Biosynthesis and Cytotoxic Activity of *In Vitro* Expressed Scygonadin Protein

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Abstract

Antimicrobial peptides (AMP) are key components of an innate immune response which represent immediate action of the defence mechanism of an organism. It is considered a novel therapeutic agent due to its abundance in nature and a broad range of defence activity against microbial. Preceding research has shown that scygonadin AMPs isolated from seminal plasma of mud crab had the potential as a novel antimicrobial agent. However, its cytotoxicity properties on cultured cells have never been experimentally addressed. In this study, the scygonadin protein was expressed *in vitro*, followed by cytotoxicity assessment via MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. A full-length sequence of the scygonadin gene of 387 bp was cloned into pBAD/Myc-His A vector and expressed in TOP10 cells. The protein expression was induced, purified and quantified before being subjected to cytotoxicity analysis. Next, an African green monkey kidney (Vero) cell was chosen to evaluate the cytotoxicity level of scygonadin *in vitro*. A total of $1x10^4$ cells/mL were seeded into a 96-well plate before being treated to various concentrations of scygonadin protein and hydrogen peroxide as a positive control for the toxicity test. The cells' viability treated with scygonadin AMP and hydrogen peroxide was also verified with fluorescent analysis. The result demonstrated that the scygonadin did not cause any cytotoxicity effects while hydrogen peroxide showed an IC_{50} value at 0.003µM and this was further confirmed by fluorescent staining analysis. The absence of scygonadin toxicity in cells indicates its potential for biopharmaceutical use.

Keywords: Antimicrobial peptide, recombinant protein, cytotoxicity

Introduction

Antibiotic-resistance microorganism has been a world health alarming issue. In 2019, approximately 1.27 million deaths caused by antibiotic-resistance microorganisms was recorded (Murray et al., 2022), leading to the urge of developing new novel and efficient antimicrobial drugs to replace the existing antibiotic (Hancock & Sahl, 2006; Egessa, 2022). The marine environment was viewed as a potential source of novel bioactive natural products due to the differences in chemical and structural compounds compared to terrestrial organism (Malve, 2016). About three-quarters of the earth's surface were encompassed by water and the ocean contains approximately 80% more diverse flora and fauna species. Aquatic life comprises microorganisms, plants, invertebrates, and vertebrate animals that are able to produce an extended range of bioactive compounds potentially with antimicrobial properties (Ely et al., 2004; Wu et al., 2021). Antimicrobial peptides (AMPs) existing in nature with the diverse, effective, and a broad spectrum of defence activity of the marine organism are the result of evolutionary success living in the harsh and diverse habitat (Nakatsuji & Gallo, 2012; Gerdol., 2017; Egassa, 2022). There was much research on the mechanism of antimicrobial peptides that were conducted since this drug discovery and the mechanism of action produce on the microbes varied differently between compound (Wu et al., 2021).

Scygonadin AMPs is an anionic peptide and were found to be highly expressed in the reproductive tract of *Scylla* crab (Wang et al., 2007; Liu et al., 2012). According to Xu et al. (2011), scygonadin was also found in female spermathecae, suggesting that it might either been originally expressed in the spermathecae or originated from the ejaculatory duct in mature males in the mating process and subsequently transmitted to

it. Preceding research has shown that scygonadin exhibit antibacterial activity against *Micrococcus leteus* and *Aeromonas hydrophila* (Huang et al., 2006; Qiao et al., 2016; Long et al., 2022) and antiviral activity against white spot syndrome virus (WSSV) by interfering with the virus replication *in vitro* (Peng et al., 2012; Qiao et al., 2016; Long et al., 2022) suggesting its potential to be further developed as antimicrobial drugs. In the process of producing such products, the promising therapeutics compound must undergo a series of screening processes and biological evaluations. In one of the processes, the toxicity of the isolated *in vitro* generated compound must first be determined.

To date, no research has been conducted on assessing scygonadin AMPs cytotoxicity on the cell line. Hence, this study aimed to evaluate the cytotoxicity level of the *in vitro* expressed proteins using Vero cells. The present study was designed by using purified scygonadin protein synthesised by an expression system of 387 bp of scygonadin gene, pBAD/myc His A vector, TOP10 competent cell and L-arabinose as an inducer (Rosli et al., 2019). The optimisation of scygonadin protein expression and purification was analysed and the purified scygonadin protein was used in the evaluation of the cytotoxicity assay to assess its potential to cause cell death in the African green monkey kidney (Vero) cell line.

Material and Methods

Scygonadin Protein Expression, Purification, and Quantification

The scygonadin protein (~17 kDa) was expressed and purified as previously described (Rosli et., 2019). Briefly, the recombinant scygonadin was expressed in TOP 10 *Escherichia coli* competent cells. The cell was cultured and induced with a series of L-arabinose concentrations (0.1, 0.2, 0.02, 0.002, 0.0002, and 0.00002%) and the cultures were induced at a different time points of 4, 8, 12, and 24 hours. Each sample of different concentrations and time points was subjected to protein extraction and purification. Besides that, the expressed scygonadin protein volume was scaled up to achieve the maximum concentration of purified protein. The expressed and purified protein was analysed by Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot. Presently, the protein band intensity was further confirmed by quantification using Image J analysis.

Cell Culture

Vero cells were grown in minimum essential medium (MEM) (Gibco, USA), supplemented with 10% fetal bovine serum (Gibco, USA), 1% penicillin-streptomycin (Gibco, USA), 1% Non-essential amino acid (NEAA) (Gibco, USA) and was cultured 5% CO_2 incubator at 37 ^{o}C. The cell was sub-cultured every two days or when achieved confluency. Phosphate buffer saline (PBS) (Gibco, USA) with pH 7.4 was used to wash the monolayer cell and TryPLE Express (Gibco, USA) enzyme reagent for cell detachment.

MTT Cytotoxicity Assay

The scygonadin protein was tested for its cytotoxicity *in vitro* using Vero cell by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. A total number of $1x10^4$ cells/mL of Vero cells were seeded into each well of the 96-well culture plate. After 24 hours of incubation, the cell was treated with various concentration of 0.0, 1.875, 3.75, 7.5, 15, 30, 60, 120, 240 µg/mL scygonadin protein and 0.0, 0.015, 0.03125, 0.0625, 0.125, 0.25, 0.5, 1 and 2 µM concentration of hydrogen peroxide (H_2O_2) as positive toxicity control. After 72 hours of post-treatment, 20 µL of MTT (1 mg/mL) was added to each well. In this study, MTT was used as it can only be reduced to purple-blue formazan by viable cells. It is insoluble in water and unable to penetrate intact cell membranes (McGaw et al., 2014). After 3 hours, 100 µL of DMSO was added to dissolve formazan crystal and further incubated for 10 minutes at 37 ^{o}C subsequently, an absorbance reading was taken by spectrum analysis (λ=562nm). Cell viability of treated cells was assessed in percentage compared to the untreated healthy cell (Correa et al., 2018).

Fluorescence staining of Acridine orange (AO) and Propidium iodide (PI)

Vero cell ($2x10^5$ cells/mL) was seeded into a 6-well plate and incubated for 24 hours prior to treatment. Vero cells were treated with 240 µg/mL of scygonadin protein, 0.003 µM of H_2O_2 as positive control and untreated cell as negative apoptosis control. A volume of 300 µL of AO/PI (1 mg/mL) solution was added to each well. The stained cell was visualised by an inverted light microscope Olympus IX73.

Results and Discussion

SDS Page and Western Blot Analysis of *in vitro* Protein Synthesis

A series of optimisation of scygonadin protein expression was executed and the result of SDS-PAGE-page and Western blot analysis was analysed by Image J software (version 1.53s). Figure 1 (A) of SDS-PAGE analysis showed that scygonadin expression levels after being induced with 0.02% of L-arabinose were higher

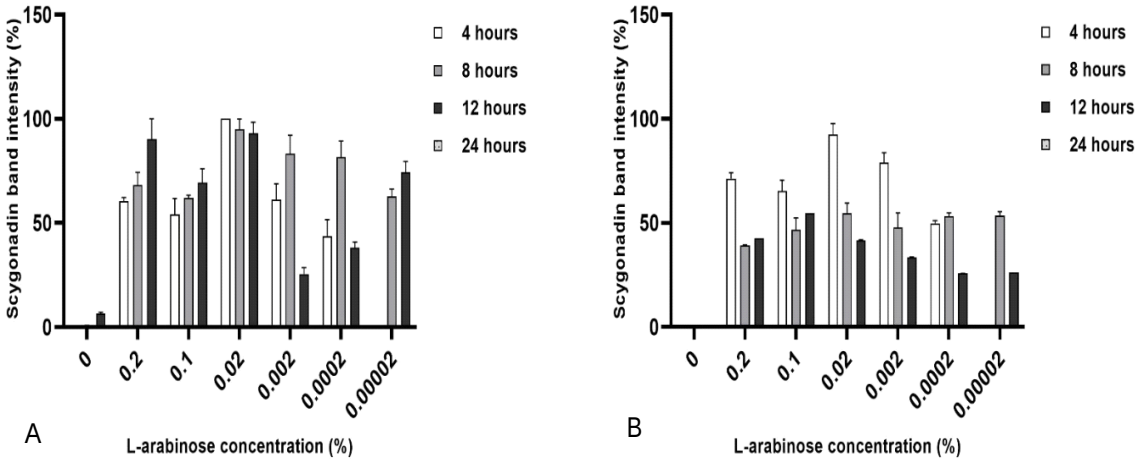

Figure 1. Percentage of scygonadin band intensity of (A) SDS-PAGE analysis and (B) Western blot of 4, 8, 12 and 24 hours post-induction with various concentration of L-arabinose (0, 0.2, 0.1, 0.02, 0.002, 0.0002, and 0.00002%). The analysis was done by Image j software.

compared to other concentrations. In addition, the expression level was slightly higher after 4 hours of post-induction compared to 8 and 12 hours. However, the expression level analysed by Western blot analysis (Figure 1 B), showed that scygonadin induced by L-arabinose after 8 hours is higher compared to 12 hours post-incubation which is approximately 55% and 40% of intensity respectively. Whilst, there is no scygonadin expressed after 24 hours of post-incubation, the scygonadin expressed after 4 hours of post-incubation showed the highest level, particularly the one with 0.02% of L-arabinose concentration.

The purification of scygonadin protein was carried out by three batches of expressed cells with a volume of 100, 500 and 1000mL. Figure 2 showed that compared to 100mL and 500mL of culture, 1000mL showed a substantial amount of purified scygonadin.

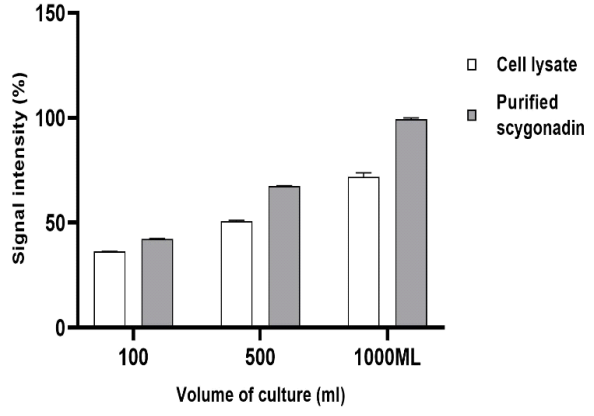

Figure 2. The signal intensity of cell lysate and purified scygonadin protein with 100, 500 and 1000mL culture volume. The analysis was done by Image J software.

This result showed that the amount of scygonadin protein expression *in vitro* is well correlated to the volume of culture used, indicating the scalability of the protein to be expressed in a large culture volume.

Cytotoxicity Assay

Healthy cells are often reflected in the rates of cell proliferation and viability and once exposed to the toxic substances, their metabolism and health rate will be impendent (Aslantürk et al., 2018). Thus, it is important to investigate the effect of the target compound on cell viability by cytotoxicity assay (Gothai et al., 2019). MTT assay was designed to assess the cytotoxicity of the cell as the reduction of MTT to formazan is correspond to the cell viability. In this study, the cytotoxicity of H_2O_2 on the Vero cell line was assessed, and the outcome was represented in Figure 3. It is demonstrated that the H_2O_2 treatment inhibits cell growth at 0.003 µM. In contrast, scygonadin protein (Figure 3 B) even at a high concentration of 240 µg/mL which is equivalent to 20.99 µM/mL showed no half maximal reduction in the cells.

The findings of scygonadin cytotoxicity were further validated through dual fluorescence staining of AO/PI. AO/PI dual fluorescence staining was conducted to differentiate the viable cells from the dead cell by examining the morphologic characteristic of the cell via microscope (Bagheri et al., 2018). The assessment of the cytotoxicity mechanism of the H_2O_2 compound on Vero cells with IC_{50} dose of 0.003 µM of the H_2O_2 compound showed that there is the presence of yellow and red colors (Figure 4 B) of nuclei resulting from the dispersion of PI into the cell. It is also indicated the ongoing late stage of apoptosis event

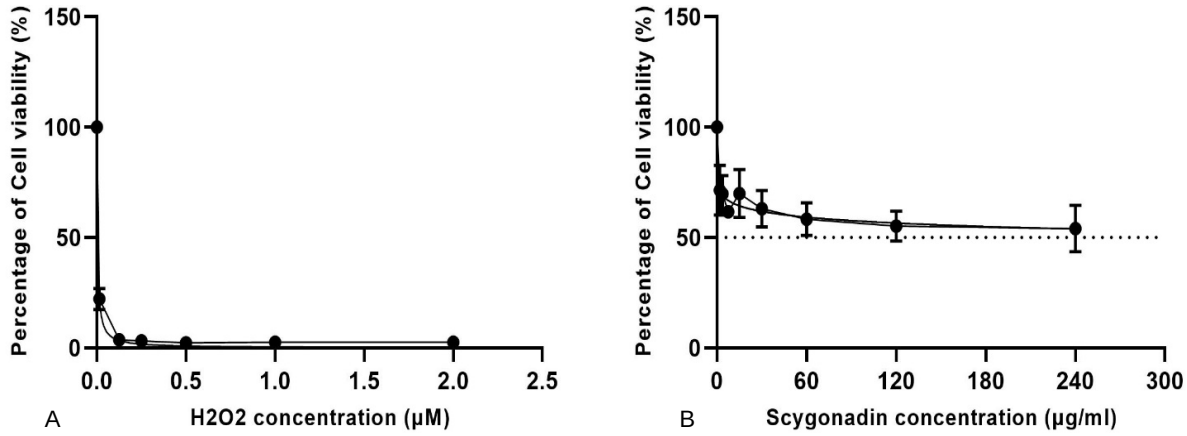

Figure 3. Graph of percentage of cell viability against hydrogen peroxide concentration. (A) The Vero cell was treated with hydrogen peroxide of 0.0, 0.015, 0.03125, 0.0625, 0.125, 0.25, 0.5, 1 and 2 µM. (B) The Vero cell was treated with scygonadin of 0.0, 1.875, 3.75, 7.5, 15, 30, 60, 120, 240 µg/mL.

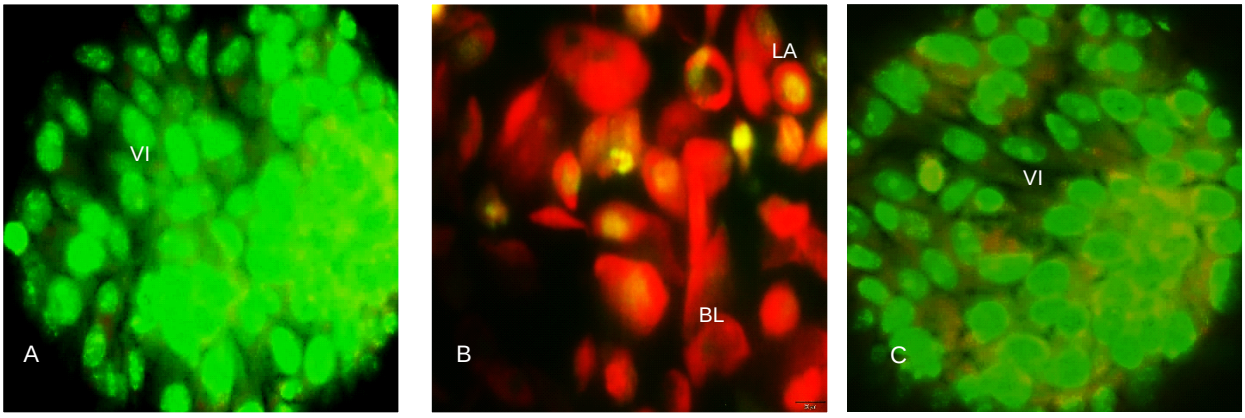

Figure 4. AOPI staining of Vero cells with (A) as untreated cells, (B) cells treated with 0.003 µM of H_2O_2 and (C) Vero cells treated with 240 µg/mL of scygonadin protein. The image was taken under an inverted light microscope Olympus IX73. Actual magnification of 400x. Abbreviations: VI, viable cells; BL, blebbing cell; LA, late apoptosis.

to the presence of apoptotic bodies, membrane blebbing, and cell shrinkage (Ismail et al., 2021). In contrast, the cells treated with 240 µg/mL of scygonadin protein (Figure 4 C) showed a fluorescence green colors with healthy intact nuclei similar to the control Vero cells (Figure 4 A) with no sign of dead cells, as the cells were only stained with membrane permeable AO dye.

Previous research by Peng et al. (2012), showed that extracted scygonadin from mud crab exhibited antimicrobial activity with 90% of cidal index against Gram-positive bacteria *M. leteus* when treated with 12 µM of scygonadin protein while the recombinant scygonadin sharing the same effects against *M. leteus* with a minimum inhibitory concentration of 7.5-15 µM. The investigation on the cytotoxic characteristics of scygonadin has been explored in this study on MTT by various doses of scygonadin of up to 20.99 µM/mL. As it has been observed in the results, all tested concentration of scygonadin showed no toxicity activity, from this, it can be deduced that scygonadin are harmless and can be used for biopharmaceutical with low risk.

Conclusion

In this study, scygonadin protein when treated in a healthy Vero cell line showed no indication of cytotoxicity compared to hydrogen peroxide as positive control which showed a cytotoxicity value of 0.003 µM. These findings were further verified by fluorescence viability assay which indicated that the Vero cell when treated with scygonadin protein exhibited no sign of apoptosis which correspond with untreated healthy Vero cell. It is concluded that scygonadin could potentially be further analysed for applications in disease treatment such as an antibacterial and antiviral drug candidate.

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Supplementary Material

Supplementary material is not available for this article.

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