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EXPRESSION AND PURIFICATION OF SNAKE ANTIVENOM PEPTIDE IN
PICHLIA PASTORIS

A Thesis

Presented to

The Faculty of the Department of Chemical and Materials Engineering

San José State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Israel Juarez Contreras

December 2019

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The Designated Thesis Committee Approves the Thesis Titled

EXPRESSION AND PURIFICATION OF SNAKE ANTIVENOM PEPTIDE IN
PICHLIA PASTORIS

by

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ABSTRACT

EXPRESSION AND PURIFICATION OF SNAKE ANTIVENOM PEPTIDE IN *PICHLIA PASTORIS*

by Israel Juarez Contreras

It has been shown that a peptide of the first 10-15 N-terminal amino acids of the lethal toxin neutralizing factor (LTNF) protein found in opossums (*Didelphis* spp.) holds promise as a low-cost therapy for snake envenomation. To date, the 11-mer has been expressed in *E. coli* and shown to neutralize snake venoms. However, possible endotoxin concerns warrant an investigation into other microbial hosts. The methylotrophic yeast, *Pichia pastoris*, shows promise as an alternative host. Active LTNF peptide was expressed and purified in *P. pastoris*. This was accomplished by subcloning a tandem repeat of the first 15 N-terminal amino acids of lethal toxin neutralizing factor (LTNF-15) into the *Pichia* expression vector, PPIC9K and transforming the yeast via electroporation. Expression of LTNF-15 from *Pichia* was verified by applying a fluorescent histidine tag stain onto a SDS-PAGE gel containing supernatant samples of *P. pastoris* clones. Purification of the LTNF-15 peptide was conducted by Ni-NTA purification. It was found that expressed LTNF-15 peptide demonstrated neutralizing activity in an *in vitro* assay using azocasein. In addition, it was determined that a protein concentration of 70 mg/L of LTNF-15 was attained during the fermentation process. Thus, showing promise of *Pichia* as a viable production host.

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CHAPTER ONE

INTRODUCTION

In the United States, around 99% of the 6,000 yearly cases of snake envenomation are from the subfamily *Crotalinae* which includes pit vipers and rattlesnakes [1].

Characteristic symptoms of crotalid envenomation include localized swelling, and tissue necrosis. If left untreated, loss of limbs, kidney failure and death can occur. Current antivenom treatment comes from a mix of Fab immunoglobulin fragments from blood of healthy sheep immunized with snake venom [2]. However, there are reported immunological effects and cost to produce is \$14 USD per vial [3].

The cost of antivenom is a significant factor, particularly in developing countries where access to treatment is limited or nonexistent. According to the World Health Organization (WHO), there are about 5 million annual cases of snake envenomation, of which 500,000 cases lead to permanent disability or death [4]. Most of these cases arise in developing countries, such as India, where the annual mortality rate due to snake envenomation is estimated to be around 46,000 people [5].

It has been reported that mammals such as *Didelphidae* (opossum) show resistance to snake envenomation [6]. Investigation into opossum serum revealed a variety of proteins capable of neutralizing components of snake venom. Several of these proteins exhibit similar characteristics, most notable of which the presence of the same five amino acid sequence, LKAMD, at the start of the N-terminus [7-9]. Further work conducted by Lipps identified a 68 kDa protein called Lethal Toxin Neutralizing Factor (LTNF) in the opossum serum. Lipps postulated that LTNF can serve as a universal and alternative

treatment for envenomation. Further work on LTNF by Lipps demonstrated that only the first 10-15 N-terminal amino acids of LTNF consisting of LKAMDPTPPLWIKTE were required for complete neutralization of snake venom activity [10]. Therefore, a synthetic peptide consisting of 10-15 amino acids of LTNF could be produced and used as a treatment for snake envenomation.

The production of recombinant proteins in microorganisms has revolutionized the field of biotechnology. Previously, large quantities of biological samples of organisms were required for the purification of small amounts of protein. The current method now involves simply incorporating a gene of interest into a microbe of choice and growing the microbe in a bioreactor. This method has produced valuable therapies including insulin, anti-malarials, cancer treatments and many others.

Current research into LTNF recently conducted by Komives confirmed Lipps' claim of synthetic LTNF neutralizing snake venom. In addition, Komives demonstrated the ability of recombinant LTNF produced in the gram-negative bacteria, *E. coli*, to partially neutralize snake venom [11]. The inability of the peptide to provide 100% neutralization was likely due to the presence of residual contaminants from the purification process, as the process had not been optimized. Furthermore, most recombinant LTNF produced in *E. coli* formed inclusion bodies, which are densely packed, denatured protein molecules in the form of particles. The process of recombinant protein recovery from inclusion bodies is labor intensive and the resulting yields are often low [12]. As a result, it is recommended to investigate other production hosts for recombinant LTNF expression.

The methylotrophic yeast, *Pichia pastoris* is a commonly used recombinant production host capable of growing to high cell densities and producing gram amounts of recombinant protein per liter of culture [13]. Purification of *P. pastoris*-produced recombinant protein is straightforward, as most protein secreted into the growth medium is recombinant protein. Purification requires a less labor-intensive process as well as an improved yield and recovery when compared to *E. coli*. In this project, recombinant LTNF protein production by *P. pastoris* was investigated together with purification of the peptide resulting in a bioactive peptide.

CHAPTER TWO

LITERATURE REVIEW

The availability of snake antivenom is a global health issue affecting many regions of the world including Sub-Saharan Africa and Southeast Asia [2]. A variety of factors limit the availability of snake antivenom in these regions, ranging from the complications involved in preparing region-specific antivenom mixtures, to the difficulty of maintaining regional production systems [2]. Currently, snake antivenom is produced by injecting a non-lethal dose of snake venom into a host animal such as a horse or sheep and harvesting the antibodies produced by the animal in response to the venom [2].

In the 1990s, Lipps identified and isolated a peptide found in opossum serum called LTNF which she erroneously reported to be effective against the venoms of several species of snake, including those with lethal neurotoxins [6]. Unfortunately, little progress had been made until 2016 when Komives was able to produce a recombinant form of the first 11 amino acids of LTNF in *E. coli* which exhibited neutralizing activity [11]. The difficulty of purifying the peptide from inclusion bodies was attributed to a low yield and partially purified product that necessitates optimization [11]. However, a team in India was able to obtain high yields and purity for the LTNF-11 from the *E. coli* strain [14]. Still, the presence of *E. coli* endotoxin in biotherapeutics produced in *E. coli* is a motivation to explore the production in other hosts [15].

The methylotrophic yeast, *Pichia pastoris*, is one of the most extensively studied yeast species due to its superior ability to produce large quantities of recombinant protein and express proteins that could not be produced effectively in other recombinant hosts

[13]. Investigation of LTNF expression in *Pichia pastoris* was carried out in this project. This was conducted by transforming *Pichia pastoris* with a recombinant plasmid, PPIC9K, containing a gene to encode a concatenated peptide chain of the LTNF-15 with a 6x-His tag. A literature review was conducted to gain a better understanding to the characteristics of *Pichia pastoris* expression system and to examine methods used in developing recombinant *Pichia* strains.

2.1 The *Pichia* Expression System

2.1.1 A Brief History

Development of the initial protocols for growing *P. pastoris* on methanol at high cell densities was conducted by the Phillips Petroleum Company [14,15]. This was mainly investigated as *P. pastoris* was considered an inexpensive source of animal feed [16]. However, due to the energy crisis of the 1970s, the cost of methanol was considerably high, ending any interest in *Pichia* for this application [16].

In the decade that followed, Phillips Petroleum collaborated with the Salk Institute Biotechnology/Industrial Associates Inc (SIBIA) to investigate the potential use of *P. pastoris* for recombinant protein expression. Key developments included the isolation of the gene and promoter for alcohol oxidase, which allows for methanol induction in many of the commercially available vectors [17-18]. Furthermore, the development of auxotrophic strains of *Pichia* allowed for direct selection of recombinant clones and provided a greater range of manipulation [19]. Most of these developments have been filed in patent literature and common strains and vectors are commercially available through Invitrogen [16]. With these developments, *P. pastoris* is now widely used as a

model eukaryote for cell biology research and is a common host for recombinant protein production.

2.1.2 Methanol Utilization

A unique characteristic of *P. pastoris* is the ability to utilize methanol as the sole carbon source [20]. Indeed, methanol has a bulk cost comparable to glucose now that new catalysts have been developed for conversion of natural gas (CH₄) to methanol [21]. Utilization of methanol by *P. pastoris* is only possible through a methanol utilization pathway consisting of several unique enzymes. The first step in this pathway is the oxidation of methanol to formaldehyde, resulting in the formation of hydrogen peroxide. For this reason, this initial reaction takes place within the peroxisome [22]. In addition, the peroxisome contains the three key enzymes responsible for methanol metabolism: alcohol oxidase (AOX), catalase, and dihydroxyacetone synthase [22]. After these initial reactions, subsequent reactions occur in the cytosol [22]. Although there are two genes present in *P. pastoris* for AOX: AOX1 and AOX2, the AOX1 gene is primarily responsible for most alcohol oxidase activity [23]. AOX1 is tightly regulated and induced by methanol to high levels [13].

Three phenotypes of methanol utilization exist in *P. pastoris*: Mut⁺, Mut⁻ and Mut^S. The Mut⁺ phenotype grows on methanol at the same rate as the wild type and requires high feeding rates of methanol in fermentations [14]. Although the Mut⁻ phenotype is unable to grow on methanol due to the deletion of both AOX genes, it may be required to produce certain recombinant proteins due to the low growth rates exhibited by this phenotype [22,23]. The Mut^S phenotype contains a deletion of the AOX1 gene, resulting

in a dependence on the AOX2 gene for methanol metabolism. Since the AOX2 gene is not expressed at the high levels of AOX1, the growth rates are slower than the Mut⁺ phenotype but may aid in production of certain recombinant proteins [22]. The common *Pichia* phenotypes used for protein production are Mut⁺ and Mut^S [24,25].

2.1.3 Expression Strains

Most strains utilized in expression experiments share some similarities and differences. For example, all *P. pastoris* expression strains are derivatives of the lab strain NRRL-Y 11430 and have a mutation in the histidinol dehydrogenase gene (HIS4). This mutation allows for auxotrophic selection of recombinant transformants [26]. Strains have been developed based on their ability to utilize methanol through the deletion of the AOX genes. There are instances where strains with deleted AOX genes express foreign proteins better than wild type strains and have the added benefit of requiring less methanol for induction [27-29], reducing the flammability risks involved when large amounts of methanol are required.

The most common strain of *P. pastoris* is GS115 which contains the wild type AOX genes and grows on methanol at the wild type rate (Mut⁺) [13]. However, recombinant protein degradation can occur due to the expression of native proteases in *P. pastoris*. This is especially true in fermentation processes where a high cell density and lysis of cells can increase the likelihood of proteases being expressed [13]. Various methods can be used to reduce the likelihood of native protease production, including using an unbuffered minimal media to create acidic conditions unsuitable for protease production or buffering the media to pH 6.0 [30]. However, if media conditions fail to prevent

protein degradation, specialized strains, SMD1163, SMD1165, and SMD1168 can be utilized [30]. As derivatives of GS115, these strains are produced from deletions to the PEP genes present in GS115, which result in the inability to produce proteases [13] and may significantly improve the yield of protein [31]. An enzymatic assay can be conducted to determine if a protease deficient strain is required [32].

2.1.4 Expression Vectors

Plasmids designed for recombinant protein expression in *Pichia* share some similarities [30]. Most expression vectors contain an AOX1 promoter, followed by a multi-cloning site, a region in the plasmid which contains specific DNA sequences that allow restriction enzymes to cleave and insert genes [13]. Many vectors contain the HIS4 gene, allowing for a selectable marker for transformation experiments. Some vectors contain the AOX1 3' flanking sequence that is used in integrating the gene directly into the *P. pastoris* genome [13]. Certain expression vectors include the alpha-factor signal sequence from *S. cerevisiae* as a means of facilitating secretion of recombinant protein [33,34]. Some vectors feature drug-resistance genes that can be used to effectively screen clones with multiple copies of the vector [30].

2.1.5 Genetic Manipulation

There are several methods for *P. pastoris* transformation with varying degrees of efficiency [35]. Transformation of yeast by the spheroplast method is done by preparing an incomplete enzymatic digestion of the cell wall and incorporating foreign DNA into the host [36]. The cell wall is then regenerated when embedded in an agar medium [36]. Although this method requires several strenuous and complicated steps, it is well studied

and has a high transformation efficiency [35]. However, even with these benefits, this method is unfavorable due to the complexity of the process.

Electroporation is another commonly used technique for transformation of yeast cells [35]. Due to the electrical pulse cells are subjected to, pores form through the cell membrane, allowing foreign DNA to be delivered [35]. The introduction of an osmotic support solution and pretreatment of cells with DTT and lithium chloride enables successful electroporation [37]. With these improvements, electroporation is on par with the spheroplast method regarding transformation efficiency while being extremely user friendly [35]. The only drawback to this method is specialized equipment is required [35].

Using either the spheroplast method or electroporation results in the integration of the vector into the *P. pastoris* genome [36]. This occurs through homologous recombination between shared sequences by the vector and genome [36]. The resulting transformants are extremely stable, even in the absence of selective pressure and can be stored for an indefinite period of time in a mixture containing glycerol at -80°C [32].

Depending on the features of the expression vector, two results may occur during homologous recombination [13]. If the expression vector contains an AOX1 3' sequence, the deletion of wild type AOX1 is replaced by the AOX1 3' sequence of the expression vector. This results in the generation of Mut^S while Mut⁺ transformants are generated from a recombination event at the wildtype HIS4 gene. This second case is rare, and a large amount of transformants may be false positive [30]. This occurs in 10-50% of

transformants and can increase if transformation is performed through electroporation [13].

Multiple gene insertion events can occur spontaneously at low frequencies between 1-10% [13]. Multi-copy *P. pastoris* colonies are of high interest, as they tend to produce large amounts of recombinant protein. Identification of multicopy-containing colonies is conducted by subjecting transformants to varying degrees of the antibiotic to which the expression vector encodes resistance. [30] This screening process effectively identifies multicopy clones and destroys false positive transformants.

2.1.6 Post-translation Modifications in *Pichia*

P. pastoris has the capacity to perform posttranslational modifications such as processing signal sequences, folding, disulfide bridge formation and O- and N-linked glycosylation. [13] In mammals, O-linked oligosaccharides are composed of a variety of sugars, whereas *P. pastoris* adds O-linked oligosaccharides composed of only mannose residues [13]. In addition, there is a small probability of O- glycosylation to proteins that are not natively glycosylated [13]. Meanwhile, N-glycosylation in *P. pastoris* is different compared to higher eukaryotes [38] Higher eukaryotes process glycosylation through a series of addition and trimming reactions to generate mixtures of different sugars whereas in *P. pastoris*, N-glycosylation can occur in a similar fashion or can process protein with additional carbohydrate, known as hyperglycosylation [30]. Hyperglycosylation poses a problem for pharmaceutical grade recombinant proteins as mammals may negatively react to hyperglycosylation and the half-life of such proteins may be deleteriously affected. [13]

2.2 Protein Production in *P. pastoris*

2.2.1 Nutritional Requirements

The majority of recombinant protein expression in *Pichia* is carried out with complex media using recipes available from Invitrogen [30]. These serve as an excellent source of nutrients for expression in shake flask but have limitations at a large scale [15]. A common example of this is the initial concentration of glucose or glycerol used must be optimized for biomass production for large scale processes [15]. In addition, common nitrogen sources such as yeast extract and peptone often have batch to batch variability [15]. Eliminating these sources of nitrogen in favor of ammonium hydroxide is common in fermentation processes [15].

A defined basal medium with a salt solution with vitamin and trace metal supplements is a common fermentation media used for large scale *Pichia* expression [39,40]. Along with being a nitrogen source, ammonium hydroxide is used as a means of pH control, but the concentration must be optimized. High concentrations of ammonium hydroxide can inhibit cell growth, whereas low concentrations aid in increased protein production [41]. Supplementing the media with other nitrogen sources such as yeast extract and casamino acids aids in protein secretion while inhibiting native secreted proteases [30]. Alternatives to the common basal medium have also been shown to improve overall protein yield but appear to depend on the particular protein being produced [42].

2.2.2 Fermentation Control

As discussed, the primary components of fermentation media are glycerol, methanol, biotin, salts, and trace elements. These are relatively inexpensive and ideal for large scale processes [15]. Although glucose is a common carbon source in bacterial fermentations, it is avoided in *Pichia* due to the by-product ethanol being a repressor to the AOX promoter [43].

Methanol monitoring in a *Pichia* fermentation is critical for successful recombinant protein production [14]. Methanol concentrations greater than 2% (w/v) in the fermentation vessel can be toxic for the cells and low levels of methanol will result in no protein expression [14, 33]. Monitoring of methanol using an off-line biochemistry analyzer or gas chromatograph is both laborious and expensive [15]. In addition, evaporation of methanol can lead to inaccurate data if there is a time lag between sampling and obtaining the analysis.

Online methods are available for methanol detection, such as sensors capable of detecting methanol vapor, but are limited to shake flask and small fermentation vessels [44]. If there is a lack of instrumentation or methods for determining methanol concentration in the culture, constant monitoring of the dissolved oxygen can be utilized to determine whether a culture has a methanol concentration that is outside the optimal range [13]. Cultures starved of methanol will correspond to an abrupt increase in DO, called a DO spike [13]. Methanol is then manually pumped at a low rate until the DO concentration stabilizes between the range of 25-40% of saturation [13].

Other factors such as pH and temperature also affect expression levels in fermentation processes. For example, recombinant protein might be unstable at particular pH values or sensitive to certain operating temperatures [45]. This may be due to the small range of temperature (25-30°C) and large range of pH conditions (2-8) in which *Pichia* can reasonably grow well [30]. Optimization is critical for the fermentation process and is approached mainly through trial and error [15].

2.3 Comparison to *E. coli*

E. coli is the preferred organism used for recombinant protein production due to fast growth and protein production rates. Although *Pichia* has comparatively fast growth rates, protein production spans on the order of days [15,30]. A major advantage of *Pichia* is its capacity to perform post-translational modifications similar to mammalian cells [30]. Intracellular mechanisms which modify proteins exist in eukaryotic cells but do not exist in prokaryotes such as *E. coli*, limiting the type of proteins that can be expressed [46]. However, eukaryotic proteins have successfully been expressed in *E. coli* although some may be produced in an unstable form [47].

Protein aggregates within the cell, called inclusion bodies, may result during recombinant protein production in *E. coli* [48]. These large, spherical particles are the result of molecular mechanisms failing to repair or remove misfolded or unfolded protein and require additional processing to remove from the cell and refold [49]. *E. coli* typically require an optimization in the purification process where the protein becomes denatured and refolded [48,49] whereas proteins secreted from *Pichia* typically require a single step to recover the protein from the culture medium [49].

2.4 Summary

Studies on recombinant protein expression in *Pichia* appear to follow general guidelines developed from the Philips Petroleum company which are widely available through the Invitrogen website. *Pichia* have an optimum growth temperature between 25°C and 30°C with optimum pH around 6. A variety of growth media for *Pichia* is readily available for both shake flask and fermentation scale processes. With standardized protocols increasingly becoming available for use, the ease of using *Pichia* as a host for protein expression has become popular. In addition, previous research has shown the peptide is active in *E. coli* indicating there is a possibility that *P. pastoris* should produce an active peptide with potentially higher yield and purity.

CHAPTER THREE

RESEARCH OBJECTIVE

3.1 Objective

The research objective of this project is to develop a strain of *Pichia* that produces a concatenated chain of LTNF-15 peptides, and to produce and purify the peptides.

3.2 Justification

There are over 5 million cases of snake envenomation, particularly in developing countries where the cost of current antivenom treatment is a barrier for adequate treatment. In order to provide low-cost antivenom treatment, a bioprocess can be developed that is economical compared to conventional antivenom production.

Recombinant LTNF-11 from *E. coli* has shown success in neutralizing snake toxins from the subfamily *Crotalinae*. However, contamination issues from *E. coli* endotoxins remain a concern. It would be beneficial to express LTNF-15 from *Pichia* since recombinant peptides are usually secreted into the culture medium along with low levels of native protein, yielding a relatively pure product. Purification of the product from the culture medium can then be carried out to obtain a pure product without the presence of residual contaminants. In addition, *Pichia* have been shown to express recombinant products at high levels, on the order of grams per liter of culture at high cell densities. Production of LTNF-15 from *Pichia* can potentially lead to a low-cost form of antivenom.

CHAPTER FOUR

MATERIALS AND METHODS

The project was conducted as follows: subcloning the gene into the PPIC9K vector, transformation of *Pichia* strains GS115 and SMD1168 by electroporation, screening the resulting transformants for appropriate phenotype, expression of the peptide, purification of the peptide from culture media, and then activity testing of the peptide.

4. 1 Growth Media Preparation

Growth media was prepared as follows: LB (1% Tryptone, 0.5% Yeast Extract, 1% NaCl, with 1.5 % agar for plates), YPD (1% yeast extract, 2% peptone, 2% dextrose, with 2% agar for plates), RDB (1M sorbitol, 2% dextrose, 1.34% Yeast Nitrogen Base without amino acids, 4×10^{-5} % biotin, 0.005% L-glutamic acid, 0.005% L-methionine, 0.005% L-lysine, 0.005% L-leucine, 0.005% L-isoleucine, 2% agar), MD (2% dextrose, 1.34% Yeast Nitrogen Base without amino acids, 4×10^{-5} % biotin, 1.5% agar), MM (0.5% dextrose, 1.34% Yeast Nitrogen Base without amino acids, 4×10^{-5} % biotin, 1.5% agar), BMG (100 mM potassium phosphate pH 6.0, 1.34% Yeast Nitrogen Base without amino acids, 4×10^{-5} % biotin, 1% glycerol), BMM (100 mM potassium phosphate pH 6.0, 1.34% Yeast Nitrogen Base without amino acids, 4×10^{-5} % biotin, 0.5% methanol), BMGY (100 mM potassium phosphate pH 6.0, 1.34% Yeast Nitrogen Base without amino acids, 4×10^{-5} % biotin, 1% glycerol, 1% yeast extract, 2% peptone), BMMY (100 mM potassium phosphate pH 6.0, 1.34% Yeast Nitrogen Base without amino acids, 4×10^{-5} % biotin, 0.5% methanol, 1% yeast extract, 2% peptone)

4.2 Construction of PPIC9K-snk6 vector

4.2.1 Vector Preparation

The gene construct was purchased by Eurofins and provided in a pEX-A2 vector. Novablue *E. coli* was transformed with the Eurofins vector by heat shock. Transformed cells were grown overnight in LB media containing ampicillin at 37°C, 180RPM. A midiprep was performed on the overnight culture using a PureLink HiPure Plasmid Midiprep Kit. The isolated pEX-A2 vector containing the gene construct was stored in a -20°C freezer until further use.

The *Pichia* expression vector, PPIC9K was transformed into BL21(DE3) *E. coli* by heat shock. Transformed cells were grown overnight in LB media containing ampicillin. Midiprep was performed on the overnight culture using a PureLink HiPure Plasmid Midiprep Kit. The isolated PPIC9K vector was stored in a -20°C freezer until further use.

4.2.2 Cloning and Subcloning

A double digest of isolated pEX-A2 vector at the AVRII and ECORI sites was performed according to the protocols provided by New England Biolabs. The linearized vector and cut gene construct were run on a 1% agarose gel at 80V for one hour. The gene construct was extracted from the gel using the QIAquick Gel Extraction Kit from Qiagen and immediately used for ligation into PPIC9K.

A sequential digest of isolated PPIC9K vector at the ECORI and AVRII sites was performed due to the short distance between the ECORI and AVRII cut sites. This short distance prevents efficient double digestion of PPIC9K with both enzymes. The isolated PPIC9K vector was initially cut at the ECORI site for an overnight duration, as described

by New England Biolabs. The following day, the reaction was heat inactivated at 65°C for 30 minutes and the cut vector was recovered by ethanol precipitation. After recovery, digestion at the AVRII site was performed. The linearized vector was run on a 1% agarose gel at 80V for one hour and extracted from the gel using the QIAquick Gel Extraction Kit from Qiagen and immediately used for ligation.

Ligation of the gene construct into the cut PPIC9K vector was performed using the Promega LigaFast Rapid DNA ligation kit. The resulting vector, PPIC9K-snk6 was then transformed into Novablue cells by heat shock. The cells containing the vector could grow on LB media containing ampicillin. Colonies containing the appropriate construct were screened by colony PCR using PPIC9K specific primers and grown in LB media containing ampicillin overnight. Midiprep was performed on the overnight culture using a PureLink HiPure Plasmid Midiprep Kit. Frozen stocks of positive colonies were prepared by preparing a 1:1 mixture of overnight culture and 50% glycerol. Frozen stocks were stored in a -80°C freezer.

4.3 Transformation of *P. pastoris*

Transformation of *P. pastoris* strains GS115 and SMD1168 was performed by electroporation according to the procedure by Wu and Letchworth [38]. PPIC9K-snk6 vector was linearized prior to transformation by an overnight restriction digest at the BglII site.

4.4 Screening for Mut^S Transformants

Screening of *P. pastoris* transformants was done according to the *Pichia* Expression Kit protocols established by Invitrogen. Transformants that were identified as Mut^S, were

subjected to a colony PCR using vector specific primers. Positive transformants were then grown in YPD at 28°C, 200 RPM overnight and frozen stocks were prepared.

4.5 Shake Flask Expression of Peptide

Shake flask expression of the peptide chain was performed according to the *Pichia* Expression Kit protocols established by Invitrogen with some modifications. Single colonies were inoculated in 50 mL of BMG or BMGY medium in a 500 mL baffled flask at 28°C, 250 RPM. After 16-18 hours of growth, the OD₆₀₀ was recorded to determine the amount of overnight culture required to inoculate 20 mL of BMM or BMMY medium to an initial OD₆₀₀ of 3.0. 1 mL samples of culture were obtained every 24 hours and centrifuged at 13,000 RPM at 25°C for 5 minutes. The cell-free supernatant was then stored in the freezer at -20°C for analysis. After the 5-day induction period ended as specified by the Invitrogen protocol, the remaining culture was centrifuged at 3,000 RPM at 25°C for 15 minutes. The corresponding cell-free supernatant was then stored in the freezer at -20°C for analysis.

4.6 Fermentation

Fermentation was conducted on the *P. pastoris* transformant that had good expression in shake flasks. All solutions were prepared according to Gleeson et al [50].

4.6.1 PTM Salts Solution

The composition is listed in Table 1. A 1,000 mL solution was prepared and filter-sterilized. The PTM salts solution was stored in room temperature.

Table 1. PTM Salts Solution Composition.

Chemical	
Cupric sulfate·5H ₂ O	2 g
Sodium Iodide	0.08 g
Manganese sulfate	3 g
Sodium molybdate·2H ₂ O	0.2 g
Boric Acid	0.02 g
Cobalt Chloride	0.5 g
Zinc Chloride	7 g
Ferrous sulfate·7H ₂ O	22 g
Sulfuric Acid	5 mL
Water	(to final volume of 1 L)

4.6.2 Biotin Solution

Twenty-five mg of biotin was dissolved in 0.02 M of KOH. An additional 0.02 M KOH was added for a final volume of 100 mL. The solution was then filter-sterilized and stored at 4°C for later use.

4.6.3 Basal Salts Medium

The composition is listed in Table 2. Once the basal media was prepared, it was autoclaved in a 3.5L bioreactor vessel. After autoclaving and letting the media cool, 4 mL/L of PTM salts and 4 mL/L of biotin solution were aseptically added.

Table 2. Basal Salts Medium Composition

Chemical	
Phosphoric Acid, 85%	13.3 mL
Calcium Sulfate·2H ₂ O	2.3 g
Potassium Sulfate	14.3 g
Magnesium Sulfate·7H ₂ O	11.7 g
Potassium Hydroxide	3.9 mL
Glycerol	40 mL
Water	To final volume of 1 liter

4.6.4 Glycerol Feed Solution

A 50% (v/v) glycerol feed solution was prepared and autoclaved. After cooling, 12 mL/L of PTM salts and 12 mL/L of biotin were aseptically added.

4.6.5 Methanol Feed Solutions

A 50% (v/v) solution of methanol and a 100% solution of methanol were prepared. Twelve mL/L PTM salts and 12 mL/L biotin were aseptically added into each solution.

4.6.6 Preparation of Seed Culture

The transformant with the best expression was streaked onto a YPD plate from frozen stock and left to grow overnight in a 28°C incubator. The following day, a single colony from the plate was used to inoculate a 500 mL flask containing 100 mL of BMGY media and left to incubate for 24 hours at 250 RPM, 30°C.

4.6.7 Bioreactor Cultivation

The pH in the bioreactor was adjusted to 5.0 with NH_4OH and the media was warmed with a heating jacket to 28°C . The temperature in the bioreactor was controlled through the Applikon ADI 1030 controller connected with both the heating jacket and a circulating water bath controlled at 4°C . The seed culture was aseptically added into the bioreactor once pH and temperature reached the desired specifications. The initial stir speed in the bioreactor was set at 500 RPM and maintained until the dissolved oxygen dropped below 30%. At this point, the stir speed was manually adjusted to maintain dissolved oxygen above 30%. In addition, an air flow was initiated and manually adjusted to maintain the desired dissolved oxygen saturation.

A glycerol fed-batch phase was initiated using the 50% glycerol solution described previously once a sudden and steady rise in the dissolved oxygen level was observed. Glycerol was fed at a rate of 500 milligrams per minute and stopped once the optical density of the culture was greater than 350.

Methanol induction was initiated using the 50% methanol solution described previously at a rate of 100 milligrams per minute. The 50% methanol solution was fed until dissolved oxygen and pH levels stabilized, indicating the cells had adapted to the methanol.

At this point, feeding of the 100% methanol solution described previously was initiated at a rate of 55 milligrams per minute for the remainder of the run. Afterwards, the fermentation culture was removed from the bioreactor and centrifuged in 250 mL flasks at 3,000 g for 30 minutes. Centrifugation was repeated until all biomass was

removed, leaving cell-free fermentation broth. The broth was then stored in -80°C until it was processed.

4.7 Analysis of Samples

Samples of cell-free supernatant from fermentation and shake flask conditions were concentrated using Amicon Ultra-15 Centrifugal Filter Units with a molecular weight cutoff of 3kDa or 10kDa. The retentate was collected and stored in -80°C for SDS-PAGE analysis.

4.7.1 SDS-PAGE Analysis

Due to the presence of native *Pichia* proteins, a 20% SDS-PAGE gel was prepared to provide better resolution of proteins less than 50kDa in size. Samples were prepared as follows: Fifty microliters of concentrated cell-free supernatant were added to an equal volume of 2X SDS-PAGE loading buffer and boiled at 100°C for five minutes. Twenty microliters of each sample and a 5-245 kDa protein marker purchased from Gold Biotechnology was then loaded onto the gel. Gels were run in a Tris-Glycine buffer at 80V for 60 minutes and then at 120V for 90 minutes. After electrophoresis, gels were washed in DI water and left overnight in Coomassie colloidal blue stain. Gels were then subjected to three DI water washes for visualization of bands. A duplicate electrophoresis run was prepared for each set of samples analyzed and stained in Pierce 6xHis Protein Tag Stain according to the manufacture's protocols. Since there is a 6xHis tag downstream from the polypeptide sequence, the band corresponding to the peptide will fluoresce under UV light after application of the stain.

4.8 Purification of Peptide

Immobilized metal affinity chromatography of cell-free culture supernatant as described by Komives et al. was used to purify the LTNF-15 peptide with some modifications [11].

A gravity column was packed with 5 mL of Ni-NTA resin. The column was equilibrated with a buffer consisting of 300 mM NaCl and 20 mM of sodium phosphate at a pH of 8.0. Afterwards, the resin was transferred into a conical tube containing cell free culture broth and allowed to incubate in an orbital shaker overnight at 4°C. The next day, the mixture of culture broth and resin was drained by gravity into a column and washed with buffer consisting of 300 mM NaCl, 20 mM Sodium Phosphate, and 10 mM imidazole pH 8.0. After washing, the resin was equilibrated in 100 mM Ammonium bicarbonate buffer and transferred into a conical tube. The resin was then incubated overnight with 200 micrograms of endoproteinase Glu-C at 4°C on an orbital shaker. The following day, the resin was drained by gravity into a column and washed with 5 column volumes of 100 mM ammonium bicarbonate. The flow-through was collected and immediately concentrated using a 10,000 MWCO ultrafiltration membrane. The resulting permeate was then collected and concentrated using a 1,000 MWCO ultrafiltration membrane. The resulting retentate was then polished using a 3,000 MWCO ultrafiltration membrane in which the resulting permeate was collected and frozen at -80°C in a freeze-drying flask overnight. The following day, the sample was then lyophilized using a Labconco FreezeZone Benchtop Freeze Dryer for 24 hours. The resulting powder was collected and stored at -30°C for analysis.

4.9 Azocasein Assay

An azocasein assay was conducted according to the protocol available from the manufacture with some modifications [51]. Solutions of 1% (w/v) azocasein, 1% (w/v) of lyophilized *C. atrox* venom, and 4% (w/v) of LTNF-15 peptide in 20 mM of Tris-HCl were prepared. Venom:peptide ratios of 1:1, 1:10 and 1:30 were prepared using 20mM Tris-HCl as a diluent. A 60 ug/mL concentration of *C. atrox* venom with 0.2% azocasein was prepared as a control. All reactions and controls were prepared in triplicate. Reactions were incubated at 37°C for 5, 15, and 30 minutes and stopped by addition of 5% (w/v) trichloroacetic acid (TCA). Samples were centrifuged for 10 min at 3,000 g. Equal volumes of supernatant and 0.5 M NaOH were mixed and analyzed in a plate reader (BioTEK ELX808) at 450 nm.

CHAPTER FIVE

RESULTS AND DISCUSSION

5.1 Plasmid Construction

PPIC9K-snk6 plasmid was constructed as described previously and transformed into Novablue cells using a standard heat shock protocol. The use of a concatemer of peptides is preferable to a single peptide because the resulting size of the peptide concatemer facilitates the purification process. Endoproteinase Glu-C cleaves only on the C end of glutamic acid when an acetate buffer is used for the cleavage step. The cells were plated on LB plates containing ampicillin to select for transformants and incubated overnight at 37°C. The next day several colonies were selected for colony PCR to identify positive transformants. The PCR products were loaded onto a 1% agarose gel for electrophoresis as seen in Figure 1. Of the eight colonies that were selected, only two had the expected PCR product size of 487 bp whereas the remaining colonies had larger than expected PCR products. This can be attributed to a non-ideal vector:insert ratio in the ligation step, which resulted in concatemer formation [53].

Figure 1. Agarose gel electrophoresis of PPIC9K-snk6 *E. coli* transformants. Lane 1 contains the molecular marker. Lanes 2 and 3 contain the appropriately sized PCR product whereas lanes 4-9 contain concatemers.

5.2 *P. pastoris* Transformation

Immediately after electroporation, *Pichia* transformants were plated on auxotrophic plates lacking histidine. Transformants that have incorporated the PPIC9K-snk6 plasmid grew on the plates due to their ability to synthesize histidine whereas wild-type lack this ability and therefore failed to grow [26].

Transformants were screened and identified for the desired Mut^S phenotype. Colonies from the auxotrophic selection plates were patched onto minimal methanol and minimal dextrose plates as described previously. Colonies that exhibited the Mut^S phenotype grew well on minimal dextrose medium but grew slowly on the minimal methanol plate. This

is due to the wild-type AOX1 gene being removed from the genome during plasmid integration via homologous recombination [22].

Clones that were identified as Mut^S phenotype from the minimal methanol plates were subjected to colony PCR to verify Mut^S phenotype. In Figure 2, Mut^S colonies were observed to have a PCR product size of around 500 bp. This is to be expected, as the primers used were the alpha-Factor and 3' AOX1 primers, which would yield a product size of 188 bp without a gene construct [33]. The LTNF-15 gene construct is approximately 292 bp, yielding a PCR product of 487 bp. In addition, Mut^S phenotype yield a single band if using an AOX1 primer whereas Mut⁺ phenotypes would yield two unique bands: wild-type AOX1 and the AOX1 fragment from the PPIC9K plasmid [33].

Figure 2. Agarose gel electrophoresis of PPIC9K-snk6 *Pichia* transformants. Lane 1 contains the molecular marker. Lane 3 contains a PPIC9K control. Lanes 2, 4-8, are colonies that have been verified to be Mut^S by PCR by patching onto minimal methanol plates.

5.3 Shake Flask Expression

After induction, cell-free broth was concentrated as described previously and loaded onto a 20% SDS-PAGE gel. Prior to Coomassie blue staining, the gel was stained with Pierce His-stain according to the manufacturer's protocol and observed under UV light as seen in Figure 3. Since the LNTF-15 peptide chain contains a 6xHis tag, the bands fluorescence under UV light, verifying that the peptide chain is indeed expressed.

Figure 3. His-tag stained SDS-PAGE gel of *Pichia* culture supernatants. Lane 1 the protein maker. Lanes 2-7 are *Pichia* clones, each exhibiting different levels of LTNF-15 expression.

After application of the 6xHis stain, the gel was then washed and subjected to an overnight staining of Coomassie blue seen in Figure 4. Various native secreted *Pichia* proteins are also present in the supernatant.

Figure 4. Coomassie blue stained SDS-PAGE gel of *Pichia* culture supernatants. Lane 1 the protein maker. Lanes 2-7 are *Pichia* clones, each exhibiting different levels of LTNF-15 expression.

5.4 Fermentation

The transformant with the best expression in shake flasks was selected for fermentation. Induction was carried out over a duration of 5 days.

5.4.1 Growth Rate Curve

A growth rate curve was constructed by measuring OD600 over time. As seen in Figure 5, the exponential phase occurs during the glycerol batch and fed-batch phases. Once methanol induction occurs, the cells are in stationary phase. This is to be expected due to the Mut^S phenotype lacking wild type AOX1 and therefore fail to metabolize methanol adequately for cell growth in comparison to Mut⁺ [33].

Figure 5. Growth curve of *Pichia* fermentation.

5.4.2 Protein Production

SDS-PAGE was performed on samples collected at various time points during the methanol induction phase of the run. As seen in Figure 6 and Figure 7, a band of around 11 kDA is present, indicating the peptide is indeed expressed. Protein production was quantified by densitometric analysis using ImageJ and lysozyme as a standard. Figure 8 shows protein production plateaus around 120 hours or 50 hours post induction.

Figure 6. SDS-PAGE analysis of fermentation samples from 70-120 hours. Lane 1 contains the protein marker. Lanes 2-5 contain lysozyme standards used for densitometric analysis. Lanes 6-15 contain supernatant samples collected from the 70th hour of fermentation through the 120th hour.

Figure 7. SDS-PAGE analysis of fermentation samples from 120-170 hours. Lane 1 contains the protein marker. Lanes 2-11 contain supernatant samples collected from the 120th hour of fermentation through the 170th hour. Lanes 12-15 contain lysozyme standards used for densitometric analysis.

Figure 8. LTNF-15 production in *Pichia* fermentation.

5.5 Purification

Flow through from the binding and wash steps were collected for SDS-PAGE analysis. Elution of LTNF-15 peptide resulted using saline buffer as described previously with the addition of 500 mM imidazole. As seen in Figure 9, flow through from the binding step (lane 2) contains LTNF-15 peptide, indicating that the resin exceeded its binding capacity. Flow through from the washing step (lane 3) contains trace contaminating proteins and LTNF-15 peptide. The wash buffer contains 10 mM imidazole, thus contaminating proteins do not require a high imidazole concentration to be washed from the resin. As described in Section 4.8, following washing, the resin is equilibrated with 100 mM ammonium bicarbonate and left to incubate with endoproteinase Glu-C before recovering the LTNF-15 peptide and performing a series of

ultrafiltration steps. However, to ensure LTNF-15 peptide was binding to the Ni-NTA resin, an imidazole elution step was conducted instead. Lane 4 shows the resulting eluent, showing a pure LTNF-15 band. Although there is a single band present in Figure 9, it is plausible to conclude that there are contaminating proteins left, as the range of detection for colloidal Coomassie blue staining is less than 250 ug [53].

Figure 9. SDS-PAGE of LTNF-15 purification using Ni-NTA resin. Lane 1 contains the protein marker. Lane 2 is the flow through from the binding step. Lane 3 is the flow through from the wash step. Lane 4 is the eluted LTNF-15 peptide using imidazole.

5.6 Azocasein Assay

Slight inhibition of *C. atrox* venom was seen with LTNF-15 peptide at a venom peptide ratio of 1:1. Inhibition of about 20% and 30% was observed with LTNF-15 peptide at venom peptide ratios of 10:1 and 30:1 respectively. By comparison, azocasein assay data in Figure 11 for LTNF-11 peptide behaves in a similar fashion. This suggests that LTNF peptides do not neutralize all the proteases present in *C. atrox* venom.

The azocasein assay is suitable for evaluating LTNF activity against *C. atrox* venom due to the venom containing metalloproteinases. Azocasein is a protease substrate which releases azo dye into the solution when the casein is degraded by a protease [55]. This yields TCA-soluble azopeptides that can be detected at an absorbance of 450 nm [55].

Figure 10. Partial neutralization of *C. atrox* venom proteolytic activity by LTNF-15 peptide

Figure 11. Partial neutralization of *C. atrox* venom proteolytic activity by LTNF-11 peptide.

CHAPTER SIX

CONCLUSION AND FUTURE WORK

6.1 Conclusion

LTNF-15 production by *Pichia Pastoris* was achieved by transforming wild type GS115 with the constructed PPIC9K-S6 by electroporation. LTNF-15 was successfully expressed in *P. pastoris* transformants in shake flasks and in a bioreactor. Active LTNF-15 peptide was confirmed by azocasein assay; however the peptide was unable to properly dissolve in solution while conducting *in vivo* assays. This can possibly be due to contaminating material that remained post purification.

6.2 Future Work

Future work will consist of expressing LTNF peptide in *P. pastoris* without the additional hydrophilic residues. This will stabilize the LTNF peptide and therefore be soluble in solution. Currently, the shorter LTNF peptide has been subcloned into the PPIC9K expression vector, and successfully expressed in shake flasks. Further work will be conducted on bioreactor cultivation and purification strategies, before assessing activity in azocasein and mice assays.

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