely remove the heat-unstable proteins. The supernatant of rAMP-$(His)_6$-Tag was then filtered through cellulose acetate filtration membrane with 0.45 μm pores and applied onto a HisTrap affinity chromatography column (2.6 × 10 cm; GE Healthcare Co. Ltd., USA), which was previously equilibrated with 50 mM Tris-HCl buffer (pH 7.5). The column was washed with 6× bed volumes of the same buffer containing 5 mM imidazole, and then eluted with a linear gradient of 5−500 mM imidazole in the same buffer (200 mL) using the FPLC System (GE Healthcare) at room temperature. The flow rate was 1.0 mL/min, and the eluent was collected with a 1.0 mL/tube. All collected imidazole eluted fractions were concentrated by freeze-drying and subjected to Sephadex G-25 gel filtration chromatography, eluted by sterile H_2O with a flow rate of 0.3 mL/min. Finally, all collected fractions were subjected to the following purity and antimicrobial activity assays.

Purification of rAMPs. After 4-day cultivation, the cultural cells and heat-unstable proteins were removed as mentioned above. The collected supernatants were concentrated against 50 mM Tris-HCl buffer (pH 7.5) using an Amicon ultrafiltration (cutting size: 0.5 kDa). The concentrated proteins were loaded onto CM-Sepharose column (2.6 × 5 cm) (GE Healthcare, Uppsala, Sweden). After being washed with 100 mL of 50 mM Tris-HCl (pH 7.5), the bound proteins were eluted with a linear gradient of 0−0.6 M NaCl in the same buffer (200 mL) using the FPLC System at room temperature with a flow rate of 30 mL/h. The absorbance at 280 nm was measured. The proteins eluted between 0.4 and 0.5 M NaCl were collected. All collected NaCl eluted fractions were concentrated by freeze-drying and subjected to Sephadex G-25 gel filtration chromatography, eluted by sterile H_2O with a flow rate of 0.3 mL/min. Finally, all collected fractions were subjected to the following purity and antimicrobial activity assays.

Purity Determination. The purity of the 4 purified rAMPs (GLFcin, GLFcin-$(His)_6$, GLFcin II and GLFcin II-$(His)_6$) were analyzed by HPLC. Twenty microlliters of each purified rAMP was applied onto the Jupiter C18 (4.6 × 250 mm, 5 μm) column (Phenomenex Co. Ltd., USA) and eluted by 50% aqueous methanol containing 0.1% trifluoroacetic acid. The elution diagram was monitored by UV absorbance at 220 nm.

Protein Concentration Measurements. Protein concentrations of the purified rAMPs were determined according to the bicinchoninic acid assay method (*34*) using bovine serum albumin as the standard.

N-Terminal Amino Acid Sequencing. The purified rAMPs were separated on 10% SDS gel and blotted onto PVDF membrane filter using 100 mM CAPS buffer (pH 10.5). The membrane was stained with CBB. After being destained, the strip containing the protein was dried. The N-terminal sequence was determined by Misihin Biotech Co. Ltd. (Taipei, Taiwan) using the method of Edman degradation (*35*).

Antimicrobial Activity Assay and Minimum Inhibitory Concentrations (MIC) Determination. The agar-well diffusion method was employed to determine the antimicrobial ability and MIC of rAMP according to Piddock (*36*) with some modifications. *Escherichia coli* BCRC 11549, *Pseudomonas aeruginosa* BCRC 12450, *Bacillus cereus* BCRC 10603, *Staphylococcus aureus* BCRC 25923, *Propioni bacterium acnes* BCRC10723, and *Listera monocytogenes* BCRC 14845 were cultivated at 37 °C in 50 mL of nutrient broth, cystine trypticase broth, tryptic soy broth, nutrient broth, reinforced clostridial medium, and tryptic soy broth, respectively. After incubating for 12 h, 0.5 mL of each culture was inoculated into 50 mL of each fresh medium and incubated for another 12 h at 37 °C. After the level of cells in broth had been adjusted to about 1.0×10^8 CFU/mL, 1.0 mL of each broth was mixed uniformly with 15 mL of prewarmed agar (45 °C; *Escherichia coli* BCRC 11549, nutrient agar; *Staphylococcus aureus* BCRC 25923, nutrient agar; *Propioni bacterium acnes* BCRC10723, reinforced clostridial agar; *Pseudomonas aeruginosa* BCRC 12450, cystine trypticase agar; *Bacillus cereus* BCRC 10603, tryptic soy agar; and *Listera monocytogenes* BCRC 14845, tryptic soy agar). Each agar was poured into Petri dishes and allowed to stand at 4 °C for 1 h. After gelation, the agar was punched by using a stainless ring with a diameter of 5 mm. Fifty microliters (at different concentrations from 1 to 20 mg/mL) of rAMP was added to the hole and incubated at 4 °C for 12 h to allow rAMP diffusion. The cultural broth concentrated from non rAMP gene transformed *Pichia pastori* SMD1168H was used as control. The assays were carried out in triplicate. The resulting samples were incubated at 37 °C for another 12 h, and the lowest concentration (mg/mL) of the rAMP in agar plates showing visible inhibition zone was defined as the MIC.

Thermal Stability. Each rAMP in desalted water (pH 6.0, adjusted by HCl) was incubated at 90 °C for 40 min with an interval of 10 min. The remaining antimicrobial activity was determined with the method mentioned above.

RESULTS

Amplification of Lactoferricin cDNA from Goat Breast mRNA. A cDNA fragment encoded goat lactoferricin was amplified from goat breast total cDNA by polymerase chain reaction. Two sets of specific primers, designed based on the open reading frame sequence of goat breast lactoferricin cDNA, as shown in **Figure 1**, were used for PCR reactions. All primers, incorporating one restriction site at the 5′ end, were designed, as shown in **Figure 1**, so that the corresponding PCR products could be inserted between the *Xba*I and *Xho*I sites of the pPICZαC expression vector. The PCR amplified products were 174, 156, 126 bp and 108 bp for GLFcin $(His)_6$-Tag, GLFcin, GLFcin II $(His)_6$-Tag and GLFcin II, respectively, (data not shown).

Construction of rAMP-pPICZαC Expression Vector. To ensure the lactoferricin cDNA fragment in a correct reading frame, the PCR amplified fragment was cloned into pGEM-T Easy cloning vector for screening and sequencing. The vector containing correct in-frame lactoferricin cDNA sequence was used to construct the lactoferricin expression vector. According to the results of the preliminary experiments, goat lactoferricin and lactoferricin II have noticeable antimicrobial activity against *Pichia pastoris* (data not shown). Accordingly, for high level expression of these rAMPs, the cDNAs were ligated with pPICZαC expression vector in *Xba*I and *Xho*I restriction enzyme sites. They were introduced in frame to downstream of *AOX 1* promoter, a kind of methanol inducible promoter, of pPICZαC vector to reduce the basal expression level before methanol induction.

Screening and Isolation of Multicopy Recombinant Colonies. It is generally believed (*37*) that the number of integrated copies of expression cassette can affect the expression quantity of recombinant protein, and the multicopy recombinant colony potentially expresses significantly higher levels of the recombinant protein. Also, the *Sh ble* gene, harbored in pPICZαC expression vector, could overcome the resistance to Zeocin and be considered to be a sensitive way for screening the multiple integration colonies. In addition, resistance to high concentrations of Zeocin permits the multiple integration colonies to be selected since the *Sh ble* gene product inactivates Zeocin in a dose-dependent manner. Owing to the above reasons, in this study, *Pichia* transformants with a high Zeocin resistance (2000 μg/mL) were selected for expression, and its cultural broth appeared to have relatively higher

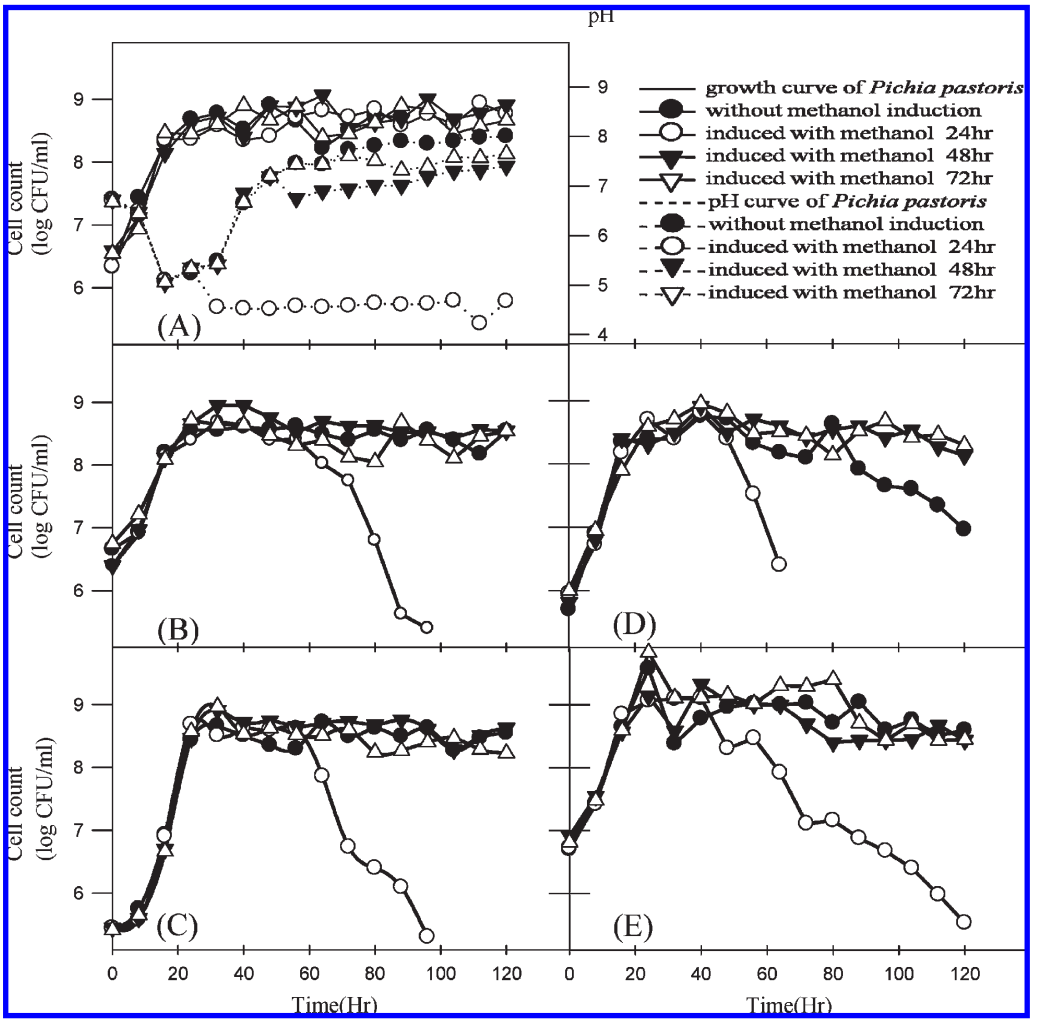

Figure 2. The effect of methanol induction on the growth and pH of *Pichia pastoris* SMD1168H, transformed without (**A**) or with GLFcin-$(His)_6$-Tag (**B**), GLFcin (**C**), GLFcin II-$(His)_6$-Tag (**D**) and GLFcin II (**E**).

Overnight cultures were diluted with the ratio of 1:100 in YPD broth. Except for the control (without methanol, —●—), methanol (2%) was added to the other test groups at the 24th, 48th and 72nd hours, —○—; the 48th and 72nd hours, —▼—; and only the 72nd hour, —▽—, respectively. Microbial growth was assessed by measuring the colony forming units (CFU)/mL at an 8 h interval during cultivation. The pH of cultural broth in each control group was recorded as dotted lines (without methanol, ---●---; treatment with methanol initiated at the 24th hour, ---○---; 48th hour, ---▼---; and 72nd h, ---▽---) in panel (**A**).

antimicrobial activity than cultural broth from low Zeocin resistance transformants (data not shown).

Effect of Methanol Induction Timing on Growth Curve and Broth pH. To investigate whether rAMPs and methanol used for induction of expression of rAMP were toxic to the expression host *Pichia pastoris* SMD1168H, the growth curve, broth pH and the opportune time of methanol induction for expression of rAMPs were measured. According to the results in **Figure 2A**, the growth curves of non rAMP gene transformed *Pichia pastoris* SMD1168H treated without or with methanol from the 24th, 48th and 72nd hours were extremely similar. However, the induction by methanol caused the decline of broth pH, especially for that induced with methanol from the 24th hour. Because the tendency of pH decline on the 4 rAMPs gene transformants was almost the same as that of non rAMP gene transformant, only the pH profile of cultural broth of non rAMP gene transformant was presented in **Figure 2A**. This phenomenon suggested that 2% methanol could not inhibit the growth of *Pichia pastoris* SMD1168H, a kind of methylotrophic yeast. Furthermore, comparing the growth curves of these 4 rAMP transformants (as shown in **Figures 2B−2E**) with the pH profile of cultural broth, the pH of sample with 2% methanol induction from the 24th hour seriously declined to acid (pH 4.6), while the viable cell counts reduced. However, those with 2% methanol induction from the 48th or 72nd hour did not dramatically decline (pH 7.2−7.6). Comparing viable cell counts with that induced with methanol from the 24th hour, those induced from the 48th or 72nd hour with higher viable cell counts might be due to the neutral pH which kept the transformants surviving. It suggested that the methanol induction of each rAMP transformed *Pichia pastoris* could express the rAMP into the cultural broth and seriously decline pH, which subsequently caused the rAMP with positive charge and damage to its host cell. Furthermore, according to the growth curves (**Figures 2A−2E**) of the controls (without methanol induction) of each rAMP transformed *Pichia pastoris*, the late-exponential phase was observed after 24 h incubation. This data suggested that the best time for methanol induction was initiated at the 24th hour during incubation with adjusting the broth pH to weak basic range to reduce the positive charge and damage of rAMPs to host cells. This strategy is able to stabilize the expression systems, and finally to achieve the overexpression of rAMPs.

Expression and Purificationof the rAMPs. Since the four rAMP cDNAs were cloned individually in pPICZαC plasmid under the

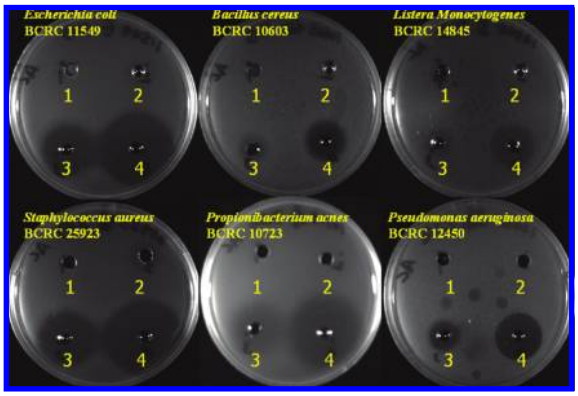

Figure 3. Assay of GLFcin-$(His)_6$-Tag antibacterial activity. (1) The control without recombinant lactoferricin; (2) 50 μL of cultural broth; (3) 50 μL of 5× concentracted cultural broth; (4) 50 μL of 10× concentracted cultural broth.

Table 1. Minimum Inhibitory Concentrations of rAMPs

	minimum inhibitory concentrations[a] (mg/mL)			
test strains	GLFcin	GLFcin-$(His)_6$	GLFcin II	GLFcin II-$(His)_6$
G(−) bacteria				
Escherichia coli	4.07	6.00	6.80	5.00
Propioni bacterium acnes	12.32	8.52	10.50	8.00
Pseudemonas aeruginosa	4.07	6.47	14.50	12.00
G(+) bacteria				
Bacillus cereus	12.32	8.52	16.00	11.50
Staphylococcus aureus	4.07	6.00	6.80	5.00
Listeria monocytogene	4.07	12.84	9.92	6.00

[a] Each assay was carried out in triplicate.

control of a strong *AOX 1* promoter, after induction by methanol, a high level of the rAMPs was expressed and secreted into the cultural broth by α-factor preprosequence during shaking cultivation. According to the results of the preliminary experiments, the 4 cationic rAMPs behaved as extremely thermally stable peptides. After 4-day cultivation, the cultural broth was centrifuged and filtrated to remove cells. The broth was then heated at 100 °C for 3 min and then centrifuged at 10000g for 10 min to completely exclude heat-unstable proteins. The resulting sample was finally collected for further purification.

Because GLFcin (p*I* 11.32, calculated from the Web site of http://www.expasy.ch/tools/protparam.html) and GLFcin II (p*I* 11.45) are cationic peptides, the recombinant GLFcin and GLFcin II could be partially purified by CM-Sepharose. To simplify the purification process, GLFcin and GLFcin II were fused with $(His)_6$-Tag to create GLFcin $(His)_6$-Tag and GLFcin II $(His)_6$-Tag, respectively. The recombinant GLFcin $(His)_6$-Tag and GLFcin II $(His)_6$-Tag could be partially purified by HisTrap affinity column chromatography, a kind of nickel column that could bind the protein fused with $(His)_6$ peptide. According to our preliminary experiments, chloric ion would neutralize the positive charge of the cationic rAMPs and destroy its antimicrobial activity. In addition, the imidazole would interfere with the antimicrobial activity assay. In order to eliminate these interferences and to further purify these rAMPs with their antimicrobial activity, the eluent from CM-Sepharose or HisTrap affinity chromatography was further purified by Sephadex G-25 gel filtration chromatography. According to the HPLC diagram, GLFcin and GLFcin II could be partially purified by CM-Sepharose following by G-25 gel filtration chromatography (data not shown), and GLFcin $(His)_6$-Tag and GLFcin II $(His)_6$-Tag could be purified by HisTrap affinity chromatography following by G-25 gel filtration chromatography (data not shown). The yield of purified rAMP amounted to 0.15 mg/mL of cultural broth.

N-Terminal Sequence. The N-terminal sequences of purified GLFcin $(His)_6$-Tag and GLFcin II $(His)_6$-Tag were found to be Ala-Pro-Arg-Lys-Asn-Val-Arg and Ser-Lys-Cys-Tyr-Gln-Trp, respectively, and they are identical to those translated from corresponding GLFcin and GLFcin II cDNA (**Figure 1**), respectively. These results suggested that the α-factor signal peptide had been excised by Kex2 and Ste 13 during the secretion process.

Antibacterial Activity and MIC of rAMPs. Antibacterial activity was assayed to determine the function of rAMPs. As shown in **Figure 3**, obvious inhibition zones appeared around the treated hole resulting from 50 μL of 5× or 10 × concentracted cultural broth of GLFcin-$(His)_6$-Tag, and their diameters varied depending on concentration, but no inhibition zones were found on the control, suggesting that GLFcin-$(His)_6$-Tag had antibacterial activity against tested bacterials, expecially for *Escherichia coli* BCRC 11549 and *Staphylococcus aureus* BCRC 25923. For quantification of antibacterial activity, the MICs of rAMP against tested bacterials were assayed and shown in **Table 1**. The 4 rAMPs behaved with antibacterial activity against tested bacteria with MICs ranging from 4.07 to 16.00 mg/mL. As shown in **Table 1**, MIC of GLFcin was lower than that of GLFcin II. Furthermore, fusion of $(His)_6$-Tag decreased the MIC of the tested rAMPs, expecially for GLFcin II-$(His)_6$-Tag. Among those MICs of rAMPs on tested indicators, they had smaller MICs against *Escherichia coli* BCRC 11549 and *Staphylococcus aureus* BCRC 25923 (4.07−6.80 mg/mL).

According to the remaining antibacterial activity of each rAMPs after various time periods of heating (0−40 min) at 90 °C (data not shown), these 4 rAMPs were revealed to be extremely heat stable. These results suggested that purification of these 4 rAMPs could be achieved by simply heating to remove most of the heat-unstable contaminants and then performing the further procedures.

DISCUSSION

Since lactoferricin possesses the advantage of a broad antibacterial spectrum without inducing resistance against antibiotics, it is promising for being used as an alternative of widely used antibiotics currently. Although lactoferricins are widely distributed in nature, their levels are low. Accordingly, it is rather difficult and time-consuming to isolate lactoferricins directly from natural sources. Now, it is very important and worth trying to realize the expression and purification of recombinant lactoferricin with low cost and high bioactivity via modern biotechnology.

To date, various expression systems, such as prokaryotic cells (*38*−*44*), yeast cells (*45*, *46*) or insect cells (*47*) have been created for overexpression of recombinant antimicrobial peptides. However, some bottlenecks have been encountered in expression of recombinant antimicrobial peptides because of their cytotoxicity to host cells (*38*), sensitivity to proteases (*48*, *49*) and low expression level (*47*). Many *E. coli* fusion expression systems, designed to decrease the toxicity of antimicrobial peptides to host cells and consequently protect the small antimicrobial peptides from proteolytic degradation, had, thus far, been developed and promoted on expression of toxic peptides (*39*−*44*). However, *E. coli* did not belong to the Generally Recognized as Safe strains, and its expressed peptides could hardly be accepted in food, cosmetics and biomedical applications.

Pichia pastoris expression systems have been used in expression of recombinant pharmaceutical proteins for quite a long time, and considered as Generally Recognized as Safe (GRAS) by the

American Food and Drug Administration (FDA). In addition, *Pichia pastoris* SMD1168H is a protease deficient methylotrophic yeast strain, and pPICZαC vector is considered to be a powerful inducible expression vector. Thus, when the cationic rAMP gene is cloned into pPICZαC vector, the expression is controlled by *AOX 1* inducible promoter and secreted into the cultural broth by α-factor signal sequence. After methanol induction of the transformant at late-exponential phase, high level secreting expression of rAMP would be achieved. Nevertheless, some issues would be encountered after methanol induction such as metabolism of methanol by the methylotrophic *Pichia pastoris* SMD1168H causing culture broth pH decline, which consequently makes the cationic rAMP carry more positive charge and damage to the expression host. The best strategy to stabilize the expression host is to reduce the toxicity of cationic rAMP by adjusting the cultural broth pH to basic range during induction and expression stages.

In this study, 4 goat lactoferricin-related gene transformed *Pichia pastoris* SMD1168H strains had been created, and a high level of active form of rAMPs were expressed as soluble form in cultural broth. These rAMPs behaved as a thermally stable peptide, retaining their antibacterial activity even after 30 min exposure at 100 °C. This unique character is highly beneficial to the further purification of these peptides and their subsequent applications.

In order to easily purify these peptides, $(His)_6$-tag was fused on the C-terminal of GLFcin and GLFcin II. After purification by HisTrap affinity chromatography and further by G-25 gel filtration, the GLFcin $(His)_6$-Tag and GLFcinII $(His)_6$-Tag could be purified. Furthermore, fusion with $(His)_6$ peptide did not affect the antibacterial activity of rAMPs, GLFcin $(His)_6$-Tag and GLFcinII $(His)_6$-Tag, since these two peptides still kept their antibacterial activities. As shown in **Table 1**, it was found that the MICs of GLFcin -$(His)_6$-Tag and GLFcin II-$(His)_6$-Tag were lower than those of GLFcin and GLFcin II. This might be because the antibacterial activity of lactoferricin resulted from its positive charge (*50*), and the histidine is cation amino acid. Accordingly, fusion with $(His)_6$ did not decrease the intensity of positive charge of the rAMPs, or even enhanced it.

In conclusion, the antibacterial activity of the recombinant goat lactoferricin could function against not only *Escherichia coli* BCRC 11549, *Pseudomonas aeruginosa* BCRC 12450, *Bacillus cereus* BCRC 10603, *Staphylococcus aureus* BCRC 25923, *Propioni bacterium acnes* BCRC10723 and *Listera monocytogenes* BCRC 14845, but also the host, *Pichia pastoris* SMD1168H. The high antibacterial activity against a broad range of microbes suggests that the recombinant goat lactoferricin-related peptides can be potential alternatives of antibiotics and can be used in the medical, food and cosmetic industries.

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