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HETEROLOGOUS EXPRESSION OF RECOMBINANT SCYGONADIN ANTIMICROBIAL PEPTIDE FROM MUD CRAB *Scylla serrata*

NURFARHANA ROSLI[1], FARAH AYUNIE MOHD ZAIN[1], SANDRA CATHERINE ZAINATHAN[2] and SITI NOR KHADIJAH ADDIS[1*]

[1]*School of Fundamental Science, Universiti Malaysia Terengganu, Terengganu*
[2]*School of Fisheries and Aquaculture Science, Universiti Malaysia Terengganu, Terengganu*
[*]*E-mail: khadijah@umt.edu.my*

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ABSTRACT

Antimicrobial peptides (AMPs) are the most common immune effectors in invertebrates that functions as the first line of defence against microbial infection. Scygonadin is an AMP which can be found in the seminal plasma of *Scylla serrata*. Preceding studies had shown that scygonadin have the ability to exhibit wide antimicrobial activities. Nonetheless, analysis of the antimicrobial properties of scygonadin is significantly dependent on acquiring sufficient amounts of the protein from mud crab, and this was proven difficult. Further functional studies of scygonadin and its commercial applications require a development of efficient, sustainable and cost-effective heterologous protein production. To address this issue, an expression plasmid containing 387 bp of scygonadin gene of *Scylla serrata* was cloned into pBAD/Myc-His A, expressed in TOP10 cells with L-arabinose as expression inducer, followed by protein purification by using immobilized metal affinity chromatography (IMAC). The optimal expression condition was determined by incubation with 0.02% of L-arabinose for 4 hours at 37°C. A total of 2 mg/ml of purified scygonadin with the molecular weight of ~17kDa was succesfully obtained. The results demonstrated that the recombinant scygonadin was successfully produced in heterologous expression system which may allow production of scygonadin in large quantities for further research and commercial application.

Key words: Antimicrobial peptide, scygonadin, *Scylla serrata*, recombinant protein, IMAC purification

INTRODUCTION

Antimicrobial peptides (AMPs), the prominent constituents in the innate immune system are crucial in defending host species from microbial intrusion (Peng *et al*., 2010). The innate immune system pertained to the host mechanism defence against microbes (Hoebe *et al*., 2004; Iwanaga & Lee, 2005). AMPs protect the host from foreign substance via protein-protein interaction to control transport system (Chen *et al*., 2012). AMPs are also regarded as promising antibiotic candidates due to its ability to decrease the target cell's resistance with extensive range of mechanism of antibacterial actions (Hancock & Patrzykat, 2002; Pushpanathan *et al*., 2013). According to Aoki & Ueda (2013), extensive range of AMPs antibacterial action includes disruption of structural membrane, inhibition of protein as well as DNA synthesis, and repression of cellular process.

AMPs affect the bacteria by folding itself to amphipathic secondary structure after binding with negatively charge bacterial membrane and hence causing bacterial death through physical, chemical or biological processes (Chen *et al*., 2012). AMPs also inhibits virus replication by incorporating with virus envelope, minimize virus's binding ability and intercept viral entry by dominating the binding receptor on host cell [reviewed by Bahar and Ren (2013)]. Interestingly, bacteria are unlikely to acquire resistance towards AMPs due to its multifaceted mechanism of actions and ability to kill within a short time. Collectively, these properties makes AMPs, an exquisite therapeutic candidate replacing existing antibiotics [reviewed by Malanovic and Lohner (2016)].

Scygonadin, an AMP originated from the seminal plasma of mud crab, *Scylla serrata* has accounted for antibacterial activities against

* To whom correspondence should be addressed.

Micrococcus luteus and *Aeromonas hydrophila* (Huang *et al*., 2006). A study conducted by Peng *et al*. (2012) demonstrated that scygonadin exhibited antiviral activity against White spot syndrome virus (WSSV) by interfering with the virus replication *in vitro*. Nevertheless, characterization of the full potential of scygonadin requires a sufficient amount of the protein in each study. Since extracting the scygonadin protein from the fresh sample is often limited due to time and requires maximum natural resource contrary to the minimum amount of protein extracted, the recombinant protein techniques were used to overcome those limitations. By selecting heterologous recombinant protein expression tool, scalable protein production can be deployed, enabling more proteins to be produced for elucidating its functional and biological properties. Hence, in this study, a recombinant scygonadin AMPs expression system was designed with optimized purified protein production.

MATERIALS AND METHODS

Cloning of scygonadin into pBAD/Myc-His A expression vector

The construction of scygonadin pBAD/Myc-His A expression cassette (Figure 1) was carried out by double digestion of full-length 387bp sequence of scygonadin gene (GeneBank Accession No. AY864802 and was synthesized by Bio-Diagnostic Sdn. Bhd) and pBAD/Myc-His A vector with restriction enzyme *Bgl*II and *Hind*III (Promega), by following manufacturer's protocol. The expression cassette contains a C-terminal polyhistidine (6xHis) tag for nickel-chelating resin purification and Myc tag to assist in detection with anti-Myc antibody in Western blot.

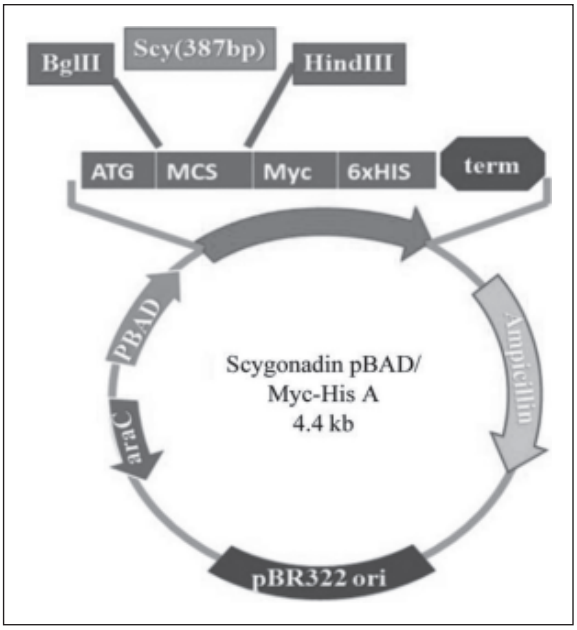

Fig. 1. Schematic representation of recombinant scygonadin pBAD/Myc His A expression plasmid.

The pBAD/Myc-His A vector was dephosphorylated by incubation with 1U Rapid Alkaline Phosphatase (RAP) at 37°C for 10 minutes, followed by inactivation of the enzyme by incubation at 75°C for 2 minutes. For ligation of *Bgl*II/*Hind*III-cut vector DNA and scygonadin gene insert, approximately 60 ng of insert DNA and 20 ng of vector DNA were incubated with 1U T4 DNA ligase (Roche) and 1X ligation buffer, in a final volume of 40 µl for overnight at 4-8°C.

The expression plasmid encoding scygonadin was transformed into chemically competent DH5α *Escherichia coli* cells using conventional heat-shock method. A total of 50 µl competent cells DH5α were thawed on the ice and mixed with 1 µl of ligation mixture. The mixture then was incubated on ice for 30 minutes followed by heat-shock at 42°C for 30 seconds and placed on ice for 2 minutes. Following that, a total of 1 ml of LB broth was added and the mixture was further incubated at 37°C for 60 minutes. A total of 100 µl of the transformed cells was spread onto 1.5 % (w/v) LB agar containing 100 µg of ampicillin (AMRESCO) per ml and incubated overnight at 37°C.

Screening of putative transformant with scygonadin pBAD/Myc-His A plasmid

Putative transformants were subjected for screening after 18 hours of incubation in LB broth and proceeded to small scale preparation of plasmid DNA by using PureYield™ Plasmid Miniprep System (Promega, USA). The putative recombinant plasmid was treated with RE (*Hind*III) to confirm the size of the plasmid. The putative plasmid with correct size was sent for sequencing for sequence verification.

Expression of recombinant scygonadin protein in TOP10 cells

Prior to protein expression, the plasmid encoding scygonadin were first transformed into chemically competent TOP10 *Escherichia coli* cells using the conventional heat-shock method.

A single colony of transformant containing scygonadin pBAD/Myc-His A plasmid was inoculated into 50 ml Falcon tube containing 10 ml LB media with the addition of 50 µg/ml ampicillin and incubated in a rotary shaker at the speed of 225 rpm for 16 hours at 37°C. The cultures were diluted and grown further for 2 hours until OD_{600}≈0.5, before being induced with various concentration of L-arabinose (Sigma-Aldrich) with the concentrations of 0.1% 0.2%, 0.02%, 0.002%, 0.0002, and 0.00002%, respectively. The cultures were left for

4, 8, 12 and 24 hours post-induction, respectively. The pre and post-induction samples were collected and were analysed with Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot.

Purification of recombinant scygonadin protein using IMAC system (Thermo Fisher)

In order to optimize the purification of scygonadin, a total of 100 ml, 500 ml and 1000 ml of expressed cell culture were initially set up and harvested by centrifugation. The cell then was suspended in binding buffer (250 mM NaH_2PO_4; 2.5 M NaCl; pH 8) and a total of 8 mg lysozyme (RBC Bioscience) was added into the solution before incubation on ice for 30 minutes. Following that, the solution was sonicated on ice using six 10-second bursts with 10-second cooling period between each burst to lyse the cell. The cell lysate was centrifuged at 3000 rpm for 15 minutes to pellet the cellular debris.

The nickel-chelating resin column was prepared by using 2 ml of resin and through a series of washing using sterile distilled water and binding buffer (250 mM NaH_2PO_4; 2.5 M NaCl; pH 8). A total of 8 ml of lysate prepared from 100 ml, 500 ml and 1000 ml culture, respectively, was added into each prepared column with nickel-chelating resin and allowed for binding overnight at room temperature using gentle agitation to keep the resin suspended in the solution. Then, the resin was washed with wash buffer (250 mM NaH_2PO_4; 2.5 M NaCl; 20 mM imidazole; pH 8 and the protein was eluted with 8ml of elution buffer (250 mM NaH_2PO_4; 2.5 M NaCl; 250 mM imidazole; pH 8). The protein sample was stored at 4°C until further analysis.

Analysis of expressed protein by SDS-PAGE and Western blot

In the SDS-PAGE procedure, the cell pellet was first lysed with lysis buffer (10 mM Tris-HCI, pH 8, 1 mM EDTA, 0.5 mg/ml lysozyme, 10 mM $CaCl_2$). A total of 40 µg of scygonadin protein from expression and purification steps were loaded into 4-12% precast gel (Novex, Invitrogen). The gel was run at 200V for 40 minutes and then stained with Coomassie Brilliant Blue R-250 for SDS-PAGE. While for Western Blot, the gel was proceeded to blotting onto nitrocellulose membrane using semi-dry blot machine (Biorad). Then the membrane was blotted with Myc tag monoclonal-Horseradish Peroxidase (HRP) conjugate antibody (diluted in 1:1000 in 10 ml of Tris-saline buffer) and developed by using 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution.

RESULTS & DISCUSSION

Recombinant scygonadin plasmid DNA extraction and purification

The generation of scygonadin pBAD/Myc His A expression vector was confirmed by agarose gel electrophoresis after enzymatic digestion with a single RE (*Hind*III). The result from agarose gel electrophoresis exhibited a linear plasmid with expected size of approximately 4.4kb (Figure 2). The presence of scygonadin gene was also verified by sequencing whereby the full-length of 387bp scygonadin are present. The obtained sequence result was also verified by BLAST (GeneBank Accession No. AY864802).

Optimisation of scygonadin pBAD/Myc His A expression system

Successful heterologous protein expression system often requires optimum amount of inducer and ideal time of induction. Here in this study, the parameter of L-arabinose inducer concentration and time of induction were carefully evaluated. Considering a possibility of toxicity of AMPs against the bacterial cells (host) and the use of pBAD/Myc His A as the backbone vector, compatible TOP10 competent cells were selected as the strain of choice in which these cells are more effective on araBAD protein expression. Some of the notable advantages of using pBAD promoter system with regulator of Ara C are that, the eminent protein expression level can be achieved using inducer and can be regulated over extensive range of inducer concentration (Guzman *et al*., 1995).

The protein expression in the competent cells containing scygonadin pBAD/Myc-His A was induced by L-arabinose with various concentrations of 0.1% 0.2%, 0.02%, 0.002%, 0.0002, and

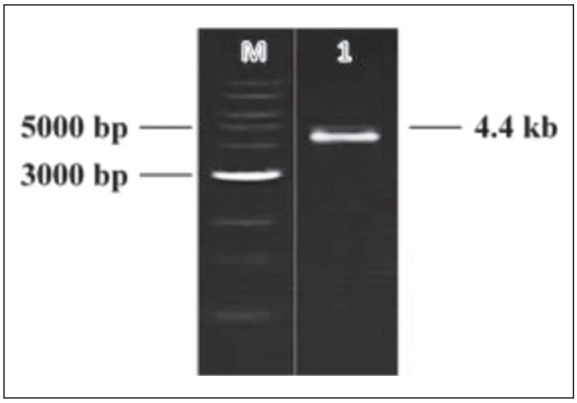

Fig. 2. Gel electrophoresis analysis for RE digested scygonadin pBAD/Myc His A expression plasmid (lane M: 1kb Quick-load DNA ladder; Lane 1: scygonadin pBAD/Myc His A digested with *Hind*III.

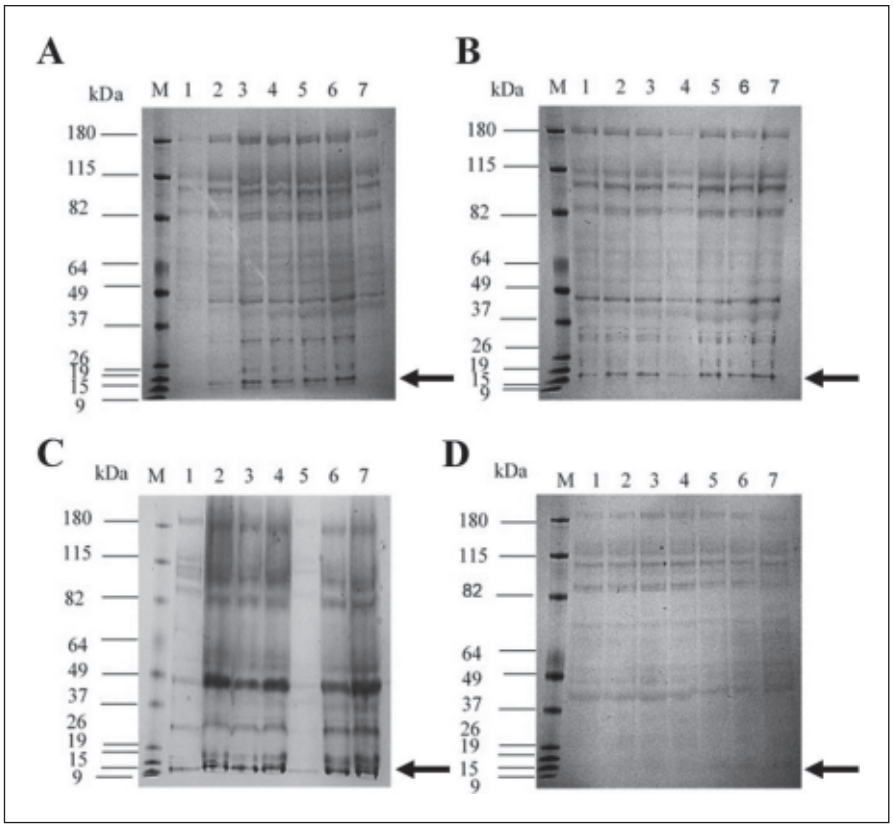

Fig. 3. SDS-PAGE analysis of the scygonadin expression following different L-arabinose inducer concentration. The sample was collected after (A) 4 hour, (B) 8 hour, (C) 12 hour, and (D) 24 hour post-induction. The gel was stained with Coomassie Brilliant Blue R-250. (M: Protein standard marker; 1: uninduced sample as control; 2-7: Post induction with 0.2%, 0.1%, 0.02%, 0.002%, 0.0002%, and 0. 00002% of L-arabinose, respectively). The protein of interest (scygonadin) is indicated in arrow with the size of ~17kDa.

0.00002%, respectively. The induction time of the L-arabinose was also optimized to 4 hours, 8 hours, 12 hours and 24 hours. Based on Figure 3, a soluble recombinant scygonadin protein around 17kDa was successfully expressed in TOP10 cells. There is an increase in the expression between the uninduced sample and the sample that has been induced with L-arabinose for 4 hours to 24 hours. From this experiment, it is found that the protein from 4 hours of post-induction were highly expressed, while the level of expressed protein decreasing over time. This is possibly due to the expression of recombinant protein contributed to a metabolic burden in the host microorganism, making significant decrease of protein production over time (Bentley *et al.,*1990; Rosano & Ceccarelli, 2014). As for the differences in inducer concentration, the scygonadin protein was highly expressed in 0.02% of L-arabinose compared to the other tested concentrations. This was evident both on SDS-PAGE as well as by protein quantitation by Bradford method (data not shown). This finding is in line with the Zhang *et al*. (2012) experiment using *pBAD* expression system, where the expression level was enhanced by 10-folds with low amount of L-arabinose inducer before the level remained constant.

Purification of scygonadin using IMAC

In this study, the recombinant scygonadin protein was purified using affinity chromatography where the method utilizes protein with high affinity for a particular chemical group (Berg *et al*., 2002). This purification method involves recombinant protein that carries a short affinity tag comprises of polyhistidine residue that will interact with transition metal ion like Co^{2+}, Ni^{2+}, Cu^{2+} or Zn^{2+} (Bornhorst & Falke, 2010).

The constructed scygonadin pBAD/Myc His A expression plasmid was purposely designed to contain C-terminal polyhistidine tag for aiding the purification step using nickel-chelating resin, while the Myc as the affinity tag for protein detection in Western blot. The final elution of the sample showed a single band of protein with the size of 17kDa, as detected using anti-Myc antibody since the recombinant scygonadin protein contain a Myc tag at the C-terminal.

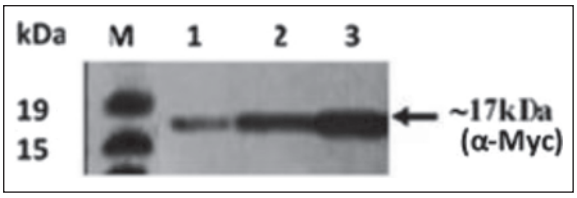

Fig. 4. Western blot analysis of purified scygonadin protein produced in different bacterial culture volumes. The protein sample was collected from the TOP10 competent cell culture after 4 hours post-induction with 0.02% of L-arabinose. (M, Protein standard marker; 1-3: purified scygonadin from 100ml, 500ml and 1000ml volume cultures, respectively). The protein of interest (scygonadin) is indicated in arrow with the size of ~17kDa. The protein was detected by using anti- Myc antibody.

In order to achieve higher protein yield, the culture volume was set up with three different volumes of 100ml, 500ml and 1000ml, respectively. As shown in Figure 4, the volume of cell culture harvested for protein purification significantly affect the amount of the eluted protein where 1000ml culture showed substantial amount of eluted protein of 2mg/ml.

CONCLUSION

This study revealed that the scygonadin pBAD/Myc His A protein expression system is a good alternative in producing scygonadin protein *in vitro*. The produced recombinant protein is able to be purified through IMAC system without the requirement of sophisticated purification setup. Due to the efficient protein expression and the quantity of the purified product, this system can be considered as a highly sustainable system which offers the potential for low-cost protein production at a larger scale. As heterologous expression is widely understood and easily manipulated, the findings reported in this study enables further functional analysis of the scygonadin with the potential for commercial applications.

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