

Recombinant expression and biological characterization of the antimicrobial peptide fowlicidin-2 in *Pichia pastoris*

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Abstract. Fowlicidins are a group of cathelicidin antimicrobial peptides that were initially identified in chickens. Fowlicidin-2, which is composed of 31 amino acids, is widely expressed in the majority of tissues in chickens and has an important role in innate immunity. In the present study, a recombinant expression system for fowlicidin-2 was successfully constructed using *Pichia pastoris* X-33 and the expression vector pPICZ α -A. Under the optimized fermentation conditions, 85.6 mg fowlicidin-2 with >95% purity was obtained from 1 liter culture medium following purification by ion exchange chromatography and reversed phase high performance liquid chromatography. The recombinant fowlicidin-2 exhibited broad spectrum antimicrobial activity and had a minimum inhibitory concentration ranging from 1 to 4 μ M. Furthermore, recombinant fowlicidin-2 exhibited hemolytic activity, promoting 50% human erythrocyte hemolysis in the concentration range of 128-256 μ M, and anticancer activity, resulting in the death of 50% of A375 human malignant melanoma cells in the concentration range of 2-4 μ M. The results of the present study suggest that recombinant fowlicidin-2 may be a promising candidate for therapeutic applications.

Introduction

The rate of emergence of drug-resistant strains has been increasing in recent years owing to the widespread and inappropriate use of conventional antibiotics. This is significant threat to human health (1), and the development of novel antimicrobial agents that are effective against antibiotic-resistance microbes is urgently required (2). Antimicrobial peptides (AMPs) are

important components of the innate host defense system and are present in virtually all species of life. AMPs have broad spectrum activity against bacteria, fungi, viruses, parasites and even tumors, and have exhibited a similar efficiency against strains that are resistant and susceptible to antibiotics (3). Since they exert nonspecific membrane-lytic activities, it is extremely difficult for bacteria to develop resistance to AMPs. Therefore, AMPs have been regarded with increasing interest for development as novel therapeutic agents for the control and prevention of infectious diseases, particularly those caused by antibiotic-resistant bacteria (4).

Among the AMPs, cathelicidins are one of the most prominent families of host defence peptides and are recognized as having a role in the defence against microbial invasion (5). The majority of cathelicidins are differentially expressed in a variety of tissues and exhibit broad-spectrum antimicrobial activity against gram-negative and gram-positive bacteria, fungi, protozoa and enveloped viruses (6,7). Fowlicidin-2 is a novel member of the cathelicidins family that was identified in chicken (8). The peptide has previously been shown to exert highly potent antibacterial and lipopolysaccharide (LPS)-neutralizing activities (8,9), and is among the most efficacious cathelicidins to have been reported (8). Due to its broad-spectrum and salt-insensitive antibacterial activities, as well as its potent LPS-neutralizing activity (8), fowlicidin-2 may be an excellent candidate as a novel antimicrobial and antiseptic agent.

There are three pathways for AMP production: Extraction from natural resources, chemical synthesis and recombinant expression. Due to the complexity, low-yield and high cost of chemical peptide synthesis (10), genetic engineering technology using microorganisms as host cells to express AMPs may be a more efficient method for AMP production. The methylotrophic yeast *Pichia pastoris* has become widely used as a heterologous gene expression system (11). Yeasts have become increasingly important in genetic engineering due to the ease by which they may be genetically manipulated, their ability to grow rapidly in inexpensive medium to high cell densities and their capability for complex post-translational modifications, including folding, disulfide bridge formation and glycosylation (12,13). The slow production of fowlicidin-2 has been, to date, a bottleneck for its development in scientific and industrial research. In our previous study, fowlicidin-2 was

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expressed in *Escherichia coli* (14); however, the yield (~6 mg/l) and the cost of production requires further improvement to meet the needs of industrial production. In the present study, fowlicidin-2 was recombinantly expressed using a *P. pastoris* expression system, and the bioactivity of the recombinant peptide was investigated *in vitro*.

Materials and methods

Strains, vectors, reagents and enzymes. The expression host, *P. pastoris* X-33, and the pPICZα-A expression vector were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). *E. coli* DH5α, *E. coli* 25922, *Staphylococcus aureus* 29213, *Pseudomonas aeruginosa* 27853 and *Salmonella typhimurium* C77-31 were preserved in our laboratory. A375 human malignant melanoma cells and RAW264.7 mice macrophages were purchased from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Chick embryo fibroblasts (CEFs) were isolated from 9-day-old chicken embryos, provided by the Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences (Harbin, China). The experimental protocol was reviewed and approved by the ethics committee of the Hospital of Northeast Agricultural University (NEAU; Harbin, China).

Construction of the expression vector. A DNA fragment encoding the mature peptide of fowlicidin-2 was designed, using DNA software (version 2.0; <http://www.dna20.com>) according to the codon bias of the methylotrophic yeast *P. pastoris*. The fragment was constructed using two complementary oligonucleotides (oligos), as follows: Sense, 5'-GAA *TTCGAAAAGAGACTTGTTCAGAGGGGTAGATTG*CGTTTCTTGCCTAAGATAAGGAGATTTAGACCCAAGGTAACCATACGATACAAGGAAGTGCCAGGTTT *[TAA]*TCTAGA-3' and antisense, 5'-TCTAGATTAAACCTGGCACTTCCTTGTATCGTAATGGTTACCTTGGGTCTAAATCTCCTTATCTTACGCAAGAAACGACCAAATCTACCCCTCTGAACAAGTCTCTTTTTCGAATTC-3'. Restriction sites for *EcoRI* and *XbaI* are indicated in the oligos in italics. A terminal *[TAA]* codon is followed by the sequence encoding fowlicidin-2 in the sense strand. GAAAAGAGA, as the codon for a glutamic acid, lysine and arginine (EKR) sequence, provide the cleavage site of Kexin protease 2 (Kex2) in the translated peptide (Fig. 1). The two complementary oligos were synthesized chemically by Sangon Biotech Co., Ltd. (Shanghai, China). A total of 20 μl of each sense and antisense oligos solution (100 μM) were mixed, heated at 95°C for 2 min and cooled down to room temperature. The annealed product was purified using the gel extraction kit (cat. no. 28704; Qiagen, Beijing, China). Following digestion by *EcoRI* and *XbaI*, the annealed product was ligated using a DNA Ligation kit (cat. no. 6022; version 2.1; Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's instruction between the *EcoRI* and *XbaI* sites of the pPICZα-A vector, which contains the *Saccharomyces cerevisiae* α-factor secretion signal and the alcohol oxidase 1 promoter. A total of 10 μl of the resultant plasmid (30 ng) was added to 100 μl of competent *E. coli* DH5α cells (Tiangen Biotech Co., Ltd., Beijing, China) and held on ice for 30 min prior to heat shocking at 42°C for 90 sec. After incubation on ice for 2 min, the cells

were diluted into 0.8 ml LB culture (0.5% yeast extract, 1% tryptone, 1% NaCl) (Hopebio, Qindao, China) and cultured at 37°C for 30 min. Then, appropriate dilutions were plated on LB plates (containing 20 μg/ml Zeocin) and incubated for 12 h at 37°C (15). Positive transformants were screened by colony polymerase chain reaction (PCR) using a *Taq* PCR Mastermix kit (cat. no. KT201; Qiagen) and the following primers: P1, 5'-GACTGGTTCCAATTGACAAGC-3' and P2, 5'-GCAAATGGCATTCTGACATCC-3'. The following thermal cycling conditions were used: 94°C for 5 min followed by 32 cycles (95°C for 30 sec, 58°C for 30 sec and 72°C for 1 min) and finally 72°C for a 5 min extension. Finally, they were verified by DNA sequencing.

Transformation of *P. pastoris* and selection of transformants. The pPICZα-A-fowlicidin-2 construct was linearized using the *SacI* restriction enzyme and transformed into *P. pastoris* X-33 cells by electroporation using a Gene Pulser apparatus (Bio-Rad Laboratories, Inc., Hercules, CA, USA) in 0.2-cm electroporation cuvettes (200 Ω, 25 μF, 1,500 V). The pPICZα-A vector was similarly linearized and transformed into *P. pastoris* X-33 as a negative control. The two types of transformed *P. pastoris* were incubated at 30°C in YPD plates (Hopebio) containing 1% yeast extract, 2% tryptone, 2% dextrose, 2% agar and 100 μg/ml Zeocin (Invitrogen; Thermo Fisher Scientific, Inc.). In order to determine the methanol-utilizing phenotype of the transformants, all Zeocin-resistant transformants cultured in YPD medium were selected on MD plates (1.34% YNB, 4x10⁻⁵% biotin, 2% dextrose and 2% agar) and MM plates (1.34% YNB, 4x10⁻⁵% biotin, 0.5% methanol and 1.5% agar) (both purchased from Invitrogen; Thermo Fisher Scientific, Inc.). Zeocin⁺ Mut⁺ phenotype colonies were further confirmed by colony-PCR using the P1 and P2 primers.

Heterologous expression of recombinant fowlicidin-2 (rfowlicidin-2). A total of 10 pPICZα-A-fowlicidin-2 transformants were cultured at 30°C in a shaking flask containing 20 ml buffered glycerol complex medium (1% yeast extract, 2% peptone, 0.3% K₂HPO₄, 1.18% KH₂PO₄, 1.34% YNB, 4x10⁻⁵% biotin, 1% glycerol, pH 6.0) to optical density (OD)₆₀₀=2.0-6.0. Cells were harvested by centrifugation at 1,500 x g for 5 min at room temperature and resuspended to an OD₆₀₀ of 1.0 in buffered methanol-complex medium (BMMY; 1% yeast extract, 2% peptone, 0.3% K₂HPO₄, 1.18% KH₂PO₄, 1.34% YNB, 4x10⁻⁵% biotin, 0.5% methanol, pH 6.0). To induce expression of rfowlicidin-2, methanol was added every 24 h to a final concentration of 0.5% (v/v), after which the resuspended culture was grown for 72 h and the supernatant was obtained by centrifugation at 12,000 x g for 5 min at 4°C. The expression of fowlicidin-2 in the supernatant was determined using Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE). Total protein yield in the culture medium was measured using a Bradford protein assay kit. The highest expressing *P. pastoris* clone identified as *P. pastoris* X33-Fow2 was singled out and used for large-scale production of rfowlicidin-2.

Purification of rfowlicidin-2. The fermentation medium obtained from the *P. pastoris* X33-fowlicidin-2 fermentation was harvested by centrifugation at 12,000 x g for 10 min at

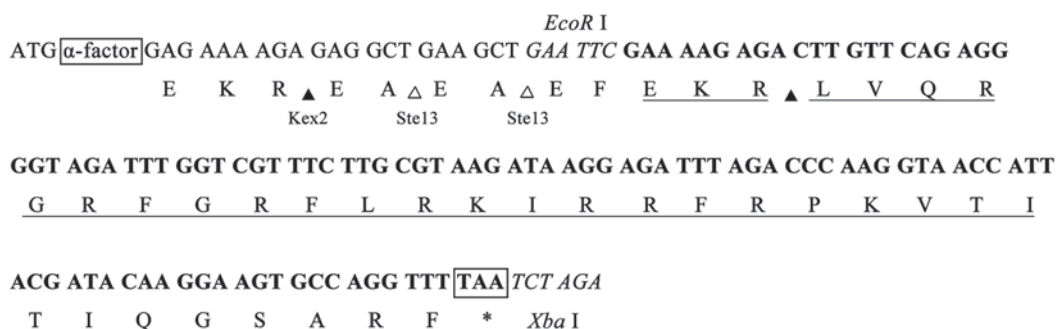


Figure 1. Nucleotide sequence of DNA encoding fowlicidin-2 and the C-terminal amino acids of the α -factor signal peptide. The Kex2 and Ste13 cleavage sites for proteolytic processing and α -factor-driven secretion of the fusion protein are indicated by arrowheads. The synthesized DNA fragment is shown in bold. The sequences of *Eco*RI and *Xba*I sites are italicized. The amino-acid and encoding sequences of fowlicidin-2 are underlined. Kex2, Kexin protease 2; Ste13, dipeptidyl aminopeptidase.

4°C. A total of 30 ml of the resultant supernatant was loaded onto a Q-Sepharose Fast Flow column (10 ml broth/5 ml resin; GE Healthcare Life Sciences, Chalfont, UK) pre-equilibrated with 20 mM sodium phosphate buffer (pH 7.2; buffer A) for purification by ion exchange chromatography. The column was washed again with buffer A, and then eluted with a linear gradient of 0.0-1.0 M NaCl in buffer A. Fractions containing fowlicidin-2 were collected and further purified by reversed phase high-performance liquid chromatography (RP-HPLC) on a C18 column (250 x 4.6 mm, 5 μ m; silica pore size, 300 Å; Supelco, Inc., Bellefonte, PA, USA), which had been pre-equilibrated using 0.1% trifluoroacetic acid (TFA). Bound proteins were eluted with a linear gradient of acetonitrile (20-45%, v/v; 1.5% per min) in 0.1% TFA. The flow rate was 1.0 ml/min and the absorbance of the eluent was monitored at 214 nm. Purification was monitored by 15% Tricine-SDS-PAGE. The purified peptide was lyophilized and stored at -20°C until further analysis. The peptide yield was determined using a Bradford protein assay kit.

Mass spectrometry and N-terminal sequencing analysis. The purified peptide (30 ng) was dissolved in a saturated solution of α -cyano-4-hydroxycinnamic acid containing 50% acetonitrile and 0.1% TFA, and then analyzed using a Matrix-Assisted Laser Desorption/Ionization Time-of-Flight mass spectrometer (Applied Biosystems; Thermo Fisher Scientific, Inc.). For N-terminal sequencing analysis, the purified peptide was run on a 15% tricine-SDS-polyacrylamide gel, and then transferred to a polyvinylidene difluoride (PVDF) membrane (EMD Millipore, Billerica, MA, USA) by semi-dry electrophoretic transfer. N-terminal sequence analysis was performed by automated Edman degradation using a PPSQ-33A Sequencing System (Shimadzu Corporation, Kyoto, Japan), according to the manufacturer's protocol.

Antibacterial activity assay. Antibacterial activity of fowlicidin-2 against various microorganisms was tested using the microbroth dilution method (16). Briefly, bacteria subcultured in the log phase were diluted to 2×10^5 - 7×10^5 colony forming units/ml with fresh Mueller Hinton Broth medium (Hopebio). Aliquots (50 μ l) from each strain suspension were distributed into the wells of a 96-well polypropylene microtiter plate, after which 50 μ l peptide was added to each

well. Cultures were grown for 18 h at 37°C with agitation. The absorbance was measured at 600 nm using a microplate reader. Experiments were performed in triplicate. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of peptide able to fully inhibit bacterial growth, as determined by OD measurements.

Hemolytic activity assay. The hemolytic activity of rfowlicidin-2 was determined using human red blood cells (hRBCs), as described previously (17). Briefly, fresh anticoagulated human blood from a healthy donor was washed thrice with phosphate-buffered saline (PBS) and diluted to a concentration of 4% (v/v) in PBS. The experimental protocol was reviewed and approved by the ethics committee of the Hospital of NEAU. The erythrocytes (90 μ l) were dispensed into a 96-well plate, followed by addition in triplicate of 10 μ l serially diluted peptides. The resulting suspension was incubated for 1 h at 37°C. Following centrifugation of the plate at 1,000 x g for 5 min at room temperature, the supernatants were transferred to a new 96-well plate and the absorbance was measured at 570 nm using an ELISA plate reader for analysis of released hemoglobin. Negative controls consisted of cells suspended in PBS only, and positive controls consisted of cells suspended in 1% Triton X-100. The percentage of hemolysis was calculated as follows: Percentage of hemolysis (%) = $[(A_{570 \text{ nm, peptide}} - A_{570 \text{ nm, PBS}})/(A_{570 \text{ nm, 1\% Triton X-100}} - A_{570 \text{ nm, PBS}})] \times 100$.

Determination of anticancer activity in vitro. The anticancer activity of rfowlicidin-2 was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method, as described previously (18). Briefly, A375 human malignant melanoma cells, RAW264.7 mice macrophages and CEFs were seeded into a 96-well plate at a density of 2.5×10^4 cells/well in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and cultured at 37°C in a humidified 5% CO₂ incubator. When the cell fusion reached more than 80%, the cells were washed once with DMEM, followed by the addition of 90 μ l fresh DMEM containing 5% FBS and various concentrations (0 to 128 μ M) of 10 μ l peptide in triplicate into each well. Cells were incubated at 37°C for 24 h, after which 10 μ l alamarBlue dye was added to each well and incubated for a further 6 h. The

fluorescence was measured at 490 nm. The anticancer activity of rfowlicidin-2 was defined as the peptide concentration that induced 50% cell death (EC_{50}).

Statistical analysis. Data were analyzed by one-way analysis of variance using SPSS version 17.0 software (SPSS, Inc., Chicago, IL, USA). Differences among groups were compared with Student's t-test.

Results

Screening of positive transformants. A fowlicidin-2 DNA fragment was inserted into the *Eco*RI and *Xba*I sites of pPICZ α -A to construct the recombinant pPICZ α -A-fowlicidin-2 vector. The pPICZ α -A-fowlicidin-2 vector was transformed into *P. pastoris* X-33 cells by electroporation, and the transformants that grew on MD plates were identified by PCR (Fig. 2). A total of 31 multicopy transformants were screened out on MD plates under selection by Zeocin (100 μ g/ml) and were confirmed by PCR amplification. A total of 10 colonies presenting the *Zeocin*⁺ *Mut*⁺ phenotype and with strong PCR amplification products of fowlicidin-2 DNA were used for the induced expression of the peptide.

Expression of rfowlicidin-2. pPICZ α -A-fowlicidin-2 transformants were analyzed for the extracellular expression of fowlicidin-2 in BMMY broth at 28°C for 72 h. The highest-expressing transformant, *P. pastoris* X33-Fow2, was selected for further high-density cultivation. Aliquots of the culture supernatant were withdrawn at 12-h intervals and analyzed by Tricine-SDS-PAGE. As shown in Fig. 3, a major protein band of ~3.7 kDa, which corresponded to fowlicidin-2, appeared following induction with methanol. The highest expression level of fowlicidin-2 was ~121 mg/l at 60 h of culture in the optimized fermentation conditions (28°C, pH 6.0, 0.5% methanol in BMMY medium).

Purification and identification of rfowlicidin-2. After 60 h of methanol induction, the cultures were harvested and the supernatants were collected for the purification of rfowlicidin-2 using a Q-Sepharose Fast Flow column and RP-HPLC. The highest peak absorbance fractions, which were pooled and detected as a single band by Tricine-SDS-PAGE (Fig. 4), contained the majority of rfowlicidin-2. The average volume of rfowlicidin-2 recovered from 1 liter culture medium was ~85.6 mg, with a purity of >95%. These results indicate that rfowlicidin-2 may be successfully purified from methylotrophic yeast, with a relatively high recovery rate and purity, using a two-step purification strategy. The purified rfowlicidin-2 was transferred to a PVDF membrane, and sequenced for up to 4 cycles. The N-terminus began with a leucine-valine-glutamine-arginine sequence, which was consistent with the predicted sequence of rfowlicidin-2 (8). This indicates that the rfowlicidin-2 peptide that was produced did not have any additional amino acids at its N-terminal end.

Antibacterial activity of rfowlicidin-2. The MICs of the peptide for several microorganisms are shown in Table I. Fowlicidin-2 showed strong and broad-spectrum antimicrobial activities against both gram-positive bacteria (*S. aureus*

Table I. MICs of rfowlicidin-2 for four tested bacteria.

Tested bacteria	MIC (μ M)
<i>Escherichia coli</i> ATCC25922	1
<i>Staphylococcus aureus</i> ATCC29213	4
<i>Pseudomonas aeruginosa</i> ATCC27853	4
<i>Salmonella typhimurium</i> C77-31	2

MIC, minimal inhibitory concentration.

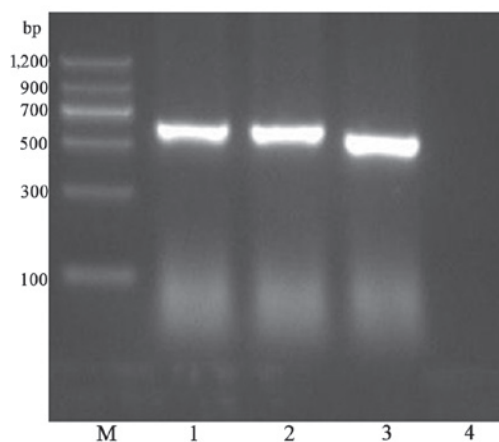


Figure 2. Polymerase chain reaction (PCR) screening of the recombinant pPICZ α -A-fowlicidin-2 expression plasmid. Lane M contains the DNA molecular weight marker, lanes 1 and 2 contain the PCR product of the pPICZ α -A-fowlicidin-2 plasmid, lane 3 contains the PCR product of the pPICZ α -A plasmid, and lane 4 is the negative control.

ATCC 29213) and gram-negative bacteria (*E. coli* 25922, *P. aeruginosa* ATCC 27853, *S. typhimurium* C77-31). For these strains, the MIC values were in the range of 1-4 μ M.

Hemolytic activities of rfowlicidin-2. To evaluate the hemolytic activity of fowlicidin-2 against red blood cells, fresh hRBCs were used. The peptide exhibited a dose-dependent hemolytic activity against hRBCs, with the EC_{50} occurring within the range of 128-256 μ M (Fig. 5).

Anticancer activity of rfowlicidin-2. The cytotoxic activity of rfowlicidin-2 was assessed against A375 human malignant melanoma cells, RAW264.7 mouse macrophages and CEFs. Concentration-response studies demonstrated that the peptide exerted a significantly higher cytotoxic activity against the human malignant A375 cells ($P < 0.01$), as compared with the two other types of cells (Fig. 6). Fowlicidin-2 showed a 2-fold higher activity against the human A375 cells (EC_{50} =2-4 μ M), as compared with the RAW264.7 cells (EC_{50} =4-8 μ M), and an 8-fold higher activity, as compared with the CEFs (EC_{50} =16-32 μ M; Fig. 6).

Discussion

The emergence of pathogenic microorganisms with resistance to conventional antibiotics has driven the search for novel types of antimicrobial agents. Although numerous

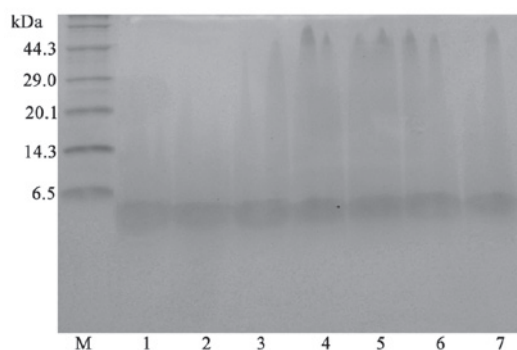


Figure 3. Tricine-sodium dodecyl sulfate-polyacrylamide gel analysis of the supernatant from fermentation broth containing secreted recombinant fowlicidin-2. Lane M contains the protein molecular weight marker, and lanes 1-7 contain the medium from cultures of fowlicidin-2-expressing *Pichia pastoris* at 12, 24, 36, 48, 60, 72 and 84 h following methanol induction, respectively.

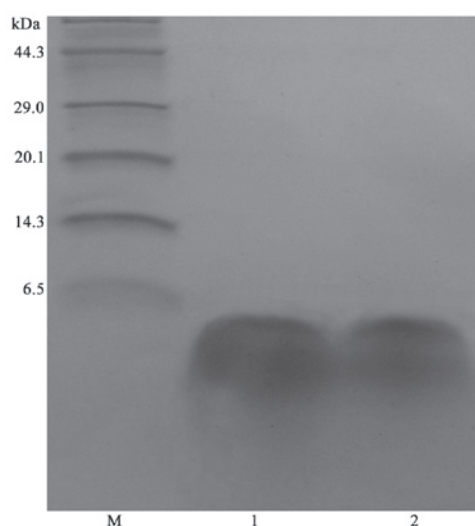


Figure 4. Tricine-sodium dodecyl sulfate-polyacrylamide gel analysis of the purified recombinant (r)fowlicidin-2. Lane M contains the protein molecular weight marker, and lanes 1 and 2 contain the purified rflowlicidin-2 from different batches.

novel strategies have been investigated, there are a number of avenues that have yet to be explored (19). AMPs are promising candidates for the development of novel antimicrobial agents, since they have been shown to be an important component of immunity and have a broad antimicrobial spectrum (3). However, the production of active AMPs in a cost-effective and scalable method is challenging. It has been recognized that recombinant DNA techniques are a potential means for producing AMPs in large quantities. Numerous AMPs have been successfully produced using genetic engineering methods (10). Various host cells have been used for the expression of AMPs; however, *P. pastoris* has emerged as one of the most efficient recombinant bioreactors (20). Similar to prokaryotes, *P. pastoris* is able to grow rapidly in inexpensive medium, and is amenable to high-cell-density fermentations (20,21). Furthermore, it is non-pathogenic and is capable of secreting functional extracellular proteins directly into the culture medium (21,22).

The present study used *P. pastoris* to express fowlicidin-2 at a high level. The Kex2 recognition site was introduced at

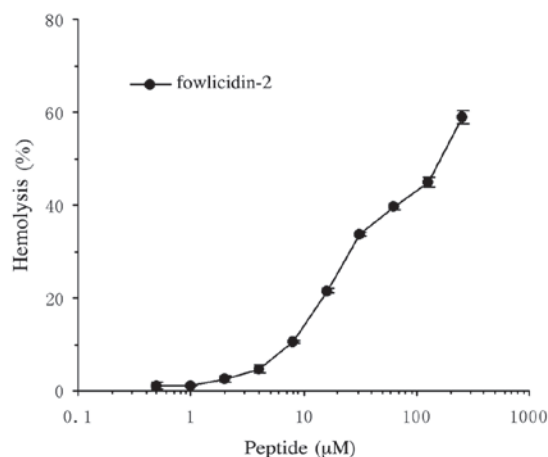


Figure 5. Hemolytic activity of various concentrations of recombinant fowlicidin-2 on human red blood cells.

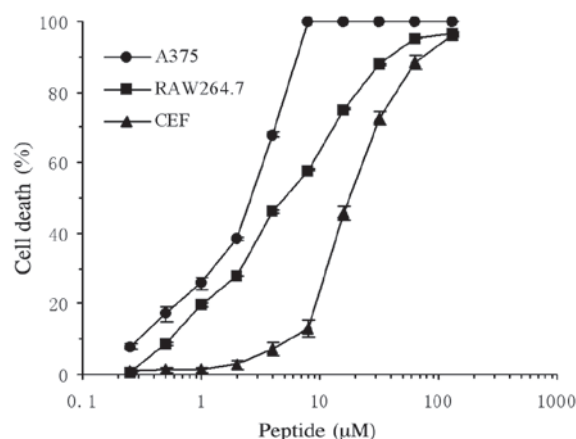


Figure 6. Anticancer activity of various concentrations of recombinant fowlicidin-2 against A375 human malignant melanoma cells, RAW264.7 mouse macrophages and chick embryo fibroblasts.

the N-terminal of fowlicidin-2. The Kex2 endoprotease is a member of the membrane-bound endoprotease family and is responsible for site-specific endoproteolysis at pairs of basic amino acid residues, in particular EKR, in the α -factor precursor peptide (23,24). The translated peptide fowlicidin-2 was successfully released by Kex2, and was shown to have the predicted amino acid sequence and molecular weight. Furthermore, sequencing revealed that there were no additional residues at the N-terminus of the peptide following Kex2 digestion, which was important since the natural N-terminus is critical for the activity of AMPs (25,26).

The antibacterial assay in the present study indicates that rflowlicidin-2 is prominent among the most potent cathelicidins discovered to date, showing activity against a variety of bacteria at concentrations of $\leq 4 \mu\text{M}$. In the present study, the lowest detected MIC of rflowlicidin-2 was towards *E. coli* 25922 ($1 \mu\text{M}$). The peptide exhibited a similar activity against *S. aureus* ATCC29213 and *P. aeruginosa* ATCC27853. These results demonstrate that the rflowlicidin-2 peptide was functional and effective against gram-negative and gram-positive bacteria. In addition, rflowlicidin-2 was shown to exert a hemolytic effect on hRBCs, with EC_{50} occurring at 128-256 μM ,

which is markedly higher than that reported by Xiao *et al* (8). The underlying mechanism is unclear and may be due to the different spatial structures of the rfwlicidin-2 obtained in the present study and the chemically synthesized fowlicidin-2 used in the study of Xiao *et al* (8).

Several cationic amphipathic AMPs or their derivatives have previously been reported to exert anticancer activity, display selective cytotoxicity against cancer cells *in vitro* and show efficacy in various *in vivo* xenograft models (27,28). However, only a small range of AMPs have been shown to have anticancer activity. Various cathelicidin-related or cathelicidin-modified AMPs, including LL-37 (29), Ceragenin CSA-13 (30), FK-16 (31) and BF-30 (32), have been reported to exhibit anticancer activity. However, to the best of our knowledge, there have been no previous reports on the anticancer activity of fowlicidin-2. Cationicity, α -helicity and amphipathicity are among the most important physicochemical parameters that determine the functional properties of α -helical AMPs (9,33). Increasing the degree of α -helicity and mean hydrophobicity of AMPs generally result in an increase in cytotoxicity against cancer cells (34). The secondary structure of fowlicidin-2 is dominated by α -helices. The N-terminal α -helix of the peptide adopts a typical amphipathic structure, whereas the C-terminal helix is more hydrophobic (9). In the present study, the anticancer activity of fowlicidin-2 was determined using the MTT method *in vitro*. The survival of cells was reduced by treatment with fowlicidin-2, and the percentage of cell death increased in a concentration-dependent manner. However, the EC_{50} of fowlicidin-2 for A375 human cancer cells was 2–4 μ M, and few A375 cells survived when treated with 8 μ M fowlicidin-2. Conversely, fowlicidin-2 showed relatively weak cytotoxic activity against RAW264.7 cells (EC_{50} =4–8 μ M) and CEFs (EC_{50} =16–32 μ M). These results suggested that fowlicidin-2 may be considered a promising candidate for anticancer applications. However, the mechanism of action of the peptide on cancer cells requires further investigation.

In conclusion, in the present study bioactive fowlicidin-2 was successfully expressed at a high level (85.6 mg/l) and high purity (>95%) using the yeast *P. pastoris* as a recombinant expression system. In addition, rfwlicidin-2 showed strong and broad-spectrum antimicrobial activity and anticancer activity. The present study was the first to demonstrate an inhibitory effect of fowlicidin-2 on cancer cells *in vitro*.

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