RECOMBINANT PROTEIN EXPRESSION OF A TICK SALIVA SERINE PROTEASE INHIBITOR

An Undergraduate Research Scholars Thesis

by

GRACE OLUWATIMILEHIN ADENIYI-IPADEOLA

Submitted to the Undergraduate Research Scholars program at
Texas A&M University
in partial fulfillment of the requirements for the designation as an

UNDERGRADUATE RESEARCH SCHOLAR

Approved by Research Advisor: Dr. Albert Mulenga

May 2017

Major: Biomedical Sciences

TABLE OF CONTENTS

ABSTRACT

Expression of a Recombinant Tick Saliva Serine Protease Inhibitor

Grace Oluwatimilehin Adeniyi-Ipadeola
Department of Biomedical Sciences
Texas A&M University

Research Advisor: Dr. Albert Mulenga
Department of Veterinary Pathobiology
Texas A&M University

The *Ixodes scapularis* tick is a medically important vector of five out of the 15 reportable human tick borne disease agents, making it a great concern to public health. In order to create vaccines against tick borne disease agents, it is imperative to understand the mechanisms of tick feeding. During feeding, ticks inject saliva containing several proteins into host, including serpins (**ser**ine **p**rotease **in**hibitors). Serpins are the largest superfamily of protease inhibitors and are believed to aid the successful transmission of disease agents into a host by allowing ticks to evade host defenses during feeding. Tick serpins inhibit host proteolytic defense pathways like coagulation, inflammation, hemostasis, and much more. In this study, yeast recombinant protein expression constructs of Serpin 1B, was prepared using *Pichia pastoris* expression system. Large-scale expression of Serpin 1B, purification, and substrate hydrolysis experiments were subsequently performed. This project successfully cloned and expressed Serpin1B using Pichia pastoris expression system. In this study, biochemical characterization showed that rSerpin1B inhibits chymotrypsin by at least 33% and trypsin by 99%.

DEDICATION

This project is dedicated to my family and most especially my mom, Adepeju Adeniyi-Ipadeola. Thank you for always encouraging me to challenge myself, for your support and prayers. I could not have made it this far without you and I am so incredibly blessed to be your daughter.

ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. Mulenga for recognizing the potential in me, and believing in my abilities for the past four years. I am honored to be your protégé and I am grateful for the learning environment that you have created for me outside the classroom. Thank you for giving me the opportunity to discover my passion for research.

I would also like to thank the current and past graduate and post-doctoral students in Dr. Mulenga's laboratory; Taylor Hollman, Tae Kim, Mariam Bakshi, Patricia Ishii, Dr. Lindsay Porter, Dr. Zeljko Radulovic, and Dr. Lucas Tirloni. Thank you for providing your time and much needed advice in the laboratory. Without your contributions to my success, progress on this project would have been more difficult.

Finally, this project would not have been possible without the funding support from the National Institute of Health: National Institutes of Allergy and Infectious Diseases. Receiving the Diversity Fellowship in Life Science Research has strengthened my decision to pursue a career in research.

CHAPTER I

INTRODUCTION

Ticks are vectors of pathogens that impact global public health, they are the second most important vectors of human disease agents after mosquitoes (Mulenga et al., 2009). Pathogens that ticks transmit cause many important zoonotic tick-borne diseases (TBD), such as babesiosis, erhlichiosis, anasplasmosis, borreliosis, among others (Dantas-Torres et al., 2002). *Ixodes scapularis*, the blacklegged tick or deer tick, is a medically important tick species, known to transmit six out of the 15 reportable human tick borne disease agents, including those responsible for anaplasmosis, babesiosis, and borreliosis (Dantas-Torres et al., 2002).

Currently, tick control has been based on acaricides, however studies have been shown that acaricide-resistant tick populations are being selected (Rajput et al., 2006). Due to this shortcoming, an alternative and more efficient control method is being considered through vaccination (Rajput et al., 2006). In order to develop effective anti-tick vaccines, it is important to identify effective vaccine target antigens and understand the mechanism of tick feeding.

The mouthparts of ticks have evolved to allow it to successfully latch onto hosts and feed for days. During feeding, the tick lacerates host tissue and small blood vessels in the skin, to create a feeding lesion, from which it and sucks up the blood that bleeds into the wounded area. This feeding mechanism has evolved to help ticks overcome host's defenses against hemostasis. Accordingly, tick saliva contains anti-clotting, anti-platelet, vasodilatory, and anti-inflammatory components to help the tick feed successfully (Franceschetti el al., 2009). Tick saliva contains several proteins, including proteins belonging to the serpin (serine proteinase inhibitor) superfamily. In arthropods, these proteins are involved in immune system, morphogenesis and

development (Meekins et al., 2017). In mammals, serpins regulate pathways that are essential to life such as blood coagulation, inflammation, complement activation, and fibrinolysis (Rau et al., 2007). Serpin encoding cDNAs have been identified and cloned in many tick species including, the blacklegged tick *Ixodes scapularis* (Mulenga et al., 2001).

From this finding, it was proposed that through serpins, ticks can evade host defenses and if disrupted, serpins can compromise a tick's ability to feed, acquire and transmit diseases (Mulenga et al., 2001). It is hypothesized that ticks use serpins to accomplish successful feeding through inhibition of protease mediators of host defense pathways (Mulenga et al., 2001).

Serpins inhibit serine proteases by a process called "suicide substrate inhibition mechanism". Through this process, serpins undergo irreversible conformational changes to inhibit their target protease. This inhibition is achieved through the formation of a covalent complex in which both serpin and protease are rendered inactive (Meekins et al., 2017). The reactive center loop (RCL) of a serpin is complementary to the protease's substrate-binding site (Meekins et al., 2017). This allows the serpin to be recognized as a substrate by target proteases. Cleavage of the RCL results in a conformational change in the serpin that leads to an irreversible inhibition of the target protease (Meekins et al., 2017). The trapped protease is rendered inactive because the loop insertion translocates the protease and distorts its active site (Meekins et al., 2017).

Studies have provided evidence suggesting the potential of serpins as targets for tick control. According to these findings, when ticks feed on animals immunized with recombinant tick serpins, they obtain smaller blood meals which leads to reduced tick fertility (Imamura et al., 2005). Previous studies have identified and cloned more than 30 serpin encoding cDNAs in some medically important tick species including, *Amblyomma americanum* (Mulenga et al., 2007), *Ixodes ricinus* (Leboulle et al., 2002), and *I. scapularis* (Ribeiro et al 2006). Identification and

characterization of at least 45 *I. scapularis* serpin genes showed that 93% of these serpins are generally considered inhibitory (Mulenga et al., 2009).

In this research, transcriptional profiling, recombinant serpin 1B cloning, recombinant expression and functional characterization was performed. Subsequent research would actively describe the role(s) of native serpin 1B at the site of tick feeding using the recombinant protein produced in this research.

CHAPTER II

METHODS

Semi-qualitative expression analysis of Serpin 1B

Semi-qualitative analysis of several cDNA transcripts was performed using actin as a housekeeping gene. The Serpin 1B coding cDNA was synthesized from total RNA obtained from midgut (MG) and salivary gland (SG) *I. scapularis* ticks that were fed on a host for 24, 48, 72, 96, and 120 hours. Semi-qualitative polymerase chain reactions (Semi q-PCR) were run using tick actin primers and gene specific serpin 1B primers. The annealing temperatures were determined for both primers (59.3 ºC for actin and 54.9 ºC for serpin 1B). The reaction for actin was run for 28 cycles while serpin 1B was run for 35 cycles.

PCR amplification of Serpin 1B mature protein open reading frame

PCR amplification of Serpin 1B mature protein open reading frame was performed using cDNA from fed larvae. To construct the expression plasmid, the Serpin 1B open reading frame (ORF) without the signal peptide coding region was unidirectionally subcloned into pPICZαC vector using primers. The primers were designed using a program in MacVector DNA sequence analysis software and diluted to make a 10 µM working solution. A mature Serpin1B protein open reading frame was sub-cloned into pPICZαC ClaI and NotI restriction enzymes sites using forward and reverse primers described in table 1.

The optimal annealing temperature was determined to be 55 ºC. Serpin 1B transcription patterns were determined in larvae and 24, 48, and 72 hours fed nymph. Amplification of the gene was accomplished through polymerase chain reaction (PCR). The PCR mixture contained Taq

polymerase (GoTaq), forward and reverse primers, fed larvae and nymph tick template cDNA (24, 48, and 72 hours fed), and water. The products of the PCR reaction were analyzed by electrophoresis. This was accomplished by running the products on a 1% agarose gel that contained 1µg/mL ethidium bromide for 20 minutes. After this process, the gel was viewed under UV light to visualize amplified DNA bands. The PCR bands were excised from the gel and purified using standard gene-clean protocols.

Primer	Sequence	Restriction Enzyme
Yeast Forward	5'-**ATCGATG** CGG TAC GAA AAT GAG ATG AGG CTT G-3'	Cla I
Yeast Reverse	5'-**GCGGCCGC** GCG GGA CAA GTC AAC CAT CTC-3'	Not I

Table 1: Sub-cloning of a mature Serpin1B protein open reading frame into pPICZαC expression vector was accomplished using these primer sequences with added restriction enzymes (bolded)

Serpin 1B cDNA cloning and sequencing

The gene cleaned Serpin 1B mature protein ORF was cloned into a pGEM-T (Promega, Fitchburg, Wisconsin, USA) TA cloning vector. The pGEM-T-Serpin1B plasmid was used to transform competent DH5α *Escherichia coli* cells using heat shock chemical transformation. The transformed *E. coli* was plated onto Luria broth (LB) agar plates containing ampicillin for specific selection of transformed *E. coli*. Two colonies were randomly selected and inoculated in SOB with ampicillin overnight at 37˚C with shaking. The bacteria cultures were subjected to routine mini-prep procedures to purify the pGEM-T-Serpin1B plasmid. After the mini-prep, a 1:500 dilution of the plasmid was performed. The dilution served as the template for a PCR to confirm the insert (insert check).

To release the Serpin1B cDNA insert, the mini-prep product was double digested using Cla I and Not I enzymes, BSA, buffer 3.1, and water (Figure 1). For maximum recovery, 3µL of

Cla I and 1µL of Not I were used for the reaction because Not I has a 100% recovery with buffer 3.1 while Cla I has a 50% recovery with the buffer. The digestion reaction mixtures were incubated over night at 37˚C. Afterwards, the reaction mixture was electrophoresed on a 1% agarose gel containing 1µg/mL ethidium bromide. The electrophoresis separated the Serpin1B cDNA insert from the plasmid backbone causing a release of the insert. The released target was extracted and cleaned up using routine gene clean protocols. During the preparation of the Serpin1B insert, the pPICZαC plasmid was digested with the same restriction enzymes and protocol as the Serpin1B insert. The gene-cleaned product of the Serpin 1B was ligated into pPICZαC expression plasmid. The pPICZαC-Serpin1B plasmid was used to transform DH5α competent E. coli cells. Following miniprep, the pPICZαC-Serpin1B plasmid was quantified and sequenced using the BigDye Sequencing Master Mix with AOX 5' and AOX 3' promoter primers using routine procedures. The sequence analysis was done using MacVector software.

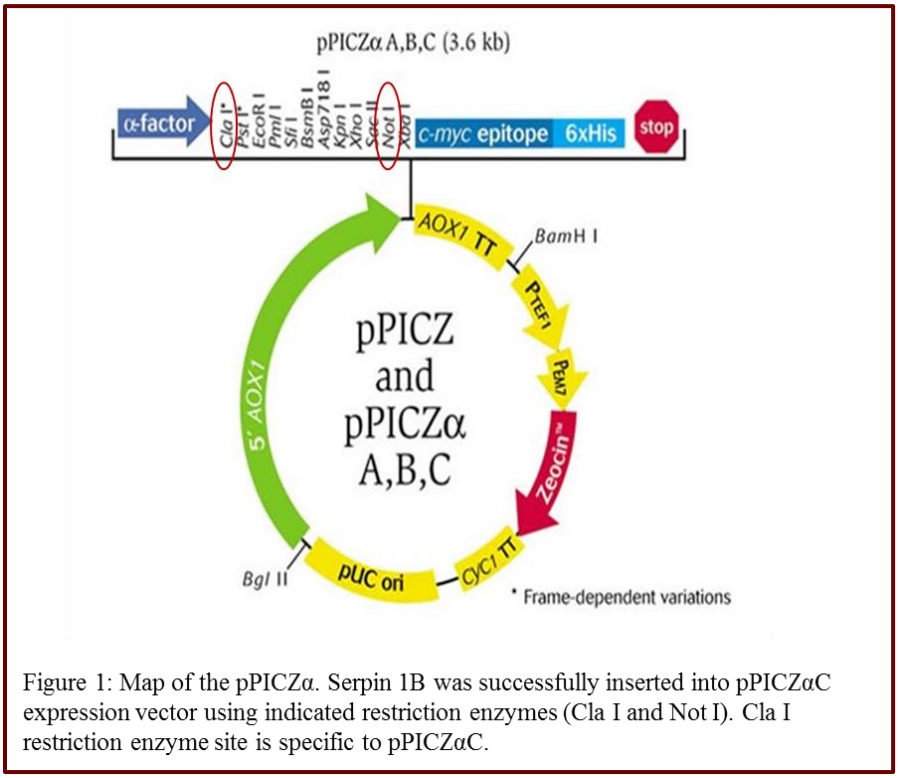

Figure 1: Map of the pPICZα. Serpin 1B was successfully inserted into pPICZαC expression vector using indicated restriction enzymes (Cla I and Not I). Cla I restriction enzyme site is specific to pPICZαC.

Transformation of yeast and induction of rSerpin1B expression

Following mini-prep, the pPICZαC-Serpin1B plasmid was linearized using Sac I restriction enzyme and used to transform *Pichia pastoris* X-33 strain (Life Technologies) by electroporation. Transformed colonies were selected on yeast extract peptone dextrose medium with sorbitol (YPDS) agar plates containing zeocin (100 µg/ ml). Positive transformants were inoculated in buffered glycerol-complex medium (BMGY) and grown overnight at 28°C with shaking (240 rpm). Subsequently, the cells were used to inoculate buffered methanol-complex medium (BMMY) to OD_{600nm} of 1.0 after which protein expression was induced by adding methanol to 0.5% final concentration every 24 h for 5 days. rSerpin 1B in spent culture media was precipitated by ammonium sulfate saturation (525 g/L of media) with stirring overnight at 4°C. The precipitate was pelleted at 11,200 g for 1 h at 4°C and re-suspended in, and dialyzed against, 20 mM Tris-HCl buffer pH 7.4. To verify expression of rSerpin 1B, western blotting analysis was performed using the horseradish peroxidase (HRP)-labeled antibody to the C-terminal hexahistidine tag (Life Technologies) diluted to 1:5000 in 5% blocking buffer (5% skim milk powder in PBS with Tween-20). The positive signal was detected using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare).

Affinity purification of rSerpin 1B

rSerpin 1B was affinity purified under native conditions using Hi-Trap Chelating HP Columns (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). Affinity purified rSerpin 1B was dialyzed against 20 mM Tris-HCl buffer pH 7.4 for downstream assays. To verify purity and background contamination, affinity purified rSerpin 1B was resolved on a 10% SDS-PAGE gel and silver stained. Samples with the least background were selected and concentrated by

centrifugation using MicroSep Centrifugal Concentration Devices (Pall Corporation, Port Washington, NY, USA).

Protease Inhibitor (PI) profiling

Inhibitory activity of rSerpin1B was tested against two mammalian serine proteases—trypsin and chymotrypsin. Reagents were mixed at room temperature in triplicate. One μM of rSerpin1B was pre-incubated with amounts of the enzyme—trypsin or chymotrypsin—for 15 min at 37°C in 20 mM Tris-HCl, 150 mM NaCl, BSA 0.1%, pH 7.4 buffer. Chromogenic substrate, N-Succinyl-Ala-Ala-Pro-Phe-pNA for chymotrypsin, and N-Benzoyl-Phe-Val-Arg-pNA for trypsin was added in a 100 μL final reaction volume and substrate hydrolysis was measured at A_{405nm} every 11 secs for 15 min at 30°C using the Synergy H1 Microplate reader (BioTek). Acquired A_{405nm} data was analyzed using Prism 7 software (GraphPad Software, La Jolla, CA, USA).

CHAPTER III

RESULTS

Experimental design

Prior to the start of the research project, a plan of action was designed with the goals of the project in mind as shown in Figure 2A and 2B. In the first step, Serpin1B translated amino acid sequence was subjected to SignalP analysis to detect the pre-protein the signal sequence. The signal peptide coding region was removed. PCR primers were used to amplify the mature protein open reading frame (ORF). This was then inserted and cloned in the recombinant protein expression plasmids in yeast cells. Subsequently, recombinant protein expression plasmids were used to transform yeast cells and the protein was purified and characterized.

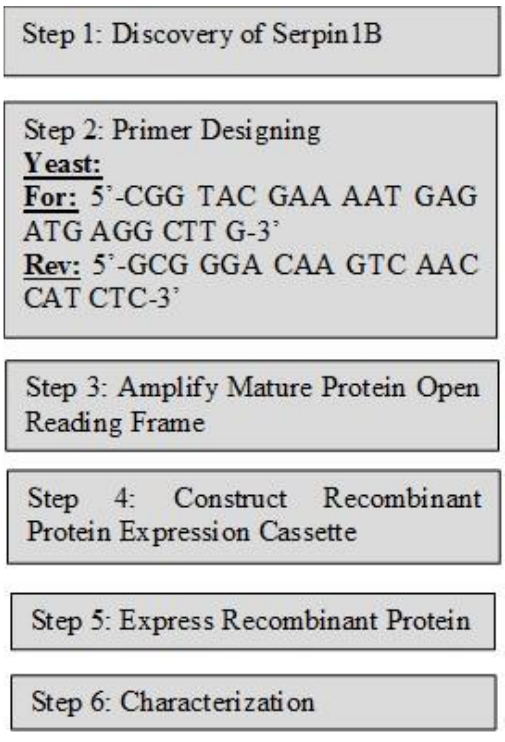

Figure 2A: Experimental Design

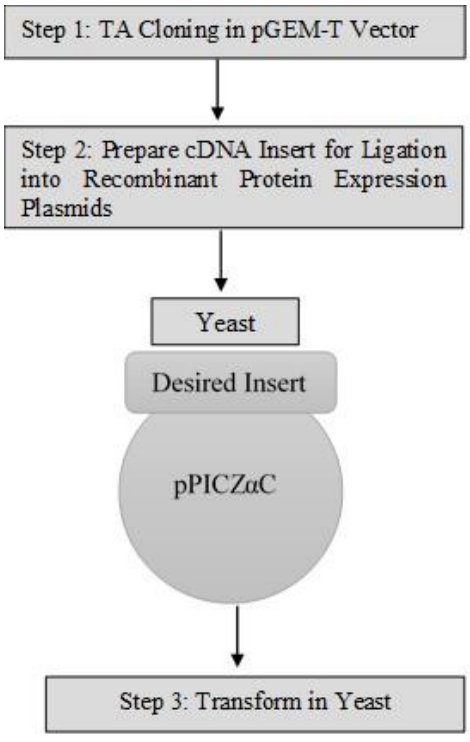

Figure 2B: Construction of Recombinant Protein Expression Plasmid

Semi-qualitative analysis of the expression of serpin 1B transcripts

According to figure 3, PCR products obtained from cDNA samples from the mid gut and salivary gland of *I. scapularis* showed that the cDNA was expressed in Serpin 1B at 35 cycles.

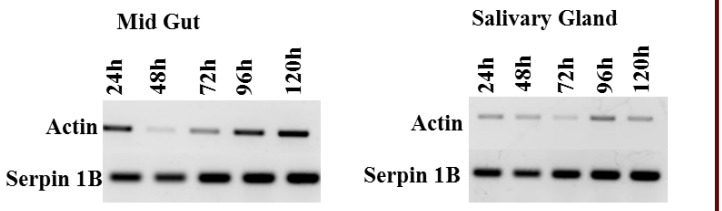

Figure 3: Semi-qualitative expression analysis of serpin 1B showed that the cDNA was expressed in Serpin 1B at 35 cycles.

Serpin1B PCR Primer amplified the expected 1000 base band

I. scapularis Serpin1B ORF, (GenBank accession number XM002415846) was cloned using PCR primers that were designed using a program in MacVector DNA sequence analysis software**.** The optimum annealing temperature for Serpin1B primer was determined by subtracting 5°C from the lowest primer's T_m. Thus, the annealing temperature was 55°C. The extension time was determined based on the expected size of the target DNA. Following PCR optimization, Serpin1B ORF primers were used to determine Serpin1B mRNA qualitative transcription pattern in *I. scapularis* larvae and nymph (24, 48, and 72hr fed) cDNA. According to figure 4, the band is strongest at larval stage demonstrating that the Serpin is highly expressed at that stage in *I. scapularis'* life cycle.

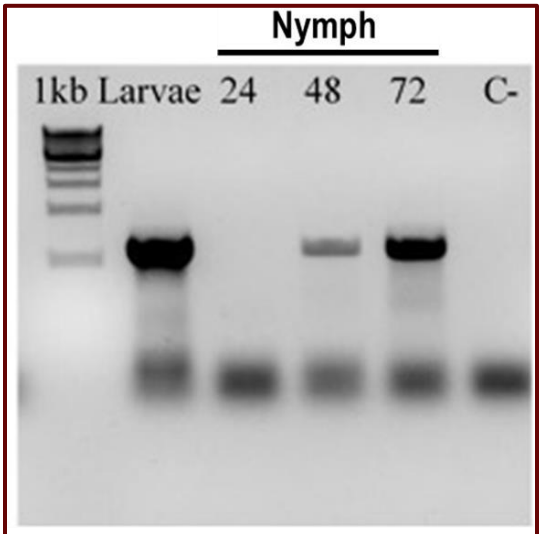

Figure 4: PCR amplification of fed larvae and nymphs (24, 48, and 72 hours after feeding). The strong band at 1000 bp for larva cDNA demonstrates that the mRNA expression of Serpin 1B is most active at the larval stage, and increases in nymphs as they feed longer.

Construction of recombinant Serpin 1B expression plasmid

After PCR amplification, Serpin1B was excised and purified from agarose gels using the manufacturer's protocol (Omega Bio-tek, Norcross, GA). The purified Serpin1B cDNA was successfully cloned into pGEMT vector. According to figure 5A, pGEMT-Serpin1B plasmid and empty pPICZαC vector were simultaneously double digested using ClaI and NotI restriction enzymes (New England Biolabs, Ipswich, MA). The purified products were ligated and transformed into DH5α E. coli cells. Transformations were plated onto LB agar containing 100 µg/ml zeocin. Figure 5B shows results of the insert check performed using Serpin1B specific primers. Colonies 2 and 10 in figure 5B were inoculated and purified with Omega miniprep kit (Figure 5C). Sequencing analysis of the isolated plasmid in figure 5C confirmed that rSerpin 1B in pPICZαC is in frame for recombinant protein expression.

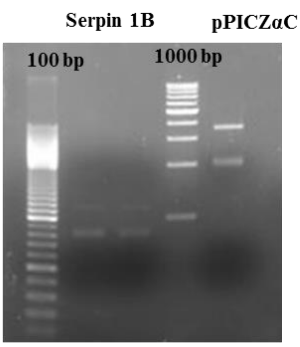

Figure 5A: Restriction enzyme digest of Serpin 1B and pPICZαC shows a release of the insert from the plasmid backbone.

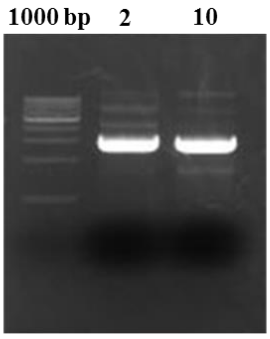

Figure 5C: Colonies 2 and 10 were selected from figure 4B for isolation. Minipreparation of Serpin1B-pPICZαC isolated the plasmid DNA from bacteria culture. The purified plasmids are supercoiled.

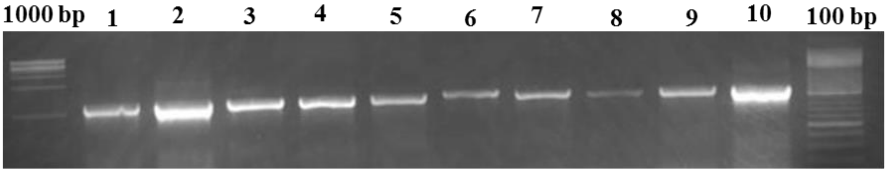

Figure 5B: Insert check of 10 randomly selected Serpin1B-pPICZαC bacteria colonies showed positive bands with colonies 2 and 10 exhibiting the strongest bands.

Recombinant Serpin 1B was expressed in *Pichia pastoris*

Figure 6 shows that rSerpin 1B was successfully electroporated into *Pichia pastoris.* Figure 7 summarizes western blotting analysis confirming that rSerpin 1B was expressed in *P. pastoris.* The pPICZαC plasmid expresses recombinant proteins that are fused with a c-terminus histidine tag. Western blotting analysis in figure 7 shows that rSerpin 1B was most secreted on the third day of expression. Affinity purification eluted rSerpin 1B from the affinity column at 50, 100 and 500mM imidazole concentration. SDS Page electrophoresis with silver staining was performed to determine protein purity.

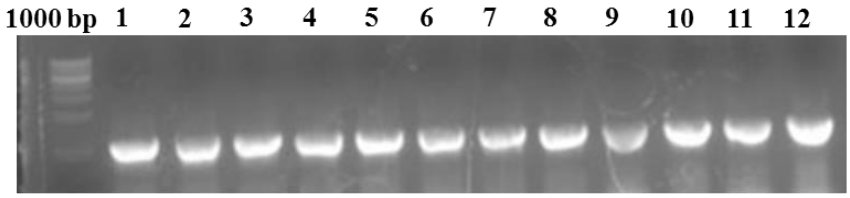

Figure 6: Serpin1B-pPICZαC X-33 insert check proves that Serpin 1B recombinant plasmid was successfully electroporated into *Pichia pastoris* X-33 strain.

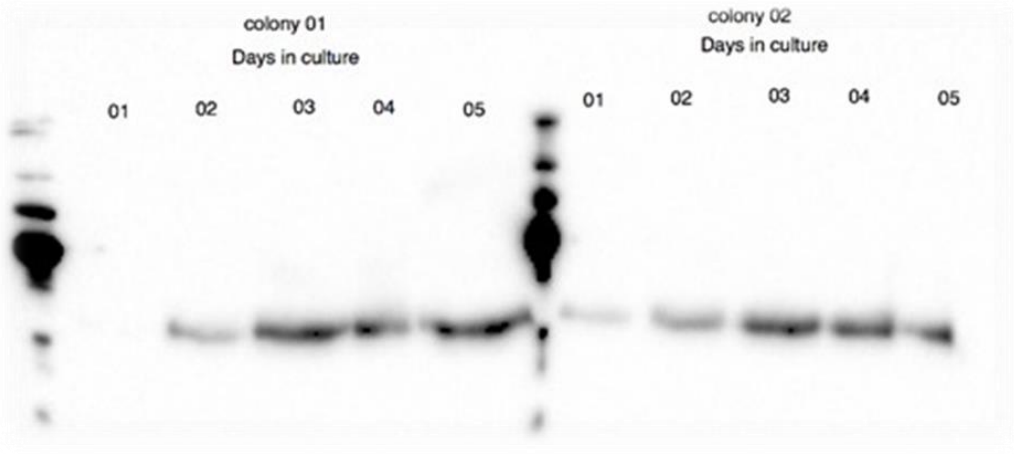

Figure 7: Western blotting analysis confirmed that rSerpin 1B was expressed in *Pichia Pastoris*. The protein was mostly expressed on the third day of culture.

Substrate hydrolysis showed inhibitory properties of recombinant Serpin 1B.

rSerpin 1B characterization with archetype enzymes, trypsin and chymotrypsin was performed via substrate hydrolysis experiments. Figure 8 illustrates inhibitory properties of rSerpin 1B. Characterization of rSerpin 1B showed a 99% inhibition of trypsin, consistently proving that rSerpin 1B likely inhibits trypsin-like serine protesases. Figure 8 also shows a 33% inhibition of chymotrypsin by rSerpin 1B.

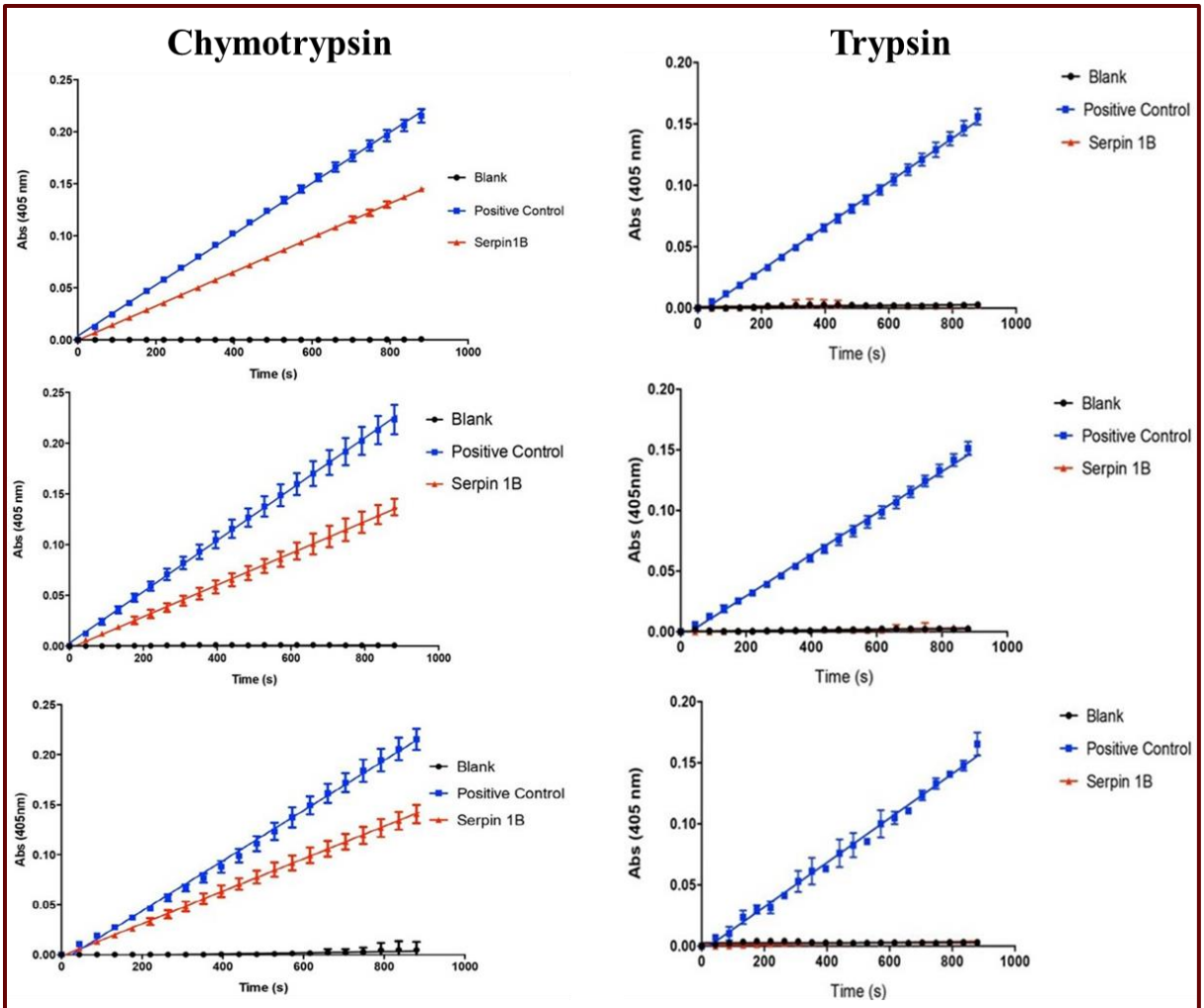

Figure 8: Characterization of rSerpin 1B shows that it partially inhibits chymotrypsin and fully inhibits trypsin. The percentage of inhibition for chymotrypsin and trypsin was 33% and 99%, respectively.

CHAPTER IV

CONCLUSION

The focus of research in tick vaccine development is currently based on identification, molecular cloning, in vitro production, and characterization of tick proteins involved with successful blood feeding, regulation of host immune responses, and pathogen transmission by ticks. The goal of this research—to clone, express and characterize rSerpin 1B—was accomplished with promising results. Subsequent research would identify trypsin-like serine proteases that may be inhibited by rSerpin 1B during tick feeding. Results from this research lays a foundation for further studies that aim to describe the role(s) of native serpin 1B at the site of tick feeding.

REFERENCES

Ibelli, A. M., Kim, T. K., Hill, C. C., Lewis, L. A., Bakshi, M., Miller, S., et al. (2014). A blood meal-induced ixodes scapularis tick saliva serpin inhibits trypsin and thrombin, and interferes with platelet aggregation and blood clotting. *International Journal for Parasitology, 44*(6), 369-379. doi:10.1016/j.ijpara.2014.01.010

Imamura, S., da Silva Vaz Junior, I., Sugino, M., Ohashi, K., & Onuma, M. (2005). A serine protease inhibitor (Serpin) from haemaphysalis longicornis as an anti-tick vaccine. Vaccine, 23(10), 1301-1311. doi:S0264-410X(04)00666-8

Kim, T. K., Tirloni, L., Pinto, A. F., Moresco, J., Yates, J. R.,3rd, da Silva Vaz, I.,Jr, et al. (2016). Ixodes scapularis tick saliva proteins sequentially secreted every 24 h during blood feeding. *PLoS Neglected Tropical Diseases, 10*(1), e0004323. doi:10.1371/journal.pntd.0004323

Leboulle G, Rochez C, Louahed J, Rutti B, Brossard M: Isolation of Ixodes ricinus salivary gland mRNA encoding factors induced during blood feeding. Amer J Trop Med Hygiene. 2002, 66: 225-233.

Meekins, D. A., Kanost, M. R., & Michel, K. (2017). Serpins in arthropod biology. *Seminars in Cell & Developmental Biology, 62,* 105-119. doi:10.1016/j.semcdb.2016.09.001.

Mulenga A, Khumthong R, Blandon MA: Molecular and expression analysis of a family of the *Amblyomma americanum* tick Lospins. J Exp Biol. 2007, 210 (Pt 18): 3188-3198. 10.1242/jeb.006494.

Mulenga, A., Khumthong, R., & Chalaire, K. C. (2009). Ixodes scapularis tick serine proteinase inhibitor (Serpin) gene family; annotation and transcriptional analysis. *BMC Genomics, 10,* 217-2164-10-217. doi:10.1186/1471-2164-10-217

Rajput, Z. I., Hu, S. H., Chen, W. J., Arijo, A. G., & Xiao, C. W. (2006). Importance of ticks and their chemical and immunological control in livestock. *Journal of Zhejiang University.Science.B, 7*(11), 912-921. doi:10.1631/jzus.2006.B0912

Rau, J. C., Beaulieu, L. M., Huntington, J. A., & Church, F. C. (2007). Serpins in thrombosis, hemostasis and fibrinolysis. *Journal of Thrombosis and Haemostasis : JTH*, *5*(Suppl 1), 102–115. http://doi.org/10.1111/j.1538-7836.2007.02516.x

Ribeiro JM, Alarcon-Chaidez F, Francischetti IM, Mans BJ, Mather TN, Valenzuela JG, Wikel SK: An annotated catalog of salivary gland transcripts from Ixodes scapularis ticks. Insect Biochem Mol Biol. 2006, 36: 111-129. 10.1016/j.ibmb.2005.11.005.

Tirloni, L., Seixas, A., Mulenga, A., Vaz Ida, S.,Jr, & Termignoni, C. (2014). A family of serine protease inhibitors (Serpins) in the cattle tick rhipicephalus (boophilus) microplus. *Experimental Parasitology*, *137*, 25-34. doi:10.1016/j.exppara.2013.12.001