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Optimized production of scygonadin in *Pichia pastoris* and analysis of its antimicrobial and antiviral activities

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ABSTRACT

The crab antimicrobial peptide scygonadin is confirmed to have antimicrobial activity against bacteria and it is probably associated with the reproductive immunity in *Scylla paramamosain*. To obtain large quantity of scygonadin for further biological assays, a 306 bp cDNA sequence encoding the mature peptide of scygonadin was cloned into a secretion vector of pPIC9K, and a high-level of the recombinant scygonadin was achieved in *Pichia pastoris*. The optimal expression condition was determined as incubation with 0.5% methanol for 48 h at 28 °C under pH 6.0, and a total of 70 mg scygonadin was expressed in 1 L culture medium. The recombinant product was purified and 97% pure scygonadin was obtained using immobilized metal affinity chromatography with a yield of 46 mg/L. The recombinant scygonadin was confirmed using SDS–PAGE analysis and MS-fingerprinting. *P. pastoris*-derived scygonadin exhibited relatively higher antimicrobial activities against bacteria than *Escherichia coli*-derived scygonadin. The antimicrobial activity of the recombinant scygonadin against pathogenic *Aeromonas hydrophila* showed salt resistant and the killing kinetics of *A. hydrophila* was time dependent. Besides, the antiviral assay demonstrated that scygonadin could interfere with white spot syndrome virus (WSSV) replication in vitro-cultured crayfish haematopoietic (Hpt) cells. Taken together, this is the first report on the heterologous expression of scygonadin in *P. pastoris*, and *P. pastoris* is an effective expression system for producing large quantities of biological active scygonadin for both research and agricultural application.

Introduction

Antimicrobial peptides (AMPs)[1] have broad activities against bacteria, fungi, viruses, parasites and even tumors. Due to their low toxicity to eukaryotic cells, their application in medicine, veterinary and aquaculture has been greatly considered and a few of AMPs have been developed as alternative agents to substitute for traditional antibiotics [1,2]. Invertebrates primarily depend on innate immunity system to protect themselves from exogenous invasion. It is known that many AMPs have been identified in invertebrates such as, penaeidin [3], crustin [4], and anti-lipopolysaccharide factor (ALF) [5,6]. Several AMPs including crustin [7], ALF [8,9], arasin-like*Sp* and GRP*Sp* [10] and scygonadin [11] have been found in *Scylla paramamosain*, which is a commercially important species in the western Pacific and Indian Oceans.

Our previous studies show that scygonadin is active against bacteria [12], particularly it is probably associated with reproductive immunity [13]. It is found to be widely expressed in other crabs, such as *Eriocheir sinensis* and *Portunus pelagicus* [14]. For further functional study of scygonadin and its future commercial application in aquaculture, heterologous expression to obtain large quantity of recombinant scygonadin is required.

In our previous study, an *Escherichia coli* expression system was constructed and the recombinant scygnodin product was obtained with activity against bacterial growth [15]. However, *Pichia pastoris*-based system has many advantages such as high-level expression of heterologous proteins, easy scale-up and cost-effective culture media, easy purification and capacity of post-translational modifications required for functionality [16–19]. Hence, *P. pastoris* may be a better choice for heterologous expression of scygonadin than prokaryotic expression system.

In the present study, a 306 bp cDNA fragment encoding scygonadin mature peptide was cloned into *P. pastoris*. The recombinant expression conditions were optimized. Furthermore, the biological activities against bacteria and virus were also investigated.

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[1] *Abbreviations used*: WSSV, white spot syndrome virus; Hpt, haematopoietic; AMPs, antimicrobial peptides; ALF, anti-lipopolysaccharide factor; pPIC9K-scy, pPIC9K-scygonadin; NH_4HCO_3, ammonium bicarbonate; ACN, acetonitrile; MIC, minimal inhibitory concentration; MBC, minimal bactericidal concentration; PDC1, plant defensin corn 1; SLPI, human secretory leukocyte protease inhibitor; nsLTP, non-specific lipid-transfer proteins.

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Materials and methods

Plasmid vectors, bacterial, fungal and virus strains, and animals

The plasmid pPIC9K and *P. pastoris* GS115 were purchased from Invitrogen (USA). Bacterial and fungal strains were purchased from China General Microbiological Culture Collection Center (China).

The red claw freshwater crayfish, *Cherax quadricarinatus*, were purchased from Zhangpu, Fujian, China, and maintained in aerated tap water at 20 °C.

White spot syndrome virus (WSSV) was kindly provided by Prof. Xun Xu (Third Institute of Oceanography, Xiamen, Fujian, China).

Vector construction

A 306 bp sequence encoding the mature peptide of scygonadin was amplified from a constructed pET28-scygonadin [15]. The forward primer (5′- GGGGAATTCGGCCAGGCACTCAACAA -3′) introduced an EcoRI site (underlined). The reverse primer was designed as (5′- CAT GCGGCCGC**TCA**ATGGTGATGGTGATGATG GTAAGAAGCAATC-CAGT -3′), with an endonuclease site NotI (underlined) and a 6× His-tag at the carboxyl terminus of scygonadin. The PCR product was digested with EcoRI and NotI, and ligated into the EcoRI/NotI-digested pPIC9K. The ligation mixture was transformed into *E. coli* DH5α. And the constructed plasmid pPIC9K-scygonadin (pPIC9K-scy) was confirmed by DNA sequencing.

Transformation of P. pastoris and PCR analysis of P. pastoris transformants

The constructed plasmid of pPIC9K-scy was linearized with SalI and transformed into competent *P. pastoris* GS115 cells by electroporation using the Bio-Rad gene pulser Xcell™. And the pPIC9K vector was also linearized and transformed into *P. pastoris* GS115 cells as a negative control. These transformants were selected on MD plates and incubated at 30 °C for 2–3 days.

Twenty positive clones (GS115/pPIC9K-scy) on MD plates were detected by a genomic PCR assay to ensure the integration of the scygonadin gene into the *P. pastoris* genome, using 5′AOX1 (5′-GACTGGTTCCAATTGACAAGC -3′), and 3′AOX1 (5′- GCAAATGGCA TTCTGACATCC -3′) as primers. *P. pastoris* genomic DNA was extracted by the kit from Tiangen (Beijing, China) and prepared as a template for PCR amplification. PCR amplification was performed as follows: initial denaturation at 94 °C for 5 min, followed by 25 cycles (94 °C, 30 s; 55 °C 40 s; 72 °C 2 min) and a final elongation at 72 °C for 8 min.

Optimization of recombinant scygonadin expression in P. pastoris

A single clone of GS115/pPIC9K-scy was first grown in 2 mL YPD medium (1% yeast extract, 2% tryptone, 2% D-glucose) for 18–24 h at 28 °C, with shaking at 230 rpm. These cells were further cultured in 50 ml BMGY medium (1% yeast extract, 2% tryptone, 1.34% YNB, 1% glycerol, 4×10^{-5}% biotin, and 100 mM potassium phosphate buffer, pH 6.0), until the culture reached an OD_{600} = 3–6(log-phase growth). Thereafter these cells were harvested by centrifugation at 1800 g for 6 min at room temperature. To induce protein expression, the pellet was resuspended in 100–400 ml BMMY medium (1% yeast extract, 2% tryptone, 1.34% YNB, 4×10^{-5}% biotin, 0.5% methanol, and 100 mM potassium phosphate buffer, pH 6.0) and grown at 28 °C with shaking at 230 rpm. A final concentration of 0.5% methanol was added for protein expression at 24 h interval. The recombinant yeast was induced for 96 h, and 0.45 ml supernatant was precipitated by trichloroacetic acid. The concentrated supernatant was analyzed by 15% SDS–PAGE gel and the expression efficiency of scygonadin was estimated by gel scanning the intensity of the target protein band with Bio-Rad Quantity One Software. To investigate the effect of pH on the expression of scygonadin, a clone of GS115/pPIC9K-scy was firstly cultured in BMGY medium, then resuspended in BMMY medium (1% yeast extract, 2% tryptone, 1.34% YNB, 4×10^{-5}% biotin, 0.5% methanol, and 100 mM potassium phosphate buffer, pH ranging from 4.0 to 8.0) with the same amount of cells and induced for 48 h. The effect of methanol concentration on the production of recombinant scygonadin was also tested by inducing the cells with various initial methanol concentrations (0.5%, 1%, 2% and 3%) for 48 h.

Preparation of recombinant scygonadin from the supernatant and purification of secreted scygonadin

After induction under optimized condition, the cultured medium was harvested by centrifugation at 10,000 g for 40 min and 500 ml supernatant was dialyzed in a 50 mM sodium phosphate buffer (50 mM PBS, 50 mM NaCl, pH 8.5) at 4 °C. After 24–36 h dialysis, the supernatant containing the secreted component of scygonadin was collected by centrifugation at 15,000 g for 40 min at 4 °C.

The collected supernatant was filtered with a 0.45 μm filter membrane and then loaded on a HisTrap FF crude column (GE Healthcare Life Sciences) equilibrated with binding buffer (20 mM PBS, 50 mM NaCl, and 10 mM imidazole, pH 8.2). The column was washed with binding buffer and then eluted with a gradient of imidazole formed by binding buffer and elution buffer (20 mM PBS, 500 mM NaCl, and 1 M imidazole, pH 8.2). The eluted fractions were collected and dialyzed twice against 20 mM sodium phosphate buffer (20 mM PBS, 20 mM NaCl, pH 8.2), and finally dialyzed in milli-Q water for 36 h at 4 °C. The purified recombinant scygonadin was analyzed by 15% SDS–PAGE gel. The protein concentration was determined as described by Bradford [20].

Characterization of the recombinant scygonadin with Matrix assisted laser desorption ionization-time of flight mass spectrometry

The recombinant scygonadin was excised from the 15% SDS–PAGE gel and washed twice with milli-Q water. Gel was destained with a solution of 50 mM ammonium bicarbonate (NH_4HCO_3) and 100% acetonitrile (ACN) (1:1) and then dehydrated in 100% acetonitrile. Thereafter, the sample was swollen in 2–4 μL trypsin protease digestion solution (25 mM ammonium bicarbonate and 10 ng/μL trypsin). After 0.5–2 h incubation at 4 °C, the gel was incubated at 37 °C for 12–15 h in 25 mM ammonium bicarbonate. After that, the trypsin protease digested scygonadin was analyzed using a MALDI mass spectrometer (ABI, USA) as described previously [21,22].

Antimicrobial and bactericidal assays

The antimicrobial activity of the recombinant scygonadin was determined against a panel of microorganisms, including Gram-positive and Gram-negative bacteria, yeast and filamentous fungi. Antibacterial assays were performed with eleven strains of bacteria including Gram-positive *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Micrococcus leteus*, *Corynebacterium glutamicum*, *Bacillus subtilis* and *Bacillus cereus*, and Gram-negative *E. coli*, *Aeromonas hydrophila*, *Pseudomonas fluorescens*, *Shigella flexneri*, *Vibrio harveyi*. The antibacterial activities of the recombinant scygonadin were tested at pH 7.4. The bacteria were cultured on Mueller–Hinton broth medium or Difco marine broth.

The minimal inhibitory concentration (MIC) for liquid growth inhibition assay and minimal bactericidal concentration (MBC) were performed by the liquid growth inhibitory or bactericidal

assays as previously described [15,23]. Briefly, bacteria were diluted in 10 mM NaPB to A_{600} = 0.003(3–6 × 10^5 CFU/mL). The assay mixture consisted of 50 μL diluted purified peptide, 30 μL diluted bacteria suspension and 20 μL culture media. After 24 or 48 h of incubation at 28 °C, MIC was calculated as the lowest peptide concentration yielding no detectable growth. This assay was performed in triplicate. The aliquots of the cultures were then plated on Nutrient Broth agar, and MBC was the concentration which killed more than 99.9% microorganisms after overnight incubation at either 37 °C or 28 °C.

Yeast was grown in YPG medium (1% yeast extract, 1% peptone, 2% glucose). Filamentous fungi was grown in 1/2 Potato Dextrose Broth [23]. Briefly, the assay mixture contained 50 μL diluted purified peptide, 30 μL diluted yeast (~10^3 CFU/well) or fungi suspension (~10^3 spores/well) and 20 μL its corresponding broth. The antimicrobial activities were evaluated after 48 h of incubation at 28 °C, by assessment of visible turbidity in each well in the plate. Each assay was performed in triplicate.

Kill-curve studies

Bactericidal effect was performed using an important aquatic pathogen, *A. hydrophila*. Recombinant scygonadin (50 μM, 1 × MBC) was incubated with *A. hydrophila* as described above [15,23]. At each time point of incubation, 6 μL was taken from the mixture, serially diluted in 10 mM NaPB and plated on nutrition broth agar. Plates were incubated at 37 °C for 24–36 h. Meanwhile, an equivalent volume of milli-Q water without recombinant scygonadin was added to the control tube. The percentage of CFU was defined relative to the CFU obtained in the control (100% cfu at 0 h).

Salt-sensitivity assays

When the effect of sodium chloride on recombinant scygonadin antimicrobial activity was studied, the MIC was determined against *A. hydrophila* in Mueller–Hinton broth with no salt or with varying concentration of NaCl. *A. hydrophila* was incubated with the recombinant scygonadin or water as described above, while supplemented with different final concentration of NaCl (0–600 mM).

Preparation of crayfish Hpt cell cultures

The crayfish Hpt cells were obtained from the healthy red claw crayfish, *C. quadricarinatus*, as previously described [24,25]. Briefly, the haematopoietic tissue was dissected from the dorsal side of the stomach and then washed with a crayfish phosphate buffer saline (CPBS: 10 mM Na_2HPO_4; 10 mM KH_2PO_4; 150 mM NaCl; 10 μM $CaCl_2$ and 10 μM $MnCl_2$; pH 6.8) and then incubated in 0.1% collagenase (type I and IV) (Sigma, Germany) in CPBS at room temperature for 45 min to dissociate the Hpt cells. Subsequently the Hpt cells were washed twice with CPBS by centrifugation at 2500g for 5 min at room temperature. The cell pellet was then resuspended in modified L-15m81 medium [24,25] and seeded at a density of 2 × 10^5 cells/500 μL at each well in a 24-well plate. After 30 min, Hpt cells were supplemented with a 2–5 μL crude astakine preparation in each well [25]. Subsequently the Hpt cells were grown at 20 °C overnight.

Inhibition of WSSV replication by recombinant scygonadin in crayfish Hpt cell cultures

To examine the effect of scygonadin on WSSV replication, 100 μL of the purified scygonadin at a final concentration of 25 μM or 50 μM was mixed with 100 μL of 2 × 10^6 copy of WSSV for 30 min at 25 °C. For viral infection, 100 μL of WSSV pre-incubated with one of the concentrations of purified scygonadin were added to the one-day-old Hpt cell culture and incubated at 25 °C for 40 min for WSSV attachment [25]. Thereafter the medium was replaced with a new 500 μL of L-15m81 medium together with 2–5 μL of crude astakine preparation and incubated for another 3 h at 25 °C followed by total RNA extraction.

Total RNA was extracted using RNAprep pure Micro Kit (Tiangen, Beijing, China) with DNase I digestion, and cDNA was synthesized with PrimeScript™ RT reagent Kit (Takara). To determine the WSSV replication, IE1 (forward 5′- GGTATTGAGGTGATGAAGAGGCG -3′; reverse 5′- TGACATGGGAACCACT GTTGAG -3′) was amplified as previously described [25]. The red claw crayfish house keeping gene, 18S rRNA (forward 5′- GCTCTCGATTGTCGGTTATGC -3′; reverse 5′-TAATTTGCGTGCCTGCTGC -3′) was used as an internal control. 1 μL of cDNA reaction was used for PCR amplified using HS™ Kit (Dongsheng Biotech, China) with the following conditions: 94 °C for 5 min; 28 cycles for 18S rRNA gene, 35 cycles for IE1 with 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s; with a final extension for 7 min at 72 °C. The PCR products were then analyzed on 2% agarose gel electrophoresis.

Results

Construction and transformation of pPIC9K-scy into P. pastoris

As shown in Fig. 1, the scygonadin mature peptide sequence (GenBank Accession No. AY864802) was amplified by PCR, and a 306 bp PCR product encoding 102 amino acids was cloned into the pPIC9K expression vector together with the secretion signal of α-mating factor peptide at the N-terminus and a 6× His-tag at the C-terminus of the scygonadin peptide. Results of the DNA sequencing confirmed that the recombinant pPIC9K-scy plasmid was constructed correctly.

The constructed plasmid of pPIC9K-scy was linearized with SalI and transformed into the genome of *P. pastoris* GS115 cells by homologous recombination. And transformants were selected on MD plates after incubation at 30 °C for 2–3 days. PCR amplification of genomic DNA confirmed the scygonadin gene integrated into the transformants and a transformant with the pPIC9K vector was used as a negative control (data not shown).

Optimization of recombinant scygonadin expression in P. pastoris

The clone of GS115/pPIC9K-scy was induced by 0.5% methanol at pH 6.0 for 96 h. A single protein band around 11 kDa was observed at 6 h and its production increased during the subsequent induction till 96 h. The molecular weight of this peptide was consistent with the size of native scygonadin [12], while the same band was absent in the culture supernatant after GS115/pPIC9K induction (Fig. 2). As shown in Fig. 3, scygonadin (4.1 mg/L) was detected after 6 h induction and its production increased to the maximum (89.2 mg/L) after 96 h induction. The study of induction time on the expression of scygonadin from GS115/pPIC9K-scy showed that the highest expression was achieved at 96 h. But as shown in Fig. 3, the expression concentration of recombinant scygonadin did not show significant difference between 48 h (2 days), 72 h (3 days) and 96 h (4 days) induction. Considering the time and energy consumption, 48 h (2 days) was selected as the optimal

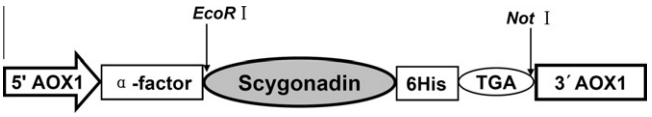


Fig. 1. Schematic representation of the vector of pPIC9K-scy.

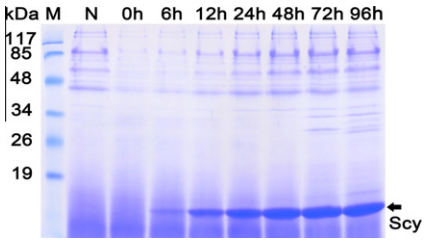


Fig. 2. SDS–PAGE analysis of the effect of induction time on the expression of scygonadin (Scy) induced with.0.5% methanol at initial pH 6.0. Samples were taken from cultures of GS115/pPIC9K-scy at 0, 6, 12, 24, 48, 72 and 96 h of induction, respectively. The supernatant (0.45 mL) was concentrated and separated by 15% (w/v) SDS–PAGE gel and stained with Coomassie Brilliant Blue R-250. Lane M, molecular weight marker (Fermentas); Lane N, Sample was taken from culture of GS115/pPIC9K at 24 h as negative control.

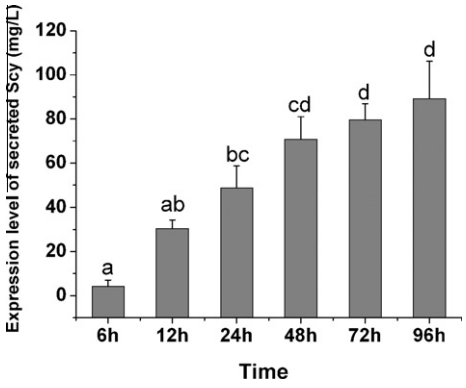


Fig. 3. The efficiency of the recombinant scygonadin (Scy) expressed in different induction time. Each histograph represents the average of three independent experiments, and the vertical bars represent the mean ± S.D. and the data were analyzed by one way ANOVA followed by Tukey post hoc test. The same letters (a, b, c, d) indicate no significant difference between different time points and different letters indicate statistically significant differences ($p \leqslant 0.05$) between induction time points.

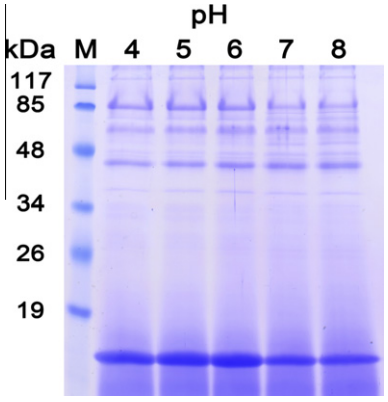


Fig. 4. SDS–PAGE analysis of the effect of initial pH on the expreesion of the recombinant scygonadin induced with 0.5% methanol for 48 h.

induction time of scygonadin expression. The induction of scygonadin expression at different initial pH displayed that the highest protein expression occurred at initial pH of 6.0 (Fig. 4). And the effect of different methanol on the expression of scygonadin was not obvious (Fig. 5).

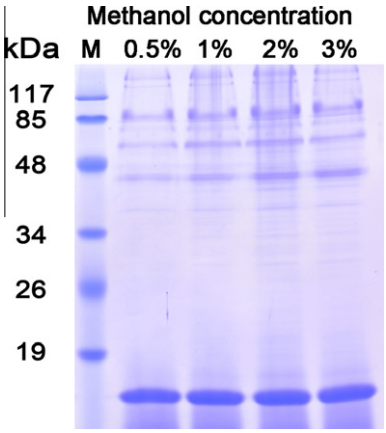


Fig. 5. SDS–PAGE analysis of the effect of methanol concentration on the expression of the recombinant scygonadin induced for 48 h at initial pH 6.0.

As a result, the optimal condition for scygonadin expression was achieved as incubation for 48 h with 0.5% methanol at 28 °C, pH 6.0.

Purification of scygonadin by IMAC and MS-fingerprint of the recombinant scygonadin

After 48 h induction with 0.5% methanol at pH 6.0, the cell culture medium (500 ml) was harvested and dialyzed in a 50 mM sodium phosphate buffer. With the 6× His-tag at the C-terminus of scygonadin, scygonadin was easily purified through a HisTrap FF crude column. Unspecific binding proteins were washed with 10 mM imidazole of the binding buffer (Fig. 6, lane 3) and a little quantity of scygonadin and unspecific proteins were then washed with 30 mM imidazole (Fig. 6, lane 4). Thereafter pure scygonadin was eluted with 300 mM imidazole (Fig. 6, lanes 5–6), which appeared as a single band of 11 kDa. The eluted fractions were collected and finally dialyzed in milli-Q water before antimicrobial activity was tested. Overall, approximate 23 mg pure recombinant scygonadin were obtained from 500 ml of cell culture medium and 97% purity was achieved by wash and elution with a gradient of imidazole (Fig. 6).

The 11 kDa recombinant scygonadin was further confirmed by MS-fingerprinting. As shown in Fig. 7, similarity search in protein databases (BLAST) revealed that the protein was found match the sequence of scygonadin (GenBank Accession No. 74836523).

Antimicrobial and bactericidal assays

The antimicrobial and bactericidal activities of the recombinant scygonadin were determined against a panel of microorganisms using MIC and MBC. Compared to the activity of scygaondin expressed in *E. coli* [15], the purified recombinant scygonadin expressed in *P. pastoris* showed similar antimicrobial spectrum. Briefly, they all showed a potent activity against bacteria (MIC 6.25–50 μM) but no activity against yeast and fungi tested (MIC > 50 μM) (Table 1). But the *P. pastoris*-derived scygonadin displayed more active against bacteria than *E. coli*-derived scygonadin. In detail, scygonadin expressed in *P. pastoris* showed similar antimicrobial activities as scygonadin expressed in *E. coli* for most of Gram-positive bacteria, such as *M. luteus* (MIC 6.25–12.5 μM), *S. aureus* (MIC 6.25–12.5 μM), *C. glutamicum* (MIC 12.5–25 μM), but more active against *B. subtilis* (MIC 25–50 μM). In addition, the recombinant scygonadin expressed in *P. pastoris* was more efficient in inhibiting growth of most of Gram-negative bacteria such as *A. hydrophila*, *P. fluorescens* and *S. flexneri* (Table 1).

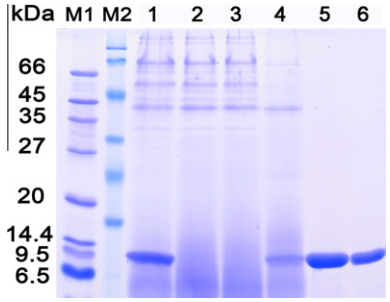


Fig. 6. SDS–PAGE analysis of the purified scygonadin by Ni^{2+} affinity chromatography. Lane M1, low range protein marker (Bio Basic Inc.); Lane M2, molecular weight marker (Fermentas); Lane 1, samples from supernatants; Lane 2, flow-through fractions; Lane 3, wash fractions with 10 mM imidazole; Lane 4, wash fractions with 30 mM imidazole; Lanes 5–6, elution fractions with 300 mM imidazole; Lanes 1–4, samples from supernatants (0.45 mL), flow-through (0.45 mL), and wash (0.45 mL) were all concentrated and separated by 15% (w/v) SDS–PAGE gel and stained with Coomassie Brilliant Blue R-250.

Kinetics of killing of A. hydrophila by the recombinant P. pastoris-derived scygonadin

As shown in Table 1, *P. pastoris*-derived scygonadin had relatively higher activity against *A. hydrophila* (MIC 12.5–25 μM) than *E. coli*-derived scygonadin (MIC 30–60 μM). Moreover, in the kinetic study, *A. hydrophila* was used to evaluate bactericidal or bacteriostatic activity of scygonadin. Over 50% *A. hydrophila* were killed after 4 h at a concentration 50 μM (1 × MBC) of scygonadin, and approximately 90% *A. hydrophila* were killed at 8 h by incubation (Fig. 8). Thus the killing of the *A. hydrophila* by the recombinant scygonadin showed time dependent.

Salt-sensitivity assays

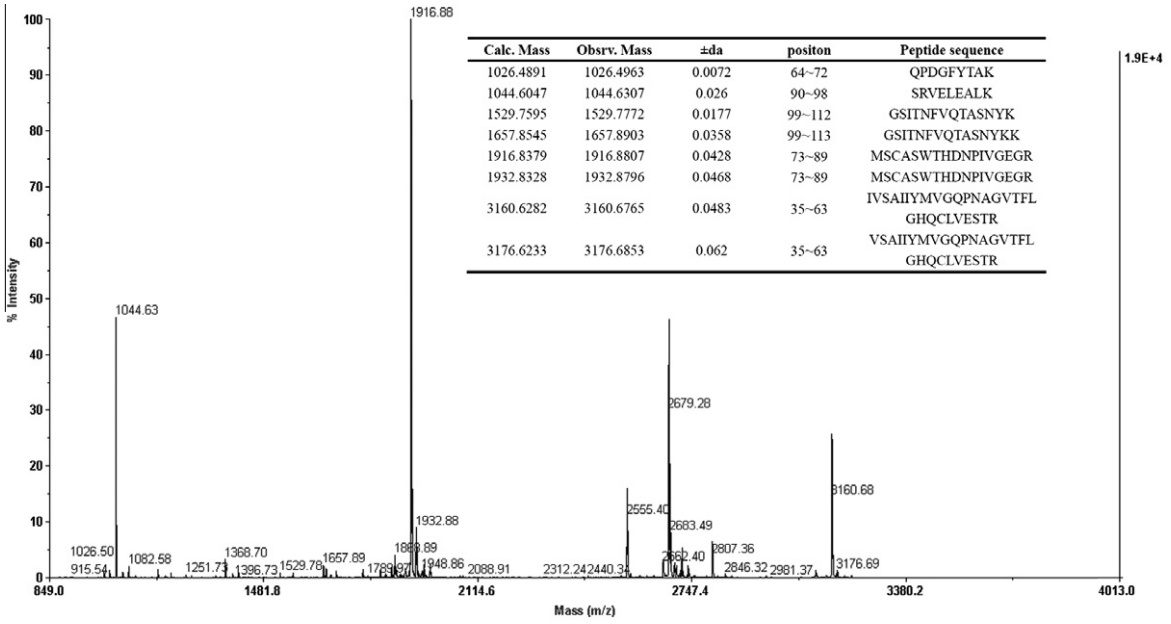
To understand if the high salinity might modify the antimicrobial activity of recombinant scygonadin, the MIC of scygonadin against *A. hydrophila* was detected at different concentrations of NaCl ranging from 50 to 600 mM. And the result showed that scygonadin retained its antimicrobial activity against *A. hydrophila* at elevated concentrations of salt up to 200 mM (Table 2).

Inhibition of WSSV replication by scygonadin in crayfish Hpt cell cultures

The Hpt cell from the red claw crayfish, *C. quadricarinatus*, has been proved to be susceptible to WSSV infection [25]. In this study, the Hpt cells were firstly dissociated and cultured with the viability over 93% by trypan blue exclusion assay (Fig. S1). The inhibition effect of scygonadin on WSSV replication was then examined in the Hpt cells. WSSV pre-incubated with recombinant scyognadin or milli-Q was added to the cell cultures for viral attachment for 40 min. After 3 h incubation, the transcripts of IE1, an immediate early gene of WSSV, were detected by RT-PCR to determine the level of WSSV replication as previously described [25]. As shown in Fig. 9, high expression of IE1 was observed in cells incubated with WSSV alone. Compared with the positive control, a significant reduction of IE1 transcripts was detected when the Hpt cells incubated with WSSV pretreated with scygonadin (50 μM). A lower reduction of IE1 transcripts was observed when the Hpt cells incubated with WSSV pretreated with less scygonadin (25 μM). This result revealed that scygonadin interfered with the WSSV replication in crayfish Hpt cells (Fig. 9).

Discussion

P. pastoris has been used as an excellent host for the expression of various kinds of heterologous proteins including some AMPs [26,27]. Previous studies have indicated that methanol concentration, pH value, and induction time are critical for the production of recombinant protein in *P. pastoris* [28–30]. In this study the optimal conditions for the secreted expression of scygonadin was determined, and it was found the pH value and induction time were the critical influencing parameters for the production of the recombinant scygonadin. The relative high quantity of recombinant scygonadin was obtained at the optimal condition of incubation for 48 h at pH 6.0 and the yield of scygonadin reached 70 mg/L. Additionally, under the optimal condition



Calc. Mass	Obsrv. Mass	±da	positon	Peptide sequence
1026.4891	1026.4963	0.0072	64~72	QPDGFYTAK
1044.6047	1044.6307	0.026	90~98	SRVELEALK
1529.7595	1529.7772	0.0177	99~112	GSITNFVQTASNYK
1657.8545	1657.8903	0.0358	99~113	GSITNFVQTASNYKK
1916.8379	1916.8807	0.0428	73~89	MSCASWTHDNPIVGEGR
1932.8328	1932.8796	0.0468	73~89	MSCASWTHDNPIVGEGR
3160.6282	3160.6765	0.0483	35~63	IVSAIIYMVGQPNAGVTFL GHQCLVESTR
3176.6233	3176.6853	0.062	35~63	VSAIIYMVGQPNAGVTFL GHQCLVESTR

Fig. 7. MS-fingerprint of scygonadin. The mass of each peptide fragment is determined by MALDI-TOF–TOF MS and displayed in the graph. In the table, ±da means the difference between the mass observed and the predicted mass, position represents the corresponding position matching to the predict scygonadin protein sequence (GenBank Accession No. 74836523).

Table 1
Antibacterial and bactericidal activities of the recombinant scygonadin expressed in *P. pastoris*.

Microorganisms	CGMCC No.[c]	*P. pastoris*-derived scygonadin		*E. coli*-derived scygonadin
		MIC[a] (μM)	MBC[b] (μM)	MIC (μM)
Gram-negative bacteria				
Aeromonas hydrophila	1.2017	12.5–25	25–50	30–60
Pseudomonas fluorescens	1.0032	12.5–25	25–50	>60
Shigella flexneri	1.1868	25–50	25–50	>60
Escherichia coli	1.2389	>50	>50	>60
Vibrio harveyi	1.1593	>50	>50	>60
Gram-positive bacteria				
Micrococcus leteus	1.634	6.25–12.5	6.25–12.5	7.5–15
Staphylococcus aureus	1.363	6.25–12.5	6.25–12.5	7.5–15
Corynebacterium glutamicum	1.1886	12.5–25	12.5–25	15–30
Bacillus subtilis	1.108	25–50	>50	>60
Bacillus cereus	1.447	>50	>50	>60
Staphylococcus epidermidis	1.2429	>50	>50	>60
Filamentous fungi				
Aspergillus niger	3.316	>50	>50	>60
Fusarium solani	3.5840	>50	>50	>60
Yeast				
Candida albicans	2.2411	>50	>50	>60
Pichia pastoris GS115	Invitrogen[d]	>50	>50	>60

The values of MIC and MBC were expressed as the interval of concentration [a] − [b], where [a] is the highest concentration tested at which microbial growth can be observed, and [b] is the lowest concentration yielding no detectable microbial growth or that killed more than 99.9% microorganisms ($n = 3$).
[a] MIC: minimal inhibitory concentration.
[b] MBC: minimal bactericidal concentration.
[c] CGMCC No. means China General Microbiological Culture Collection Center.
[d] *Pichia pastoris* GS115 was purchased from Invitrogen.

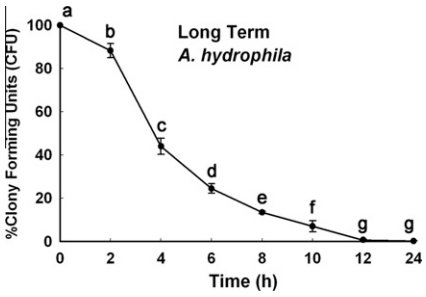


Fig. 8. Kinetics of killing *A. hydrophila* by the recombinant *P. pastoris*-derived scygonadin. The recombinant scygonadin at 1 × MBC (50 μM) or milli-Q water (control) was added to a log phase culture of *A. hydrophila*. The percentage of CFU was defined relative to the CFU obtained in the control (100% at 0 min). Each point represents the average of three independent experiments, and the vertical bars represent the mean ± S.D. and they were analyzed by one way ANOVA followed by Tukey post hoc test. The same letters (a, b, c, d, e, f, g) indicate no significant difference between different time points and different letters indicate statistically significant differences ($p \leqslant 0.05$) between time points.

Table 2
Effect of sodium chloride on scygonadin activity against *A. hydrophila*.

Final NaCl concentration	MIC (μM)
0 mM	12.5–25
50 mM	12.5–25
100 mM	12.5–25
200 mM	25–50
300 mM	>50
600 mM	>50

MIC was determined with varying concentrations of sodium chloride (0–600 mM). The results of two independent experiments are shown.

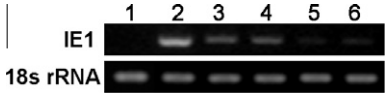


Fig. 9. Antiviral effect of scygonadin on WSSV replication in vitro-cultured crayfish Hpt cells. Lane 1, Hpt cells without WSSV infection as negative control; Lane 2, WSSV were incubated with sterilized water for 30 min before infection of Hpt cells as positive control; Lanes 3–4, WSSV were incubated with scygonadin (25 μM) for 30 min before infection of Hpt cells; Lanes 5–6, WSSV were incubated with scygonadin (50 μM) for 30 min before infection of Hpt cells. After 3 h incubation, the mRNA transcripts of IE1 were detected by RT-PCR, and 18S rRNA was used as an internal control.

relatively less amount of host own proteins secreted from *P. pastoris* and scygonadin possessed 65% of the total secreted proteins (Fig. 2), which facilitated the purification of scygonadin. Thus compared to the purification of scygonadin expressed in *E. coli* [15], a higher level of 97% purity of *P. pastoris*-derived scygonadin was achieved using one-step nickel affinity chromatography with a gradient of imidazole elution (Fig. 6).

Similar to *E. coli*-derived scygonadin [15], the *P. pastoris*-derived scygonadin exhibited obvious antimicrobial activity against both Gram-positive and Gram-negative bacteria and still no obvious activity against yeast and fungi tested. Meanwhile, the activity of *P. pastoris*-derived scygonadin against *M. leteus* was consistent with that of the native scygonadin [12]. It has been reported that some recombinant AMPs produced in *P. pastoris* have relatively stronger activity than those produced in *E. coli*, such as plant defensin corn 1 (PDC1) [31], human secretory leukocyte protease inhibitor (SLPI) [32], and non-specific lipid-transfer proteins (nsLTP) [33]. Similarly, *P. pastoris*-derived scygonadin showed relatively higher activity against bacteria than *E. coli*-derived scygonadin. Importantly, scygonadin expressed in *P. pastoris* had higher activity against *A. hydrophila* (MIC 12.5–25 μM) and *P. fluorescens* (MIC 12.5–25 μM), which are those of the important pathogens to aquatic animals. The bactericidal activity of scygonadin against bacteria, especially to these

two aquatic pathogens, suggested that scygonadin could be an efficient bacteriostatic and bactericidal agent. These results implied that *P. pastoris*-derived scygonadin is biologically effective and might fold properly by the post-translational modification while expressed in the *P. pastoris* expression system.

Besides, we demonstrated that the recombinant scygonadin could interfere with the WSSV replication in vitro-cultured crayfish Hpt cells. WSSV has been known as one of the most serious pathogens in cultured shrimps, and often causes up to 100% mortality within 3–10 days after infection. This virus has a broad host range including shrimps, lobsters, crabs and crayfish [34,35]. Several antimicrobial peptides in crustaceans are reported to have antiviral activity against WSSV, such as penaeidin, crustin and ALF [6,36–38]. In this study, scygonadin was also found to have the capacity of inhibiting WSSV replication in vitro. However, the effect of scygonadin against WSSV infection of *S. paramamosain* in vivo and the antiviral mechanism of scygonadin needs further investigation.

In summary, our study indicated that the antimicrobial peptide scygonadin can be effectively expressed in *P. pastoris*. The antimicrobial and antiviral activities suggested that scygonadin might play a role in the crab immune defense against pathogenic bacteria and viruses, thus scygonadin could be as a promising antibiotic candidate commercially used in aquaculture. In addition, due to the efficient expression and desirable antimicrobial activities of the recombinant scygonadin, GS115/pPIC9K-scy is thought suitable for scygonadin production and the *P. pastoris* expression system can be applied for a large-scale production of scygonadin in future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.pep.2011.11.008.

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