CHARACTERIZATION OF AN *IXODES SCAPULARIS* SERINE PROTEINASE INHIBITOR

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Characterization of an *Ixodes scapularis* Serine Proteinase Inhibitor

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The blacklegged tick, *Ixodes scapularis* is a medically important tick species that transmits six of the fifteen human tick-borne disease agents in the United States. Without successful feeding, ticks cannot cause damage to the host nor transmit any disease agents. Thus, a deeper understanding of how ticks accomplish feeding could lead to the identification of proteins to target for development of innovative tick control methods. Members of the serpin superfamily (serine protease inhibitors) are among proteins thought to facilitate tick feeding. In order to complete feeding, ticks have to overcome serine protease mediated pathways that are controlled by serpins. This honors thesis describes the characterization process of *I. scapularis* tick Serpin 1A. In addition to providing qualitative and semi-quantitative RT-PCR analysis of Serpin 1A in the nymph and female adult development stages at different feeding intervals (unfed, 24h, 48h, 72h, 96h, and 120h). The Serpin 1A mature open reading frame (ORF_{1A}) was successfully subcloned into the yeast recombinant protein expression plasmid, pPICZαC, which was effectively used to transfect *Pichia pastoris* strain X-33, yeast cells. Efforts to induce recombinant Serpin 1A protein failed, therefore halting the biochemical characterization of this protein. Further research into the

expression of recombinant Serpin 1A is needed for follow-up experiments to characterize the role(s) of Serpin 1A in tick feeding. Even though, characterization of the protein was halted, the qualitative and semi-quantitative data revealed that Serpin 1A mRNA was expressed at different feeding intervals and highly expressed during the first day and second day of feeding in the salivary gland. These findings suggest that Serpin 1A is associated with the *I. scapularis* tick feeding physiology. Moreover, it implies that this serpin is mostly associated with the early stages of tick feeding in nymph and female adult tick organs. Furthermore, these results propose that Serpin 1A may play a role in pathogen development, since the salivary gland is considered to be the site for pathogen development in tick anatomy. Most tick borne pathogens spread by this species are transmitted after the tick has for more than one day by the early life states and female adult stage. Thus, this information on Serpin 1A could be utilized to target this serpin in an anti-tick vaccine that is aimed at preventing tick borne disease transmission.

DEDICATION

I would like to dedicate this research to my mother, Luana Golden, an influential, independent, and strong-willed role model in my life. This dedication in this paper and future papers to come is my way of lifting you just like you raised me to be the women I am today.

ACKNOWLEDGEMENTS

I would like to thank my research advisor, Dr. Albert Mulenga for the mentorship, knowledge, support, and resources he has provided through the course of this research and during my time at the Mulenga Laboratory.

I would like to show gratitude to the all of the graduate and post-doctoral researchers in the Mulenga Laboratory for teaching me all the techniques over the course of four years, Dr. Lucas Tirloni, Dr. Zeljko Radulovic, Dr. Mariam Bakshi, Ms. Taylor Hollmann, Ms. Lauren Lewis, Ms. Lindsey Porter, and Mr. Tae Kim.

CHAPTER I

INTRODUCTION

The tick species *Ixodes scapularis* belongs to the family Ixodidae also known as the hard tick family [1]. Most hard ticks are three-host ticks, feeding on a different host in each one of their mobile life stages (larva, nymph, and adult) [1, 2]. In the *I. scapularis* life cycle, the mobile life stages that are the most likely to bite animals and humans are the larval, nymph, and female adult stages [2]. The nymph and larval stage are more likely to transmit tick-borne pathogens because of their small size going undetected for an extended period of time [1, 2]. This undetected period of time is important since it takes 24 hours or more for a tick to transmit a disease while feeding on a host [1, 2]. In the case of Lyme disease, the causative agent, *Borrelia burgdorferi* spread by *I. scapularis* is transmitted primarily at 36 to 48 hours or more during tick feeding [2].

Ticks have characteristics that make them ideal vectors of arthropod-borne disease to a wide variety of host worldwide, posing an economic and health concern [3, 4]. *I. scapularis* spreads about forty percent of the pathogens that cause human tick-borne diseases (TBDs) in the United States: Lyme disease, *Borrelia mayonni*, *Borrelia miyamotoi*, babesiosis, anaplasmosis, and Powassan virus disease [5]. Typically acaricides are used to control tick populations to decrease the spread of pathogens that cause TBDs. However, this control method has been found to negatively affect the environment and has resulted in the increase of pesticide resistant ticks [6,7]. Anti-tick immunization has been demonstrated to be a better alternative tick control method [8, 9]. Since transmission of disease agents occur during tick feeding, a deeper understanding of the tick feeding physiology is necessary to find weaknesses that can be targeted for anti-tick vaccine development [10]. Ticks accomplish feeding by disrupting the host tissue and sucking up

blood that bleeds into the wound referred to as the feeding site [10]. Under normal host physiology, the tick feeding style is expected to trigger host defenses such as inflammation, blood clotting, and the complement pathway which, stop further blood loss, tissue repair, and prevent tick-borne disease agent colonization. However, the tick completes feeding by injecting various immuno-modulating proteins that evade the host immune system, counteracting the host defenses, which allows effective pathogen transmission [11, 12]. The host defense pathways are serine protease mediated and tightly controlled by serine protease inhibitors (serpins) [13]. Serpins are found in all animal kingdoms [14, 15, 16, 17, 18]. The diverse biochemical and biological features of these proteins are what make them so versatile and abundant in nature [10, 13, 17, 19]. Some of these features have led to the inhibition of the serine proteinases of the chymotrypsin family, various cysteine proteinases, hormone transport, and blood pressure regulation [17, 19, 20]. On this basis, tick encoded serpins were projected to represent important target antigens for anti-tick vaccine development [21]. Tick serpins have been found to mediate inflammation, anticoagulation, and anti-hemostasis as well as cause the inhibition of various enzymes [22]. Several serpins that may aid in the *I. scapularis* feeding process have been identified [23]. The long-term goal is to identify serpins that are essential to *I. scapularis* tick feeding and transmission of tick-borne disease agents. To aid in this long-term goal, the purpose of this paper was to characterize Serpin 1A in the *I. scapularis* tick feeding physiology as well as analyze its expression in tick tissues (nymph, female adult salivary gland, and female adult midgut) over the course of five days.

CHAPTER II

METHODS

(a) PCR Amplification of Serpin 1A Open Reading Frame

Templates of cDNA were synthesized from total RNA of fed *I. scapularis* larvae, whole nymphs (unfed, 24 hours, 48 hours, and 72 hours), dissected salivary glands (SG) and midguts (MG) of unfed female adult ticks and those that fed from 24 to 120 hours. The polymerase chain reaction (PCR) mixture contained: ORF_{1A} forward primer, ORF_{1A} reverse primer, Promega GoTaq Green Master Mix, *I. scapularis* cDNA templates described prior, and nucleus-free water. Table 1 summarizes the thermocycler conditions used to process the PCR reaction steps 2 through 4 were repeated for thirty-five cycles. Electrophoresis was performed on the PCR products using a 1% agarose gel containing 1µg/mL ethidium bromide, and set to run for 25 minutes at 100 volts. After electrophoresis, the bands of the amplified ORF_{1A} were visualized in an agarose gel using ultraviolet (UV) light. The PCR product was then extracted and purified using the Omega E.Z.N.A. Gel Extraction Kit.

Table 1. Serpin 1A Open Reading Frame Amplification PCR Conditions.

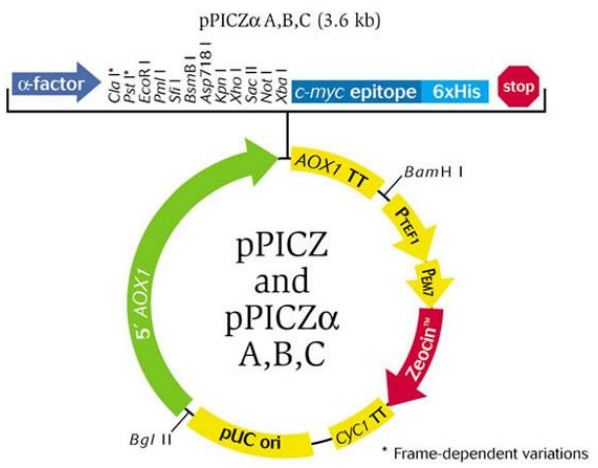
Steps	Name of Condition	Temperature of Condition	Time of Condition
1	Initial denature	95ºC	3 minutes
2	Denature	95ºC	30 seconds
3	Annealing	55ºC	30 seconds
4	Extension	72ºC	1:30 minutes
5	Final Extension	72ºC	5 minutes
6	Hold	12ºC	∞

Table 2. Open Reading Frame Primer Information

Primer	Sequence
Serpin 1AORF_{1A} Forward	5'-ATGCGACGTTTGATCATCTTCGTG-3'
Serpin 1A ORF_{1A} Reverse	5'-GTTCGCTGGACAGGTCAATAATCTT-3'

(b) Serpin 1A cDNA Cloning and Sequencing

Only the amplified product from the larvae stage was utilized to proceed to pGEM-T ligation. The ORF_{1A} was cloned into the pGEM-T cloning vector using T-A cloning (Promega pGEM-T Vector System). Then the recombinant plasmid was introduced into DH5α *Escherichia coli* host cells by transformation using the heat shock method. The transformation product was plated on low salt Luria-Bertani (LB) agar plates treated with ampicillin and placed in an incubator at 37 ºC overnight. To verify the ligation into the pGEM-T vector was successful an insert check was performed by selecting ten colonies from the LB agar plates grown overnight to be utilized as templates for a PCR using the same conditions described in figure 1A above. Electrophoresis was performed using the same terms and materials mentioned before, except the template was a sample from each colony. This electrophoresis was used to verify that the pGEM-T cloning vector now containing ORF_{1A} was present in the selected DH5α *E. coli* colonies. Only two of the positive colonies ($1A_1$ and $1A_4$) were utilized to grow culture in LB overnight at 37 ºC on an incubated shaker at 220 rpm. The Omega Plasmid Mini Kit was used to obtain a purified plasmid DNA (pDNA) from the DH5α *E. coli* present in the culture grown on the shaker overnight. Even though, the Omega Plasmid Mini Kit only calls for one elution there were two elution's performed on each sample miniprep column to make sure most of the pDNA was collected from the miniprep column. The sample obtained from both elutions was speed vacuumed to get a high concentration. The eluted product was used in a double restriction enzyme digest was performed using the New England Biolabs double digestion method for the ClaI and NotI restriction sites.



Invitrogen, 2010

Figure 1. Vector Maps for pPICZα plasmid. The circled restriction sites in the figure demonstrate the location of cleavage after the double restriction enzyme digestion.

The double digestion product was placed in an incubator at 37 ºC overnight. The restriction enzyme digest was utilized to obtain the replicated ORF_{1A} from the origin of replication. This product was then run on a 1% agarose gel under the same conditions mentioned prior. Then the released insert was cut out of the agarose gel and extracted using the Omega E.Z.N.A. Gel Extraction Kit. The obtained product was quantified to verify if the concentration of the product was enough to proceed to T4 ligation. The T4 ligation reactions were set up using the New England Bio Labs Inc T4 DNA Ligase Kit. The T4 ligase reaction included: T4 buffer, T4 ligase, pPICZαC, Serpin 1A DNA, and nucleus-free water. The T4 ligase reactions were set to incubate at 16 ºC overnight. These same reactions were denatured at 65 ºC for ten minutes the next day. The heat shock transformation procedure utilized prior was repeated on these reactions. LB agar plates treated with zeocin were prepared, and ten colonies were collected using the same techniques as

before. There was in insert check done on these ten colonies. Only two of the colonies (1A1 and 1A3) were selected to be grown in LB zeocin culture overnight on an incubated shaker at 37ºC. Again, the Omega Plasmid Mini Kit was used to obtain pDNA. This pDNA obtained from the miniprep was quantified to verify if the concentration of the samples was high enough to proceed to a big dye reaction. The Big dye reactions were set up using the Big Dye Terminator v3.1 Cycle Sequencing Kit. This reaction included: big dye, AOX forward primer, AOX reverse primer, and pDNA of Serpin 1A. The pDNA was sequenced using the Sanger sequencing method.

(c) Serpin 1A Expression Plasmid Construct

The Invitrogen by Life Technologies pPICZα C Pichia expression vectors for selection on Zeocin™ and purification of secreted, recombinant proteins protocol was used to subclone the ORF_{1A} pPICZα C expression plasmid. The Omega Plasmid Mini Kit was utilized to retrieve the recombinant pDNA.

(d) Transfection into *Pichia pastoris* X-33

To linearize this pDNA a restriction digest was performed using the restriction enzyme, Sac I. An aliquot of 5µL of this digested product was analyzed using agarose gel electrophoresis to ensure complete linearization. The linearization reaction was stopped by the heat inactivation method. After ceasing the linearization the solution was centrifuged to pellet the pDNA, then the pellet was washed with 80% ethanol, dried, and resuspended in 10µL of deionized water. To prepare *P. pastoris* for electroporation 5mL of the *P. pastoris* X-33 strain was grown in yeast extract peptone dextrose (YPD) at 30º overnight. Then 0.5mL of this overnight culture was inoculated into 500mL of media and grown more overnight. The next day the optical density (OD) was measured to ensure the growth of the cells were between 1.3-1.5 OD. Once, the density was confirmed the culture was then centrifuged at 1500 x g for 5 minutes at 4ºC to obtain a pellet. This

pellet was resuspended with 500mL of ice-cold sterile water. This centrifuge and resuspension step were repeated about 3-4 times, but instead of using water for resuspension 1M of sorbitol was used. The electroporation method was used to transfect the linearized pPICZα C DNA by using a mixture of 7µL of the linearized pDNA and 80µL of *P. pastoris* X-33 cells. Then 100µL of this culture was spread on YPD plates treated with zeocin. The plates were incubated at 30ºC for three days. On the 4th day, four colonies were selected to purify, culture, and process over the course of 5 days. A sample from each colony's culture was taken on each day. The daily samples retrieved were used to check for the expression of recombinant protein using the Western blot method.

(e)Transcriptional Profile of Serpin 1A Gene in Tick Tissues

Before proceeding to the PCRs described below the cDNA templates being used needed to be quantified in the Tecan's Infinite® 200 NanoQuant to ensure even amounts of cDNA were being utilized for each PCR. Then PCRs were run to investigate the tissue-specific expression of Serpin 1A for different daily tick feeding interval using gene-specific primers and ran in the thermocycler using the conditions in Table 3 below. The tissues being investigated came from cDNA of the nymph development stage (unfed, 24h, 24h, and 72h) and the female adult development SG and MG (unfed, 24h, 48h, 72h, 96h, and 120h).

This reaction was conducted using: gene specific Serpin 1A forward primer, gene specific Serpin 1A reverse primer, Promega GoTaq Green Master Mix, *I. scapularis* cDNA templates described former, and water. The reaction was set to repeat for thirty-five cycles in the thermocycler. Five µL of these PCR reactions were electrophoresed on a 2% agarose gel that was treated with 1 µg/mL ethidium bromide.

Next, a semi-quantitative RT-PCR was conducted, using the cDNA described in the former and Serpin 1A gene specific primers. The normalizer was created by utilizing tick actin primers,

and the cDNA described before. Information on each of these primers is given in Table 3 below. Reaction 1 included: tick β-actin forward primer, tick β-actin reverse primer, Promega GoTaq Green Master Mix, *I. scapularis* cDNA templates described prior, and water. Reaction 2 included: Serpin 1A gene specific forward primer, Serpin 1A gene specific reverse primer, Promega GoTaq Green Master Mix, *I. scapularis* cDNA templates described prior, and water. Reaction one was set to repeat for twenty-eight cycles under the conditions in Table 4 and reaction two was configured to run for the same number of cycles utilizing the terms in Table 5. The number of cycles was decreased to prevent saturation, so the tissues that exhibited significant expression of Serpin 1A could be easily distinguished from one another. Electrophoresis was preformed on 5µL of these PCR reactions on a 2% agarose gel that was treated with 1 µg/mL ethidium bromide. Lastly, the quantity feature on the Bio-Rad Gel Doc Imaging system was used, by finding the tick actin band intensity of each feeding interval, as a reference to compare the Serpin 1A gene band intensity to normalize the expression data collected. The data generated was then converted into a Microsoft Office Excel spreadsheet, and the connected scatterplot feature was used to plot the normalized expression of serpin 1A in feeding day versus band density x-y plane.

Table 3. Semi-quantitative PCR Primers.

Primer	Forward Primer Sequence	Reverse Primer Sequence
Serpin 1A Gene Specific	5'-CGCGTCTCCTTCAACGCTCCT-3'	5'-GGTGACGGACCGGATCAAC-3'
Tick Actin	5'- GGCGCCGAGGTGAAGAA-3'	5'-CCTTGCCGTCCACCTTGAT-3'

Table 4. Serpin 1A Gene Specific Primer PCR Conditions.

Steps	Name of Condition	Temperature of Condition	Time of Condition
1	Initial denature	95ºC	3 minutes
2	Denature	95ºC	30 seconds
3	Annealing	55.9ºC	30 seconds
4	Extension	72ºC	45 seconds
5	Final Extension	72ºC	5 minutes
6	Hold	12ºC	∞

Table 5. Tick actin Primer PCR Conditions.

Steps	Name of Condition	Temperature of Condition	Time of Condition
1	Initial denature	95ºC	3 minutes
2	Denature	95ºC	30 seconds
3	Annealing	59.3ºC	30 seconds
4	Extension	72ºC	45 seconds
5	Final Extension	72ºC	5 minutes
6	Hold	12ºC	∞

CHAPTER III

RESULTS

(a) Summary of Protocols

Two distinct experimental plans were created to obtain the results from the two objectives of this research and are summarized below in Figure 1A and 2A. The first plan Illustrates the protocol used to clone ORF_{1A} into the pGEM-T cloning vector, followed by the sub-cloning of the ORF_{1A} into the pPICZαC expression plasmid, and lastly, transfection into the pDNA obtained into the *P. pastoris* X-33 in order to obtain recombinant protein. The second plan was used to analyzed the intensity of the expression of this serpin in the tick tissues: nymph development stage (unfed, 24h, 24h, and 72h) and the female adult development SG and MG (unfed, 24h, 48h, 72h, 96h, and 120h). Step 1 through 3 were conducted before this experiment and entailed: getting permission from veterinarians to allow ticks to feed on rabbits, collecting the ticks after feeding had occurred, and extracting total RNA to making cDNA from the samples collected from the ticks before and after feeding.

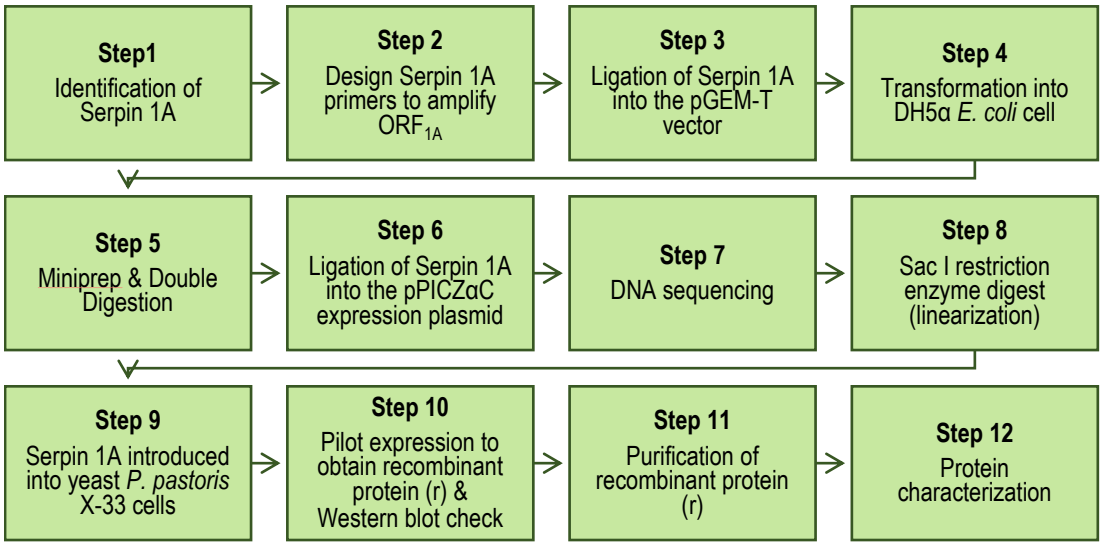


Figure 1A. Protein Characterization Plan.

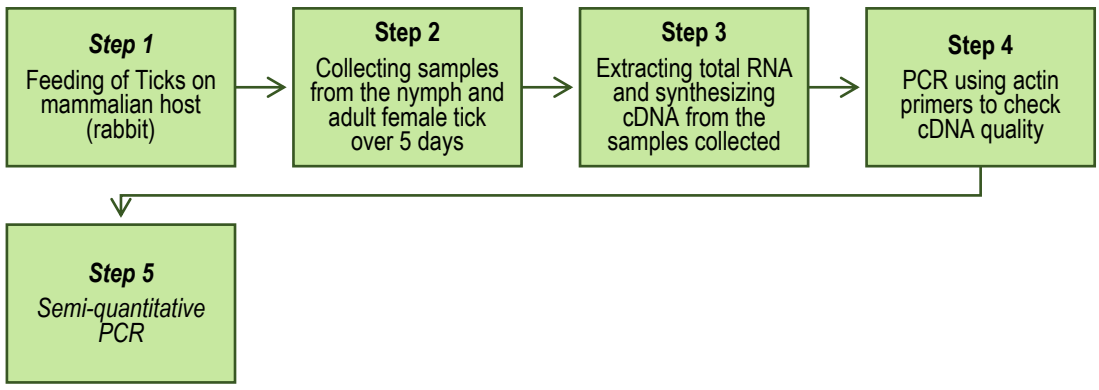


Figure 2A. Plan to Analyze Serpin 1A Expression in Tick Tissues.

(b)Serpin 1A expression in larval versus nymph

Serpin 1A mature protein ORF_{1A} primers were used to find the Serpin 1A mRNA transcription patterns in *I. scapularis* cDNA from larvae and tick nymphs fed over the course of three days. Figure 3 showcases a robust band at the larvae life stage and a faint band during the third day of feeding at the nymph life stage. The strong band shows that this Serpin was highly

expressed earlier in the feeding cycle. On the other hand, the faint band highlights this Serpin was also disclosed later on during feeding.

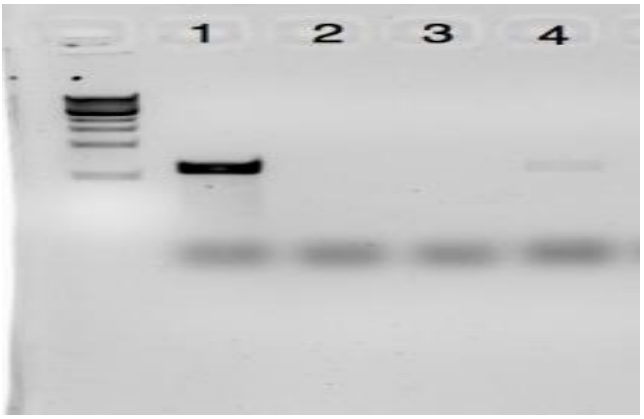


Figure 3. OFR$_{1A}$ PCR. The order of the gel from left to right: larvae, nymph 24h, nymph 48h, and nymph 72h. A strong band and faint band are present just above the 1000 bp mark for the larvae and 3rd day of nymph feeding.

(c) Cloning and DNA sequence analysis

After amplification of ORF$_{1A}$, insertion into the pGEM-T cloning vector and the introduction of this cloning vector into the DH5α *E. coli* cells was successful. The outcome of this transformation can be viewed in Figure 4. All ten colonies from the transformation of ORF$_{1A}$ into the *E. coli* cell was successful as deemed in Figure 5. Since all colonies contained ORF$_{1A}$, only two of the colonies were selected to perform the miniprep protocol explained prior. Moreover, the two miniprep products also deemed to be positive in containing the insert displayed in Figure 6. A double digestion shown in Figure 7 promotes the positive results of the double restriction enzyme digest. Figure 8 showcases the successful sub-cloning of ORF$_{1A}$ into the pPICZαC expression vector. Figure 9 notes that about 90% of the colonies obtained during this step were positive for containing the sequence of interest. Since almost all of the colonies were positive, only two

colonies were utilized to proceed to a second miniprep purification. As shown in Figure 10 the miniprep purification of these two colonies was effective. Quantification of the miniprep product from Figure 10 ensured that the samples were ready to be digested with the SacI digestive enzyme for linearization. The complete linearization of the pDNA is confirmed in Figure 11. Even though, ORF_{1A} was successfully sub-cloned into the yeast recombinant protein expression plasmid, pPICZαC; unfortunately, the recombinant protein of this serpin was not obtained as shown in Figure 12. Since the recombinant protein was not retrieved, the characterization process did not proceed.

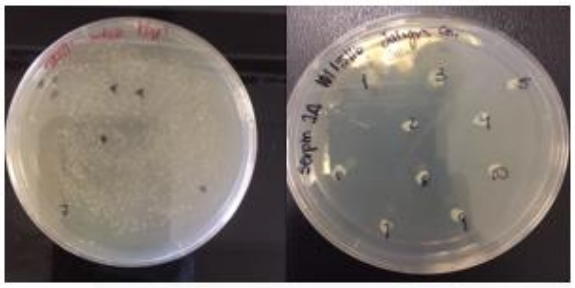


Figure 4. Transformation into DH5α *E. coli.* These plates represent the colonies that grew after transforming the pGEM-T vector containing the ORF_{1A} into DH5α *E. coli* cells for replication.

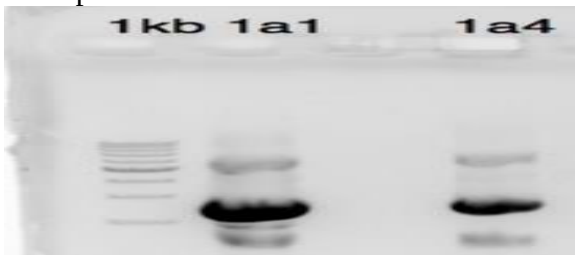


Figure 6. Serpin 1A pDNA. Shown here is the successful retrieval and purification of plasmid DNA using the Omega miniprep protocol. There was a high band expressed at just above 2 kb, showcasing the insert was inside of the plasmid.

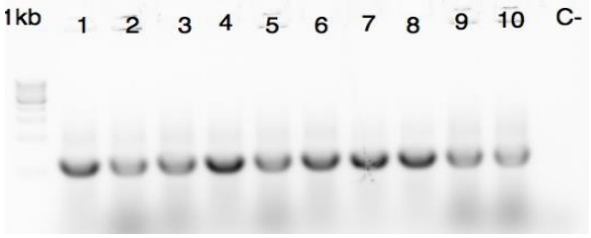


Figure 5. Insert Check of Transformation DH5α *E. coli*. There are strong bands present just above the 1000bp mark for all ten colonies. Colonies 1 and 4 were chosen to purify and retrieve the pDNA.

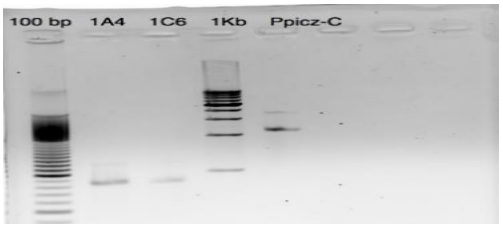


Figure 7. Double Digestion of Serpin 1A and pPICZ*α*C. The bands in the gel picture display that the double digestion was successful at obtaining the plasmid DNA of cloned ORF_{1A}. A sample of this product was proceeded and sent for sequencing. The sequencing data indicated that the cloning of the serpin 1A was successful.

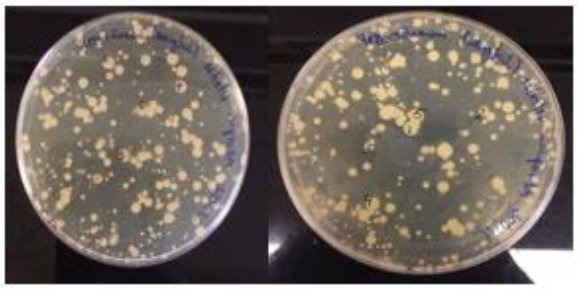


Figure 8. Subcloning Serpin 1A in pPICZαC Vector. The growth of colonies on the plates indicated that the ligation and transformation of Serpin 1A into the pPICZαC vector was completed correctly.

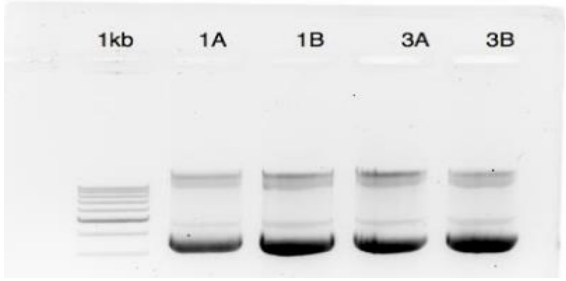


Figure 10. Miniprep of Serpin 1A. Colonies 1 and 3 from Figure 10 still possessed the insert after being purified.

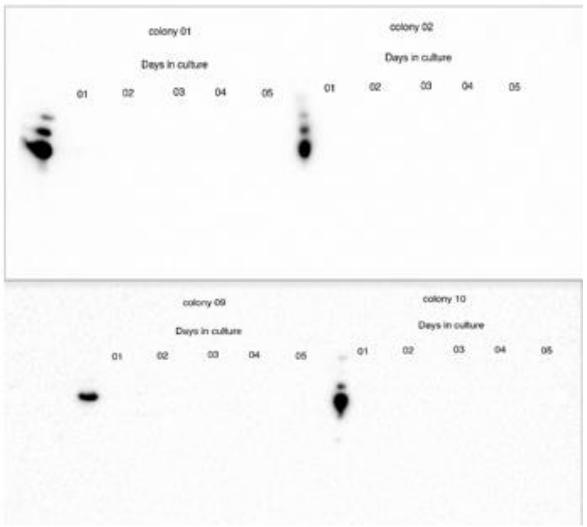


Figure 12. Protein expression in *P. pastoris* X-33 cells. The pictures above attests that the recombinant protein was not expressed in *P.pastoris* X-33. Therefore, the amino acid sequence could not be analyzed for the characterization of Serpin 1A.

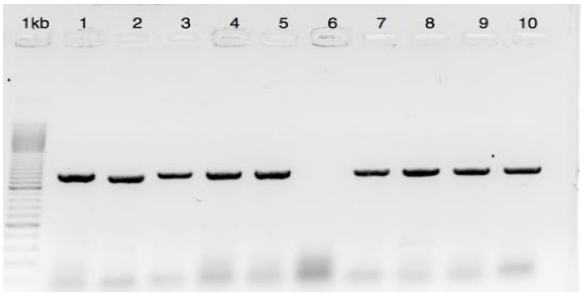


Figure 9. Insert Check of Serpin 1A in pPICZαC. The picture shown above displays that almost all of the colonies that grew on the zeocin treated plate in Figure 8 were positive for containing the target pDNA.

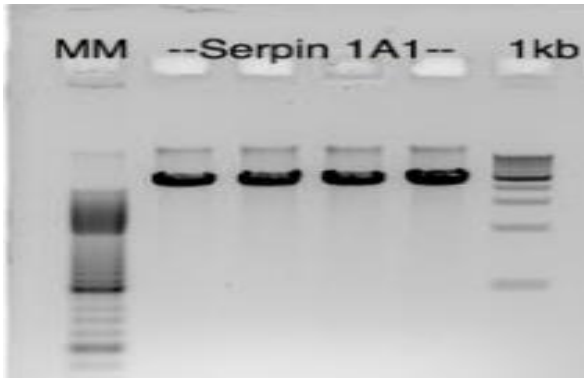


Figure 11. SacI Serpin 1A Digestion. Sample 1A from Figure 10 was digested and purified; the picture above indicated the successful linearization of the plasmid. This successful result meant that it was okay to proceed to transfection of Serpin 1A into the *P.pastoris* X-33.

(d) Transcriptional Profile of Serpin 1A

Overall, qualitative analysis of the expression of the Serpin 1A amongst the tick tissues stated prior, showed that the most expression took place in the female adult SG and nymph stage during the different feeding intervals. The lowest amount of expression was indicated when the tick had not been introduced to the host and in the female MG tissue. This qualitative analysis of each of three tick tissues can be found in Figures 13, 14, and 15. The semi-quantitative analysis of the Serpin 1A gene in reference to tick actin showed that during the nymph feeding intervals the expression was 0.85-fold when unfed, 3.15-fold during the1st day, 0.16-fold when feeding during the 2nd day, and 0.33-fold during the 3rd day. Furthermore, quantitative analysis of the daily feeding interval of the female adult SG tissue revealed the Serpin 1A gene was expressed 1.55-fold during the 1st day, 1.96fold during the 2nd day, 0.52-fold during the 3rd day, 0.54-fold during the 4th day, and 0.77-fold during the 5th day in reference to tick actin. Lastly, quantitative analysis of the daily feeding interval of the female adult MG tissue deemed the Serpin 1A gene was not expressed during the 2nd or 3rd day, but present 3.09-fold during the 1st day, 0.09-fold during the 3^{rd} day, and 0.11-fold during the 5^{th} day in reference to tick actin. All quantitative analysis data is graphed in Figures 16, 17, and 18.

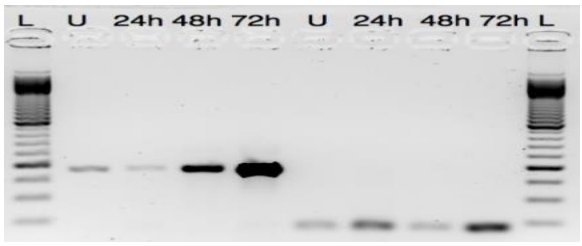


Figure 13. Serpin 1A Semi qualitative PCR analysis Nymph. The order of the picture: tick actin on the left and Serpin 1A on the right. The Serpin 1A transcript is mainly present during the 3rd day of tick feeding in the nymph stage. There is expression of the serpin 1A gene for every feeding interval.

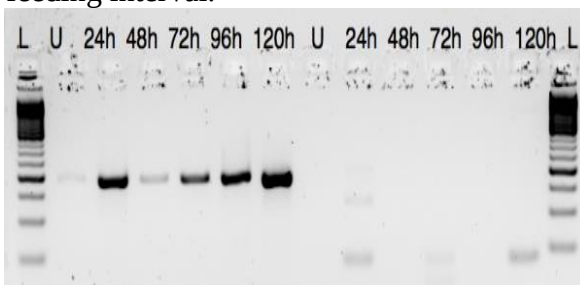


Figure 15. Serpin 1A Semi-qualitative PCR Analysis Adult Female MG. There was a low expression of Serpin 1A overall in the female adult MG; however, the majority of this low expression took place on the 1st, 3rd, 4th, 5th day.

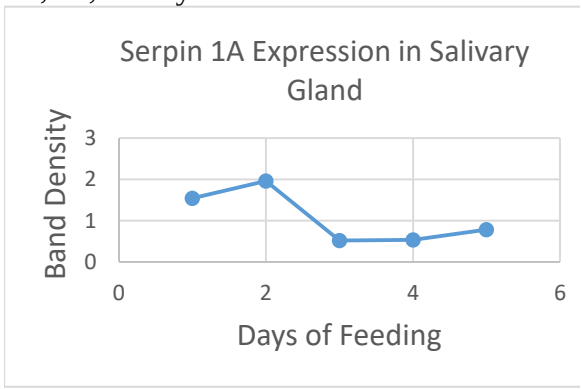


Figure 17. Serpin 1A semi-quantitative PCR Analysis of Adult Female SG. The SG of the female adult stage expressed the transcript the most during the 1st and 2nd day of feeding. There was also expression during the 4th and 5th day of feeding, with little expression during the 3rd day in reference to tick actin.

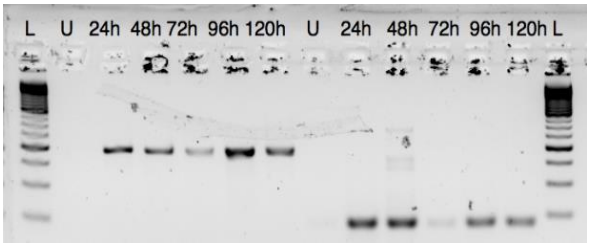


Figure 14. Serpin 1A Semi-qualitative PCR analysis Adult Female SG. The Serpin 1A transcript is mainly present during the 1st and 2nd day of feeding. There was also high expression during the 4th and 5th day of feeding, with little expression during the 3rd day.

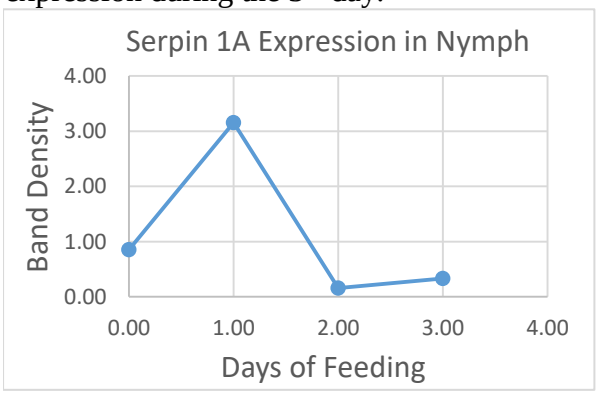


Figure 16. Serpin 1A Semi-quantitative PCR Analysis of Nymph. Here there is a high expression associated with the 1st day of feeding in reference to tick actin.

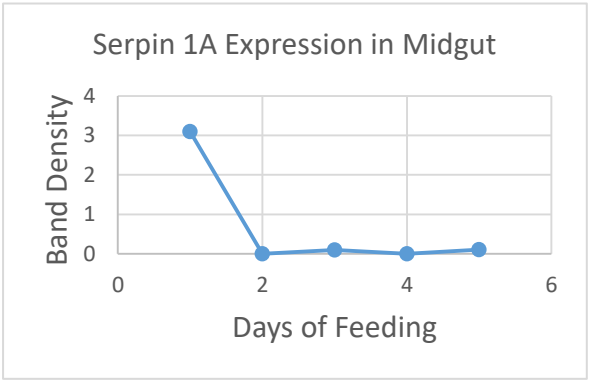


Figure 18. Serpin 1A Semi-quantitative PCR Analysis of Adult Female MG. The data above shows a high expression associated during the 1st day of feeding and little expression during the 3rd day in reference to tick actin.

CHAPTER IV

DISCUSSION

The first objective of this research, to produce a recombinant protein (r) of an *I. scapularis* Serpin 1A in *P. pastoris* was not successfully accomplished. Therefore, more experiments need to be conducted to produce the recombinant protein (r) of *I. scapularis* Serpin 1A in *P. pastoris* or other yeast species. The second objective of this research, to verify the transcriptional profile of Serpin 1A was accomplished. It is interesting that in the nymph stage the Serpin 1A gene expression was the highest during the third day of feeding, since *Borrelia burgdorferi* is typically transmitted by this vector primarily from 36 to 48 hours or more during tick feeding. It is often cited that the salivary gland is a key factor in vector competence and the site for pathogen development [1]. In this paper, Serpin 1A was expressed more in the SG than MG which may further aid in the importance of this location in reference to pathogen development in tick anatomy.

Follow-up experiments after this research can improve the outcome of obtaining recombinant protein by trying different media conditions (pH, temperature, etc.), using different strains of *P. pastoris* other than X-33, using different plasmids (pPICZαA or B, etc.), and using a different system (insect cells, mammalian cells, etc.). Also, resynthesizing cDNA from total RNA of unfed ticks could help in obtaining clearer result for transcriptional profile analysis before the tick is introduced to the host. Moreover, experiments that proceed this research can investigate if Serpin 1A is a functional serine or cysteine protease inhibitor as well as determine the role of Serpin 1A during tick feeding. Lastly, future research can find a way to utilize RNAi to silence the expression of the proteins used during tick feeding to visualize that proteins function. Visualizing the function of the protein allows for that protein to be targeted for future experiments that can

obtain recombinant protein to create a more effect vaccine against agents spread by ticks. The primary purpose of this research was to find the transcriptional profile and introduce the process to characterize of a specific tick serpin, Serpin 1A.

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