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Soluble expression and purification of a crab antimicrobial peptide scygonadin in different expression plasmids and analysis of its antimicrobial activity

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ABSTRACT

Scygonadin is an anionic antimicrobial peptide recently identified from the seminal plasma of *Scylla serrata*. To gain more detailed information on its antimicrobial activity, scygonadin mature peptide was expressed in *Escherichia coli* in order to obtain a large quantity of biologically active product. An approximately 43 kDa fusion protein CKS-scygonadin was obtained in a highly stable and soluble form. The soluble component of the fusion CKS-scygonadin was purified by immobilized metal affinity chromatography (IMAC). A single 11 kDa recombinant scygonadin was cleaved from CKS-scygonadin and purified from the cleavage mixture using an affinity chromatography column with a yield of 10.6 mg/L. Alternatively, a recombinant scygonadin was purified from pET28-scygonadin by one-step Ni^{2+} affinity chromatography and 65.9 mg/L pure recombinant scygonadin was obtained which was higher than that purified from pTrc-CKS/scygonadin in bacteria culture. The recombinant scygonadin was confirmed using SDS–PAGE analysis and MS-fingerprinting. Both recombinant products of scygonadin from different expressed plasmids showed the activity against both Gram-positive and Gram-negative bacteria, but no activity against yeast and fungi tested. The kinetic studies showed that the recombinant scygonadin was strong active against *Staphylococcus aureus* and the killing of *S. aureus* appeared time and dose dependent. Considering the quantity of recombinant product and the applicability of purification, the pET28-scygonadin expression system is a better choice to produce large quantities of recombinant scygonadin for commercial use in future. This is the first report on the heterologous expression of antimicrobial peptide scygonadin in *E. coli*.

Introduction

Antimicrobial peptides (AMPs)[1] are the major component of the innate immune system of many organisms and play an important role in protecting the host species from microbial invasion. Like other crustaceans [1], *Scylla serrata* may rely solely on innate immune mechanism to survive in a complex marine environment. In crustaceans, there have been several antimicrobial peptides so far reported which were isolated from the shore crab *Carcinus maenas* [1,2], the blue crab *Callinectes sapidus* [3] and from the Pacific white shrimp *Litopenaeus vannamei* [4], etc. However, only a few AMPs have been identified so far from *S. serrata*, including scyllin from hemolymph [5], scygonadin [6,7] and a 20-kDa protein from the seminal plasma of *S. serrata* [8], and crustin from *Scylla paramamosain* [9]. Therefore, elucidation of AMPs or their homologs will provide valuable data for us in depth understanding of the mechanism of innate immunity in *S. serrata*.

Scygonadin which was originally isolated from the seminal plasma of *S. serrata*, has shown antibacterial activity against *Micrococcus leteus* and *Aeromonas hydrophila* [6] and was thought it is an anionic AMP [6,7]. Its cDNA and genomic DNA have been revealed and this peptide shows a specific expression in male crab [7]. To characterize this peptide in details, it is necessary to elucidate its antimicrobial spectrum. However, it was difficult to obtain sufficient amounts of scygonadin for determination of its antimicrobial spectrum and for study of its other potential biological functions using our previous purification procedure of this native protein from *S. serrata* [6]. Thus, heterologous production of scygonadin is required.

It is reported that some antimicrobial peptides have been expressed in prokaryocyte or eukaryocyte systems and recombinant products were obtained for examining their antimicrobial activity, such as penaeidin in *Saccharomyces cerevisiae* [10], a scallop big defensin in *Pichia pastoris* [11], a human hepcidin in *Escherichia coli* [12], and an insect cecropin in *E. coli* [13]. Due to the ease of transformation, cloning and high cell density cultivation, *E. coli* is by far the most widely used microorganism for the production of recom-

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[1] *Abbreviations used:* AMPs, antimicrobial peptides; IMAC, immobilized metal affinity chromatography; IPTG, isopropyl-β-D-thiogalactopyranoside; TrxA, thioredoxin; LMM, light meromyosin; MIC, minimum inhibitory concentration.

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binant proteins and enzymes [14,15] and it is a useful tool to obtain recombinant protein through a prokaryotic expression system [16]. Recombinant AMPs can be used to analyze antimicrobial activity against bacteria and probably no difference in antibacterial activity was observed among the recombinant and natively purified AMPs. This was confirmed in the previous study of shrimp panaeidin [10].

This study aims to express the mature peptide of scygonadin in *E. coli*, establish a high prokaryotic expression system to obtain large quantities of the recombinant scygonadin, and also examine the antimicrobial activity of scygonadin targeting pathogenic organisms.

Materials and methods

Plasmid construction

A 306 bp sequence coding the mature peptide of scygonadin was inserted into pTrc-CKS (Gene Power Lab Ltd.) through the BglII and XbaI sites. The expression vector pTrc-CKS carries a recombinant 3C protease recognition site: Leu Glu Val Leu Phe Gln↓Gly Pro, and a His-tag at the C-terminus. The resulting pTrc-CKS/scygonadin was confirmed by PCR and DNA sequencing.

The inserted interest encoding the mature peptide of scygonadin was based on the cDNA sequence of scygonadin, which was amplified by RT-PCR [7]. The primers were based on the analysis of multiple cloning sites of the expression vector pTrc-CKS. The forward primer (5′-GCCGAGATCTGGCCAGGCACTCAACAA-3′) introduced a BglII site (underlined). The reverse primer was designed as (5′-GCGTCTAGAGTAAGAAGCAATCCAGT-3′), with an endonuclease site XbaI (underlined), corresponding to nucleotides 129–145 bp and 418–434 bp of scygonadin cDNA (GenBank Accession No. AY864802), respectively. The PCR product was digested with BglII and XbaI, and ligated into the BamHI/NheI-digested pTrc-CKS. The interest fragment inserted into pTrc-CKS included both the scygonadin mature peptide sequence and the coding sequence for some residues at the N-terminus of CKS, plus a C-terminal hexa-histidine tag. The resulting pTrc-CKS/scygonadin was verified by PCR amplification of clones and DNA sequencing.

Alternatively, another expression vector pET-28a (Novagen) was adopted for constructing a high expressed plasmid. Scygonadin mature sequence was amplified by PCR. The primers used were 5′-GCGCCATGGGCCAGGCACTCAACAA-3′ (forward primer with an endonuclease site NcoI) and 5′-GGGCTCGAGGTAAGAAGCAATCCAGT-3′ (reverse primer with an endonuclease site XhoI). The PCR products were digested by NcoI and XhoI, and then cloned into pET-28a digested by the same restriction enzymes. The expressed plasmid pET28-scygonadin was constructed with a C-terminal hexa-histidine tag and confirmed by DNA sequencing.

Expression of CKS-scygonadin and scygonadin(pET)

A single colony of *E. coli* TOP10F′ transformed with pTrc-CKS/scygonadin was inoculated into LB medium containing 120 μg/mL ampicillin, and then cultured at 37 °C until OD600 reached 0.6. A single colony of *E. coli* BL21(DE3)pLysS with pET28-scygonadin was grown in LB medium containing 50 μg/mL kanamycin and 0.5% glucose at 37 °C until OD600 reached 0.2–0.3. Both CKS-scygonadin and scygonadin(pET) were induced by adding 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to the culture. These medium were incubated at 28 °C for 3 h and shaken at 180 rpm. The cells were harvested and subsequently the suspension was lyzed using sonication (180× for 5 s, interval 10 s, 180 w) and the lysate was centrifuged at 10,000 rpm for 15 min. The supernatant was collected for analysis of fusion protein using SDS–PAGE.

Preparation of CKS-scygonadin and scygonadin(pET) from the supernatant and its absorption on Super Chelating Resin

A highly expressing soluble component of the recombinant pTrc-CKS/scygonadin or pET28-scygonadin colony, which was carefully selected by analysis of the quantity in the supernatant, was inoculated into 2–3 L of LB medium. The cells were harvested and resuspended in pre-cold PBS. Then, the suspension was cooled with ice and lyzed in an ice/water bath using sonication. The supernatant containing the soluble component of CKS-scygonadin or scygonadin(pET) was collected by centrifugation at 12,000 rpm for 15 min at 4 °C. The collected supernatant was filtered with a 0.45 μm filter membrane to be prepared for purification using IMAC.

The Purification of CKS-scygonadin using IMAC

The purification procedure was carried out using an AKTA Purifier 100 (GE Healthcare Life Sciences). The collected supernatant was filtered with a 0.45 μm filter membrane and then passed through an affinity chromatography column (GE Healthcare Life Sciences) equilibrated with binding buffer (20 mM PBS, 500 mM NaCl, and 10 mM imidazole, pH 8.0). The column was washed with binding buffer to remove contaminating proteins and the recombinant fusion proteins were eluted by 150 mM imidazole. The eluted fractions were collected and dialyzed in a 50 mM Tris–Cl buffer (50 mM Tris, 50 mM NaCl, pH 8.0). The protein concentration was determined as described by Bradford [17].

3C protease (P3C) cleavage

To release scygonadin from the fusion protein CKS-scygonadin, P3C (Novagen) was added to cleave the fusion protein in the ratio of 1 U/100 μg fusion protein in 50 mM Tris–Cl buffer. The reaction mixture was incubated at 4 °C for 24 h.

Purification of the recombinant scygonadin from the fusion protein using IMAC

The mixture proteins including CKS and scygonadin were loaded on the affinity chromatography column equilibrated with binding buffer (20 mM PBS, 500 mM NaCl, and 10 mM imidazole, pH 8.0). The unbound proteins were washed by 10 mM imidazole and 60 mM imidazole. The recombinant scygonadin was eluted by 250 mM imidazole. The eluted fractions were collected and finally dialyzed in a 20 mM Tris–Cl buffer (20 mM Tris, 20 mM NaCl, pH 8.0) before antimicrobial activity was tested.

The purification of scygonadin(pET) using IMAC

Recombinant scygonadin(pET) was purified using the procedure similar to the second purification for the recombinant CKS-scygonadin. The unbound proteins were washed by 10 mM imidazole and 60 mM imidazole. And finally dialyzed in a 20 mM Tris–Cl buffer (20 mM Tris, 20 mM NaCl, pH 8.0) before antimicrobial activity was tested.

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

Analysis of the recombinant scygonadin was carried out on a Bruker (Bremen) Biflex MALDI-TOF mass spectrometer as described previously [6].

Antimicrobial assays

Antibacterial assays were performed with ten strains of bacteria including Gram-positive *Staphylococcus aureus*, *Staphylococcus epidermidis*, *M. leteus*, *Corynebacterium glutamicum*, *Bacillus* subtilis and *Bacillus cereus*, and Gram-negative *Escherichia coli*, *A. hydrophila*, *Vibrio harveyi*, *Vibrio alginolyticus*. The antibacterial activities of the recombinant scygonadin were tested at pH 7.4. The bacteria were cultured on Mueller–Hinton broth medium and Difco marine broth was used. Determination of minimum inhibitory concentration (MIC) values was performed by the liquid growth inhibition assays as previously described [18,19]. Briefly, bacteria were diluted in 10 mM NaPB to A600 = 0.003(3–6 $\times$ 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 h of incubation at 28 °C, MIC was calculated as the lowest peptide concentration yielding no detectable growth. This assay was performed in triplicate.

Yeast was grown in YPG medium (yeast extract 1%, peptone 1%, glucose 2%). Filamentous fungi was grown in 1/2 Potato Dextrose Broth [19]. Briefly, the assay mixture contained 50 μL diluted purified peptide, 30 μL diluted yeast ($\sim10^3$ CFU/well) or fungi suspension ($\sim10^3$ 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 or bacteriostatic effect was performed using *S. aureus* as described previously [19,20]. Recombinant scygonadin(pET) (30 or 60 μM, 2× MIC or 4× MIC) was incubated with *S. aureus* as described above. 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 h. Meanwhile, an equivalent volume of 20 mM Tris–Cl buffer 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 min).

Results

Construction of recombinant expression vectors of pTrc-CKS/scygonadin and pET28-scygonadin

Scygonadin protein consists of a putative NH_2-terminal signal sequence (1–24) and a mature peptide (25–126) [7]. The scygonadin mature peptide sequence was prepared by PCR, and the 306 bp PCR product coding 102-aa peptide was cloned according to the scygonadin cDNA sequence (GenBank Accession No. AY864802). Considering a possibility of high toxicity of antimicrobial peptide against host cells as it is expressed in *E. coli* with soluble form, fusion partners are normally used such as intein [21,22], light meromyosin (LMM) [23], and thioredoxin (TrxA) [13,24]. In this study an expression vector pTrc-CKS was adopted, in which CKS as a fusion protein is a soluble partner (CMP-3-deoxy-D-manno-octulosonate cytidylyltransferase) at the upstream and a 6× His-tag as a purification utility at the downstream (Fig. 1a). The mature peptide sequence encoding 102 amino acids was ligated into a pTrc-CKS expression vector to construct an expressed plasmid in which the *E. coli* TOP10F′ harboring pTrc-CKS/scygonadin chimeric plasmid that will express a 43 kDa fusion polypeptide. Another pET28-scygonadin plasmid expressing a single 11 kDa scygonadin was designed using the similar procedure (Fig. 1b).

Expression of CKS-scygonadin and scygonadin(pET)

CKS-scygonadin was induced with 0.5 mM IPTG at 28 °C for 3 h. A fusion protein CKS-scygonadin (about 43 kDa) was expressed in *E. coli* TOP10F′ and it was highly soluble as shown in Fig. 2. And approximately 89.1 mg soluble CKS-scygonadin was obtained from 1 L of bacteria culture (Table 1). Similarly, a soluble component of the recombinant scygonadin(pET) around 11 kDa polypeptide was expressed in *E. coli* BL21(DE3)pLysS as shown in Table 2 and approximately 97.5 mg soluble scygonadin(pET) was obtained form 1 L of bacteria culture.

Purification of CKS-scygonadin and scygonadin(pET) by IMAC

In order to easily obtain the soluble component of the fusion protein CKS-scygonadin, the supernatant was collected from the sonicated crude extract of fusion protein after centrifugation, and purified through a Ni^{2+}-chelating-Sepharose column (GE Healthcare Life Sciences). To collect the fusion protein conjugated with Ni^{2+} in the affinity chromatography column, a higher concentration of imidazole was selected for washing through the column in order to harvest the fusion protein elution peak. Because the collected fusion protein mixture contained a higher concentration of imidazole, which may be toxic to bacterial cells, it is necessary to remove the imidazole completely from the fusion protein collection. After dialysis, the imidazole was removed.

The fusion protein CKS-scygonadin was completely digested after cleaved by 3C protease. Two clear bands approximately 32 and 11 kDa were detected as shown in Fig. 3, corresponding to the estimated molecular weight for CKS and scygonadin, respectively. Subsequently, the recombinant scygonadin was purified using Ni^{2+}-chelating-Sepharose column. As shown in Fig. 4, CKS protein was washed in 10 mM imidazole of the loading buffer (Fig. 4 fraction A, lane 1), and a little CKS and unspecific binding proteins were then washed with 60 mM imidazole (Fig. 4 fraction B, lane 2). Pure scygonadin was eluted with 250 mM imidazole (Fig. 4 fraction C, lane 3). The eluted fractions were collected and

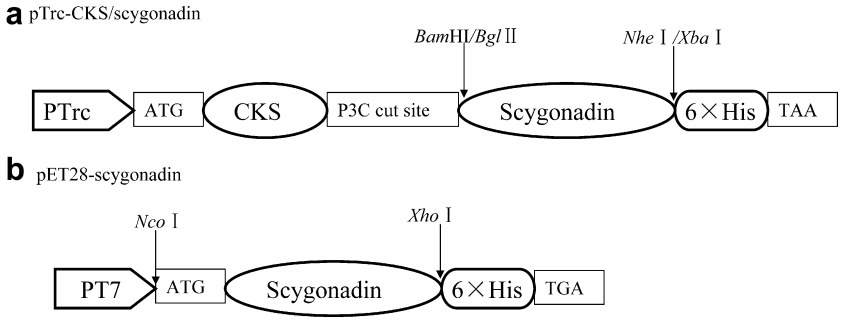


Fig. 1. Construction of the expression vector pTrc-CKS/scygonadin (a) and pET28-scygonadin (b).

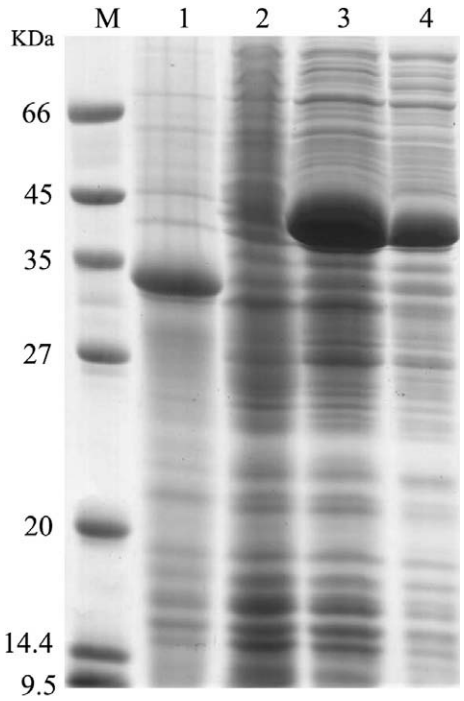


Fig. 2. SDS–PAGE analysis of CKS-scygonadin expression and the soluble product in *Escherichia coli* TOP10F′ was carried out in a 15% (w/v) separation gel. The gel was stained with Coomassie Brilliant Blue R-250. Lane M, low range protein marker (Bio Basic Inc.); lane 1, the fusion partner CKS expression induced with IPTG for 3 h; lane 2, CKS-scygonadin expression induced with IPTG for 0 h as control; lane 3, CKS-scygonadin expression induced with IPTG for 3 h; lane 4, sonicated supernatant (soluble component) of induced cells.

finally dialyzed in a 20 mM Tris–Cl buffer (20 mM NaCl, 20 mM Tris, PH 8.0) before antimicrobial activity was tested. Overall, approximately 10.6 mg pure recombinant scygonadin were obtained from the expressed fusion protein CKS-scygonadin in 1 L of bacteria culture (Table 1).

The recombinant scygonadin(pET) was purified by one-step Ni^{2+} affinity chromatography. Approximately 65.9 mg pure recombinant scygonadin were obtained, which was six times as much as the production of pure scygonadin from pTrc-CKS/scygonadin in 1 L of bacteria culture (Table 2, Fig. 5).

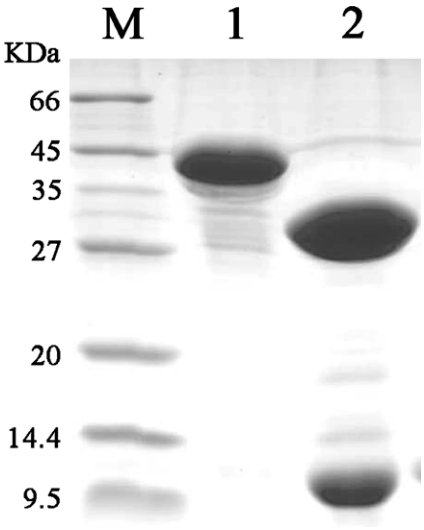


Fig. 3. SDS–PAGE analysis of 3C protease cleavage of the recombinant fusion protein CKS-scygonadin. Lane M, low range protein marker (Bio Basic Inc.); lane 1, fusion protein CKS-scygonadin after purification; lane 2, protein sample after cleavage reaction, upper band represents CKS, lower band represents the recombinant scygonadin.

MS-fingerprint of the recombinant scygonadin

The 11 kDa purified recombinant scygonadin was sent for MS-fingerprinting. The MS-fingerprinting result for scygonadin is shown in Fig. 6, which corresponds with our previous determination of this peptide [6], demonstrating that the pure recombinant protein is the expected scygonadin.

Antimicrobial assay

The antimicrobial activities of the recombinant scygonadin were determined against a panel of microorganisms using MIC. The recombinant CKS-scygonadin fusion protein showed the value of MIC over 60 μM (data not shown in table) indicating no activity against all the tested bacterial strains. The purified recombinant scygonadin from pTrc-CKS/scygonadin showed a potent activity against *A. hydrophila* (30–60 μM) and some Gram-positive bacteria (7.5–30 μM) but no activity against yeast and fungi tested

Table 1
Summary of approximately yield of total and the recombinant scygonadin from pTrc-CKS/scygonadin after purification with affinity chromatography (1 L *E. coli* culture).

Purification step	Total protein[a] (mg)	Fusion protein (mg)	Purity[b] (%)	Yield (%)
Sonicated supernatant	270	89.1	33	100
The first nickel affinity chromatography step	78.3	69.7	89	78
After dialysis and cleavage, the second affinity chromatography step	11	10.6	96	12

[a] Protein concentration was determined using Bradford's method.
[b] Protein or peptide purity was estimated by SDS gel scanning.

Table 2
Summary of approximately yield of total and the recombinant scygonadin from pET28-scygonadin after one-step purification with affinity chromatography (1 L *E. coli* culture).

Purification step	Total protein[a] (mg)	Fusion protein (mg)	Purity[b] (%)	Yield (%)
Sonicated supernatant	250	97.5	39	100
One-step affinity chromatography and after dialysis	69.4	65.9	95	68

[a] Protein concentration was determined using Bradford's method.
[b] Protein or peptide purity was estimated by SDS gel scanning.

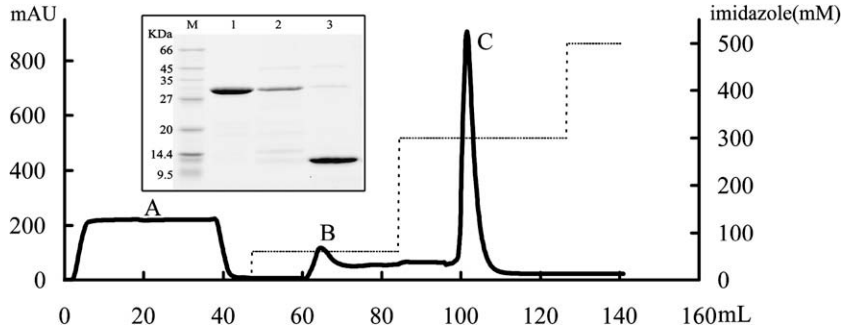


Fig. 4. Purfication of His-tagged scygonadin from the recombinant CKS-scygonadin. HPLC elution of scygonadin by Ni^{2+} affinity chromatography showing the programmed imidazole gradient and the resulting A280 nm reading and SDS–PAGE gel showing HPLC purification of scygonadin.

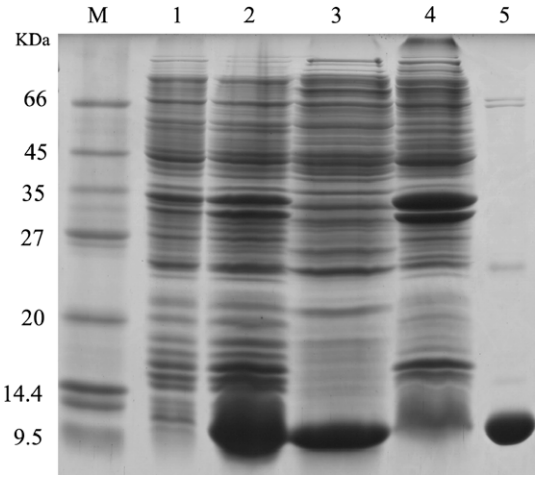
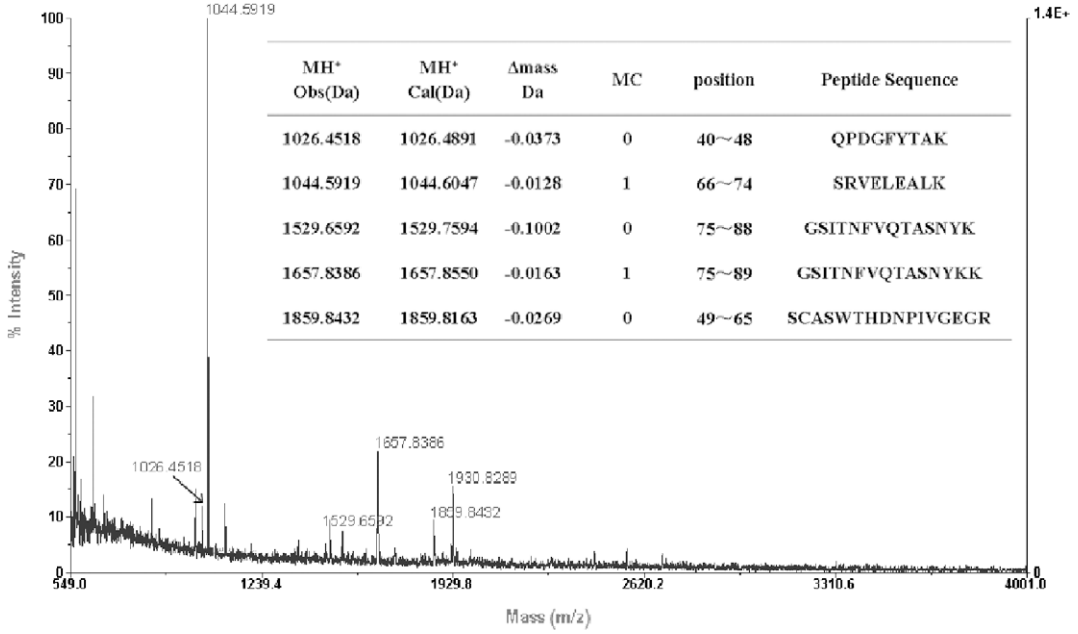


Fig. 5. SDS–PAGE analysis of the recombinant scygonadin(pET) expressed in *Escherichia coli* BL21(DE3)pLysS. The purified soluble product was carried out in a 15% (w/v) separation gel. The gel was stained with Coomassie Brilliant Blue R-250. Lane M, low range protein marker (Bio Basic Inc.); lane 1, scygonadin(pET) expression induced with IPTG for 0 h as control; lane 2, scygonadin(pET) expression induced with IPTG for 3 h; lane 3, sonicated supernatant (soluble component) of induced cells. Lane 4, product of inclusion bodies lane 5, scygonadin(pET) after purification.

(>60 μM) (Table 3). The purified recombinant scygonadin from pET28-scygonadin showed similar antimicrobial activity but more active against *S. aureus* than scygonadin from pTrc-CKS/scygonadin (Table 3). The results indicate that the antimicrobial activity of scygonadin is prone to target-specific.

Kinetics of killing of S. aureus by the recombinant scygonadin

In the kinetic study, a sensitive bacterial strain *S. aureus* was used to evaluate bactericidal or bacteriostatic activity of the purified recombinant scygonadin from the pET28-scygonadin plasmid. *S. aureus* was incubated with the recombinant scygonadin at a concentration 2–4 times over the MIC value (30–60 μM). The bactericidal activity of this peptide was assessed by plating cultures and counting CFUs after incubation at 37 °C for 24 h (Fig. 7). In the long term analysis, when *S. aureus* was incubated with the purified recombinant scygonadin at a concentration 30 μM (2× MIC) and 60 μM (4× MIC), respectively, over 97% *S. aureus* were killed after 15 min (Fig. 7). Subsequently, the killing kinetics of scygonadin was measured within a 15-min incubation period. In the short term analysis, approximately 50% *S. aureus* were killed after 3 min at a concentration 60 μM (4× MIC) and nearly 6 min at 30 μM (2× MIC). And approximately 90% *S. aureus* were killed at



MH⁺ Obs(Da)	MH⁺ Cal(Da)	Δmass Da	MC	position	Peptide Sequence
1026.4518	1026.4891	-0.0373	0	40~48	QPDGFYTAK
1044.5919	1044.6047	-0.0128	1	66~74	SRVELEALK
1529.6592	1529.7594	-0.1002	0	75~88	GSITNFVQTASNYK
1657.8386	1657.8550	-0.0163	1	75~89	GSITNFVQTASNYKK
1859.8432	1859.8163	-0.0269	0	49~65	SCASWTHDNPIVGEGR

Fig. 6. MS-fingerprint of scygonadin. The mass of each peptide fragment was determined by MALDI-TOF MS and is displayed in the graph. The matrix used in the analysis is α-cyano-4-hydroxycinnamic acid. In the table, Δ mass means the difference between the mass observed and the predicted mass, MC is the number of the missed cleavage for the peptide. Position represents the corresponding position matching to the predicted scygonadin mature peptide sequence (GenBank Accession No. Q5D710).

Table 3
Antimicrobial activity of recombinant scygonadin.

Microorganisms	CGMCC No.[c]	Scygondin from pTrc-CKS/scygonadin MIC[d] (μM)	Scygonadin from pET28-scygonadin MIC (μM)
Gram-negative bacteria			
Aeromonas hydrophila	1.2017	30[a]–60[b]	30–60
Escherichia coli	1.2389	>60	>60
Vibrio harveyi	1.1593	>60	>60
Vibrio alginolyticus	1.1833	>60	>60
Gram-positive bacteria			
Micrococcus leteus	1.634	7.5–15	7.5–15
Staphylococcus aureus	1.363	15–30	7.5–15
Corynebacterium glutamicum	1.1886	15–30	15–30
Staphylococcus epidermidis	1.2429	>60	>60
Bacillus subtilis	1.108	>60	>60
Bacillus cereus	1.447	>60	>60
Filamentous fungi			
Aspergillus niger	3.316	>60	>60
Fusarium solani	3.5840	>60	>60
Yeast			
Candida albicans	2.2411	>60	>60
Pichia pastoris GS115	Invitrogen[e]	>60	>60

MIC values are 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($n = 3$).
[c] CGMCC No. means China General Microbiological Culture Collection Center.
[d] MIC, minimal inhibitory concentration.
[e] *Pichia pastoris* GS115 was purchased from Invitrogen.

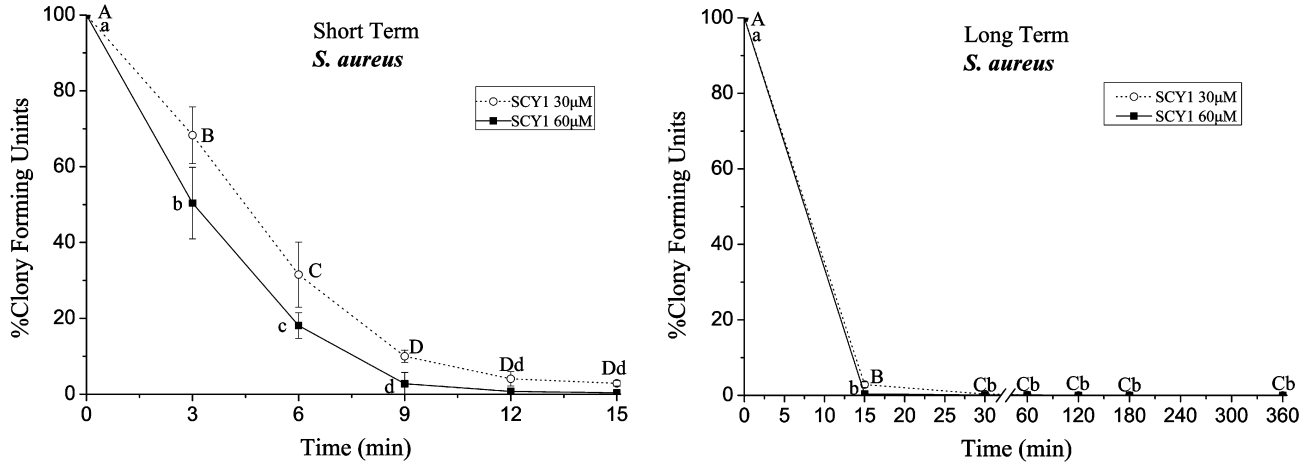


Fig. 7. Kinetics of killing *S. aureus* by recominant scygonadin. Recombinant scygonadin at 2MIC (30 μM), 4MIC (60 μM) or 20 mM Tris–Cl buffer (control) was added to a log phase culture of *S. aureus*. The percentage of CFU was defined relative to the CFU obtained in the control (100% at 0 min). Each point represents the average of three independent experiments, and the vertical bars represent the mean ± SD and they were analyzed by 2-way ANOVA followed by Tukey post hoc test. The same letters (a, b, c, d, e, f) or (A, B, C, D, E) indicate no significant difference between different time points and different letters indicate statistically significant differences ($p \leqslant 0.05$) between time points.

9 min by incubation with both 30 μM (2× MIC) and 60 μM (4× MIC) (Fig. 7). Thus the killing of the *S. aureus* by the recombinant scygonadin showed time and dose dependent.

4. Discussion

Due to the difficulty of purifying native scygonadin from the gonads of *S. serrata,* large production of this AMP in an *E. coli* system is required in order to allow further investigation of its antimicrobial spectrum and commercial use in aquaculture in future. *Escherichia coli* is a good choice as an expression host but the recombinant AMPs might be active against it. To avoid the toxicity of the recombinant scygonadin to the host cells and to obtain the soluble expressed recombinant product, an expression vector pTrc-CKS was used in this study, in which the CKS protein proved to be stable and a highly soluble partner to fuse with the target protein [25,26]. As expected, the resulting recombinant CKS-scygonadin was highly expressed in *E. coli* TOP10F′ and the soluble component was obtained as shown in Fig. 2. The result showed that the production of purified scygonadin from pTrc-CKS/scygonadin is not as high as we expected, which lead us to try to construct another expression vector pET28-scygonadin. The result showed that the recombinant scygonadin(pET) is higher soluble, most of scygonadin existed in sonicated supernatant rather than in inclusion bodies (Fig. 5). The production of the pure scygonadin from pET28-scygonadin was six times as much as that from pTrc-CKS/scygonadin, and also the purification procedure of the recombinant scygonadin from pET28-scygonadin was simpler with only one-step Ni^{2+} affinity chromatography (Tables 1 and 2) than that from pTrc-CKS/scygonadin. Thus, it would be a better choice to express scygonadin in pET28-scygonadin vector. In the study, we observed

that the soluble scygonadin(pET) would reach the highest production when the host cells were induced at OD600 0.2–0.3. If the cell density was over 0.6 at OD600, the soluble scygonadin will be obviously decreased (data not shown).

In the study, the purified recombinant scygonadin exhibited obvious antimicrobial activity against *M. leteus, S. aureus, C. glutamicum* and *A. hydrophila* (Table 3), demonstrating that this peptide possesses the characteristic of activity against both Gram-positive and Gram-negative bacteria, but no activity against yeast and fungi tested. As observed in previous studies on the activity of some AMPs, such as dermaseptins [27] or penaeidin [10], scygonadin had a specific antibacterial action which highly depended on the bacteria tested. We previously found that the native purified scygondin was active against *M. leteus* and *A. hydrophila* [6]. The purified native scygonadin (~12 μM) showed antibacterial activity (with a killing index of nearly 90%) against the Gram-positive bacterium *M. leteus* using liquid growth inhibition assays. In this study, the purified recombinant scygonadin displayed a similar activity against *M. leteus* (MIC 7.5–15 μM). The result of activity against *M. leteus* obtained from the recombinant scygonadin is consistent with our previous observation using the purified native scygonadin [6]. We presumed that the 6× His-tag at C-terminus might have not significant influence to the antimicrobial activity of the recombinant scygonadin. In view of this, expressing the recombinant scygonadin through a prokaryotic expression system would be an applicable way to be used for assessment of its antimicrobial activity.

To our knowledge, no information concerning the in vitro antimicrobial activity was derived from the sex-specific AMPs of crustaceans. The *E. coli* expression system developed in this study was shown to be appropriate for the production of large amounts of active scygonadin. Scygonadin can be highly expressed in a soluble form through the recombinant constructs of pTrc-CKS/scygonadin and pET28-scygonadin expression vectors. The recombinant product can be easily purified through IMAC. Considering the quantity of recombinant product and the applicability of purification, we suggest that the pET28-scygonadin expression system is much better for commercial use in future. This is the first report on the heterologous expression of antibacterial peptide scygonadin in *E. coli*. This recombinant scygonadin displayed antibacterial activity similar to the native one against bacteria which allowed us to perform a study of its antimicrobial activity.

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