FUNCTIONAL ANALYSIS OF A CROSS-TICK SPECIES CONSERVED

Amblyomma americanum SERINE PROTEASE INHIBITOR 39

An Undergraduate Research Scholars Thesis

by

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TABLE OF CONTENTS

ABSTRACT

Functional Analysis of a Cross-Tick Species Conserved *Amblyomma americanum* Serine
Protease Inhibitor 39. (May2014)

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Hard ticks such as *Amblyomma americanum* are pool feeders that accomplish feeders by lacerating host tissue and then sucking host blood that bleeds into the wounded area. Host defense mechanisms to tick feeding activity are serine protease mediated pathways, blood clotting and inflammation that are controlled by serpins. It's hypothesized that ticks utilize serpins to evade host defense mechanisms to successfully feed and transmit disease agents. As part of a long-term goal to understand role(s) of *A. americanum* serpins in tick feeding regulation, the goal of this research was to produce a recombinant (r) of a highly cross-tick species conserved *A. americanum* serpin, AAS39 in yeast and insect cells. The mature protein open reading frame was amplified and successfully cloned into the yeast expression plasmid, but the not insect cell plasmid. The yeast plasmid was used to transform *Pichia pastoris* yeast cells and rAAS39 was successfully expressed and affinity purified. Results from this research sets up the stage for follow up experiments to characterize the role(s) of rAAS39 in tick feeding regulation.

ACKNOWLEDGEMENTS

CHAPTER I

INTRODUCTION

Ticks are among the most important vectors of human and animal diseases and ranked second to mosquitoes in terms of the public health impact of tick borne diseases (Sonenshine, 1993). In the United States (USA) most reported human vector borne diseases are tick borne. Some of the most important human tick borne diseases (TBD) in the USA include Lyme disease, Rocky Mountain spotted fever, Human babesiosis, Anaplasmosis and Ehrlichiosis (Dantas-Torres, 2012; Gratz, 2006; Subramanian et al., 2012; Anderson and Armstrong, 2012; Burri et al., 2011; Michalski et al., 2006, Anderson et al., 1993). In livestock production, important TBD includes babesiosis, anaplasmosis, theileriosis, and heartwater (Jongejan et al., 2004).

There are no effective vaccines against animal and human TBD infections. Currently, control of ticks and prevention of TBD infections depends on use of acaricides. Acaracides are effective in the short-term, but they do not offer a permanent solution because of limitations such as ticks developing resistance and acaricides contaminating the environment. To solve this problem immunization of animals against tick feeding has been advocated. The pre-requisite to development of effective anti-tick vaccines is identification of effective vaccine target antigens. One such group of proteins, are members of the serine protease inhibitors (serpins) family. Mammalian serpins regulate pathways that are essential to life such as blood coagulation, complement activation, inflammation, fertilization, and food digestion (Huntington 2006, Huntington and Church, 2007; Silverman et al., 2010; Gettins 2002). To initiate and complete feeding, ticks must overcome serpin-regulated pathways of inflammation, blood coagulation, and complement activation in the host (Ribeiro et al., 2006; 2011). Thus, it was hypothesized that ticks utilize serpins to disrupt host defense responses and accordingly, that immunizing host

3

against or targeting these proteins for chemotherapy will reduce the tick's ability to successfully feed (Mulenga et al., 2001).

Serpins are highly conserved across taxa. Serpin encoding cDNAs have been cloned in many tick species including, the Lone Star tick, *Amblyomma americanum*. Recent studies by Dr. Mulenga at Texas A&M University indicate that the *Amblyomma americanum* may produce at least 122 unique serpin transcripts (Porter et al., in review). The next phase in the research is to understand role(s) of candidate *A. americanum* serpins (AAS) in tick feeding and to select those that are suitable for anti-tick vaccine development. In this research recombinant AAS39 was expressed in *Pichia pastoris* and affinity purified. The remaining follow up research is to gauge insight into role(s) of native AAS39 at the tick-feeding site using the recombinant protein produced in this research.

CHAPTER II

METHODS

(a) PCR amplification of AAS39 mature protein open reading frame

The AAS39 coding cDNA was discovered in data mining of unfed and fed male and female tick *A. americanum* transcriptome (Porter et al., in review, BMC Genomics). Primers were designed using a primer design program in MacVector DNA sequence analysis software. The primers were reconstituted and diluted to a 10µM working solution. To determine the appropriate annealing temperature, the gradient PCR was used with annealing temperature ranging from 50 ˚C to 65 ˚C. The optimized annealing temperature of 62 ˚C was used in further amplification experiments. In the first step AAS transcription patterns were determined in 24, 48, 72, 96 and 120h whole ticks. The PCR reaction mix contained the Gotaq PCR master premix, forward primers, reverse primers at 1µM, 24 or 48 or 72 or 96 or 120h fed female tick template cDNA, and water. PCR products were electrophoresed on a 2% agarose gel that contained 1µg/mL ethidium bromide. This was run for 25 minutes at 100V and then viewed under a UV light to visualize amplified DNA bands. PCR bands were extracted from the gel using routine gene clean protocols.

(b) AAS39 cDNA cloning and Sequencing

The gene cleaned AAS39 mature protein ORF was cloned into the pGEM-T TA cloning vector. The pGEM-T-AAS39 plasmid was used to transform competent DH5ȁ E. Coli cells using heat shock chemical transformation. Transformed E. coli was plated onto agar plates containing ampicillin for selection of transformed E. coli, and X-gal for the routine blue and white colony

selection. To verify if AAS39 cDNA was ligated into pGEM-T, ten white colonies were selected

and subjected to insert PCR. In this PCR a touch of selected white colonies was used as template.

Colonies that were confirmed to contain AAS39 cDNA inserted were inoculated in SOB with

ampicillin overnight at 37˚C with shaking. Bacteria cultures were subjected to routine mini-prep

procedures to purify the pGEM-T-AAS39 plasmid. The pGEM-T-AAS39 concentration was

determined using the spectrophotometer and ~230μg/mL and was subjected to sequencing PCR

using pGEMT specific SP6 and T7 promoter primers. DNA sequences were analyzed using the

MacVector DNA analysis program.

(c) Construction of rAAS39 expression plasmid

Recombinant AAS39 protein was expressed using the *Pichia pastoris* and pPICZα plasmid

expression system. The AAS39 expression plasmid was constructed using unidirectional cloning

methods. PCR primers (Table 1) with added restriction enzymes sites (Figure 1) were used to

amplify the AAS39 mature protein open reading frame using plasmid DNA from above (in

section b) as template. The AAS39 coding cDNA was initially cloned in pGEMT using TA

cloning methods, and the pGEMT-AAS39 plasmid purified using miniprep as described above.

To release the AAS39 cDNA insert, the mini-prep product was sequentially digested Not1

enzyme, followed by Kpn1 enzyme. Digestion reaction mixtures were incubated overnight at 37

˚C. Subsequently the digestion reaction mixture was electrophoresed on a 2% agarose gel

containing 1μg/mL ethidium bromide. This electrophoresis separated the AAS39 cDNA insert

from the plasmid backbone. The separated AAS39 cDNA insert was then cleaned up using a

standard PCR clean up protocol. Alongside preparation of the AAS39 insert preparation, the

pPICZAα plasmid was digested with restriction the same restriction enzymes to prepare for

ligation of AAS39 into pPICZAα expression plasmid. The pPICZAα-AAS39 plasmid was used

to transform DH5α competent E. coli cells. Following miniprep, the pPICZAα-AAS39 plasmid

was linearized and used to transform X-33.

Table 1: Primer Sequences

Primer	Sequence	Restriction Enzyme
Yeast Forward	5'-GGT ACC CAG CCC AAC AAC CGT GTG G-3'	KpnI
Yeast Reverse	5'-GCGT GCC GCC AGA TCA GTG ACG CGT CC-3'	NotI
Insect Cell Forward	5'-AAG CTT GAC ATG GCT TCA AGT CCG CTT-3'	HindIII
Insect Cell Reverse	5'-GGA TCC CAG ATC AGT GAC GCG TCC GA-3'	BamHI

Primers were designed to ensure that the melting temperature is ideal for PCR.

Figure 1: pPICZα plasmid with restriction enzymes
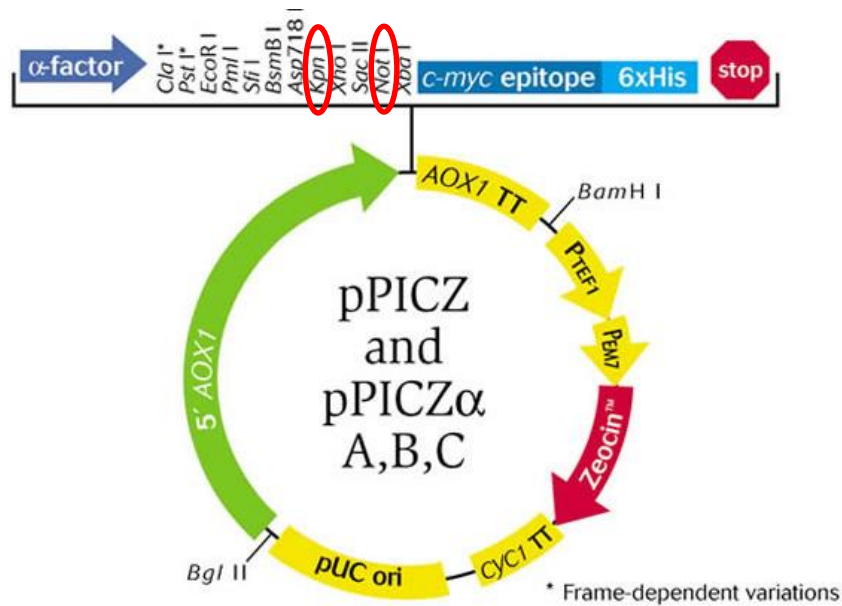
pPICZα plasmid with list of restriction enzymes.

(d) Transformation of yeast and induction of rAAS39 expression

Recombinant AAS39 protein was expressed using the *Pichia pastoris* and pPICZα plasmid

expression system (Life Technologies). The pPICZα-AAS39 expression plasmid was constructed

by unidirectional sub-cloning of mature AAS39 coding domain into pPICZαA KpnI and NotI sites, using forward primers with added restriction enzyme sites circled as seen in Table 1 and Figure 1. The pPICZαA-AAS39 expression plasmid was linearized with PmeI and used to transform *Pichia pastoris* X-33 strain by electroporation. Transformed colonies were selected on Yeast Extract Peptone Dextrose Medium with Sorbitol (YPDS) agar plates with zeocin (100μg/μl), and then selected for methanol utilization on Minimal Methanol (MM) agar plates, both incubated at 28°C. Transformants were induced to express rAAS39 by adding methanol to 0.5% final concentration every 24h for five days for up to 5 days. The pPICZαA plasmid contains a α-factor secretion signal that directs the recombinant protein for secretion in the media. To obtain the secreted recombinant protein, yeast culture spent media was centrifuged at 5000g for 10 min at room temperature. The recombinant protein in the supernatant was precipitated by ammonium sulfate saturation (525g/1L of media) with stirring overnight at 4°C. The precipitate was pelleted at 11,200g for 1h at 4°C. The pellet was re-suspended in and dialyzed against phosphate buffered saline (PBS) pH 7.4.

To verify expression the spent media precipitate was subjected to western blotting analysis using horse-radish peroxidae (HRP)-labeled antibody to the C-terminus hexa histidine tag (Life Technologies) diluted to 1:5000 in 5% blocking buffer (5% Skim milk powder dissolved in PBS w/Tween-20). The positive signal was detected using metal enhanced DAB chromogenic substrate kit (Thermo Scientific). Subsequently, rAAS39 was affinity purified under native conditions using Hi-Trap Chelating HP Columns (GE Healthcare). Affinity purified putative rAAS39 was dialyzed against 1X PBS pH 7.4 for downstream assays.

CHAPTER III

RESULTS

(a) Experimental Design

Before the research project began, 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, AAS39 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 to amplify the mature protein open reading frame (ORF). This was then inserted and cloned in the recombinant protein expression plasmids in yeast and insect cells. Subsequently recombinant protein expression plasmids were used to transform yeast cells or transfect insect cells and the protein would be purified and characterized.
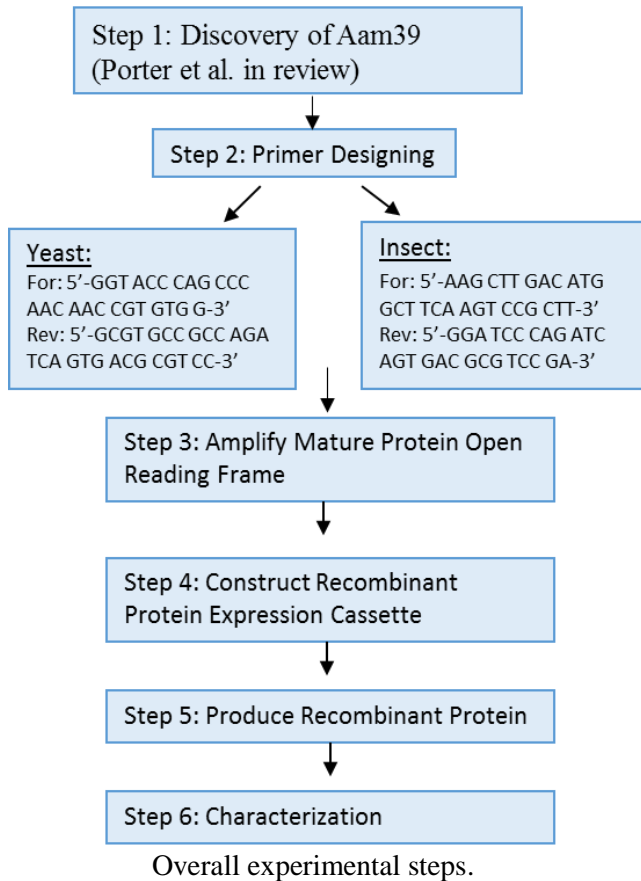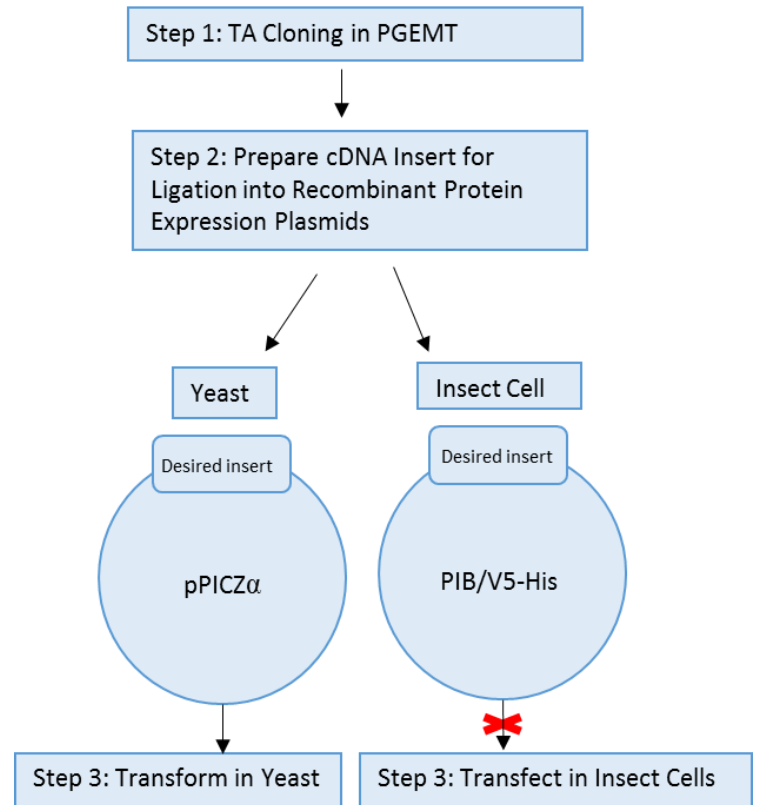
Figure 2A: Experimental Design

Step 1: Discovery of Aam39 (Porter et al. in review)

Step 2: Primer Designing

Yeast:
For: 5'-GGT ACC CAG CCC AAC AAC CGT GTG G-3'
Rev: 5'-GCGT GCC GCC AGA TCA GTG ACG CGT CC-3'

Insect:
For: 5'-AAG CTT GAC ATG GCT TCA AGT CCG CTT-3'
Rev: 5'-GGA TCC CAG ATC AGT GAC GCG TCC GA-3'

Step 3: Amplify Mature Protein Open Reading Frame

Step 4: Construct Recombinant Protein Expression Cassette

Step 5: Produce Recombinant Protein

Step 6: Characterization

Overall experimental steps.

Figure 2B: Construction of Recombinant Protein Expression Plasmid

Step 1: TA Cloning in PGEMT

Step 2: Prepare cDNA Insert for Ligation into Recombinant Protein Expression Plasmids

Yeast

Insect Cell

Desired insert

Desired insert

pPICZα

PIB/V5-His

Step 3: Transform in Yeast

Step 3: Transfect in Insect Cells

9 Experimental steps for cloning of both yeast and insect cell.

(b) AAS39 PCR primers amplified the expected 1200 base band

Figure 3 summarizes gradient PCR to determine the optimum annealing temperature for AAS39 mature protein ORF primers. After designing primers, the PCR reactions were run on gradient PCR to determine the ideal annealing temperature for the reaction. The gradient PCR performs the PCR reaction at different temperatures on the same reaction recipe. For the reactions the range of 50-65°C was picked due to it being within range of the annealing temperature of the primers. As can be seen in Figure 3, the optimum annealing temperature for AAS39 primer was determined to 62°C. This can be determined by the intensity of the band at 62°C. This is the strongest band found at the expected band length of 1200 bp indicated by arrowhead.


Figure 3: Gradient PCR

Each sample undergoes PCR at a different annealing temperatures to determine which temperature is the best to use for future PCRs. The sample that underwent PCR at 62 °C shows the strongest band at the desired length about ~1200bp.

(c) AAS39 mRNA is expressed at 120h, but not 24h feeding time point

Following PCR optimization, AAS39 ORF primers were used to determine AAS39 mRNA qualitative transcription pattern in *A. americanum* cDNA that were fed for 24 and 120h after feeding on a host to determine at what time the serpin is mostly expressed (Figure 4). The band is strongest at 120h demonstrating that serpin is highly expressed later in the feeding cycle of the tick.

Figure 4: PCR at 24 and 120 hours
after feeding
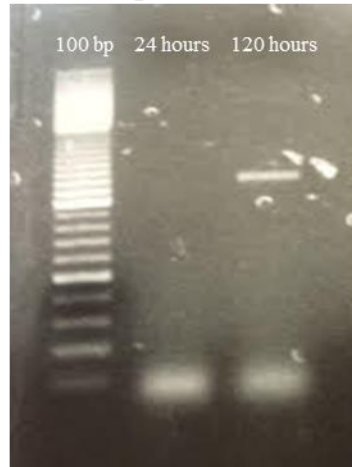
The strong band at 1200 bp for 120 hour cDNA is
strong and demonstrates that the serpin is active at this
point in the tick feeding process.

**(d) Cloning and DNA sequence analysis**

Following PCR amplification, AAS39 cDNA was purified from agarose gels using standard gene

clean protocols. The gene cleaned AAS39 cDNA was then cloned into pGEMT using TA cloning

method. To verify sequence identity of cloned AAS39 cDNA, the pGEMT-AAS39 plasmid was

subjected to DNA sequence PCR using pGEMT specific SP6 or T7 promoter primers (Figure 5A

and 5B). Following sequencing the MacVector DNA analysis software was used to compare

cloned and the sequences that was discovered in data mining of the transcriptome. The sequence

analysis showed that the cloned sequence was correct (not shown).

Figure 5A: Yeast Insert Check
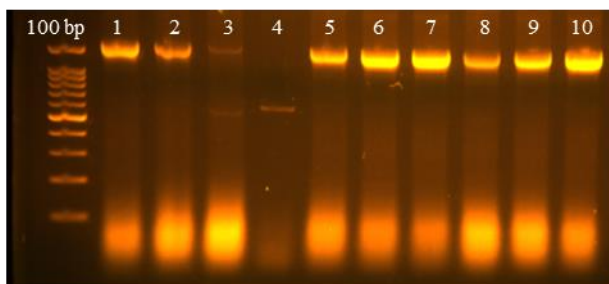
Colonies 1, 2, 5, 6, 7, 8, 9,
and10 all have strong bands at
the desired protein base pair
length. Colony 3 shows a light
band at the correct length.
Colonies 1 and 7 were chosen
to send to sequencing.

Figure 5B: Insect Cell Insert Check
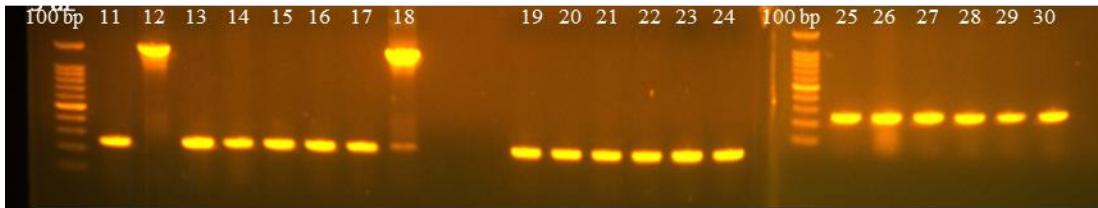
Colony 12 and 18 show the desired protein at 1200 bp. These colonies
were picked to send to sequencing.

(e) AAS39 mature protein ORF successfully cloned into yeast, but not insect cell expression plasmid

Following sequence identity verification, AAS39 mature protein ORF was subc-cloned into yeast
(pPICZAα) and insect (pIB/V5) cell expression plasmids. Sequence analysis verified that AAS39
ORF cloned in frame in pPICZAα but not in pIB/V5. After several attempts to sub-clone in pIB/V5
failed, efforts to express in insect cells were abandoned.

(f) Recombinant (r)AAS39 was expressed in *Pichia pastoris*

Figure 6 summarizes western blotting analysis validating that rAAS39 was expressed in *P.
pastoris.* The pPICZAα plasmid expresses recombinant proteins fused with a c-terminus histidine
tag. As shown in figure 6, the antibody to the histidine tag confirming that rAAS39 was expressed.
In preliminary affinity purification, rAAS39 eluted from the affinity column at 50, 100 and 500mM
imidazole concentration. The next step is to perform SDS PAGE electrophoresis with
silverstaining to determine the protein purity. This test is much more sensitive than a western blot
and will hopefully show bands at the other elution strengths.

Figure 6: Yeast Western Blot
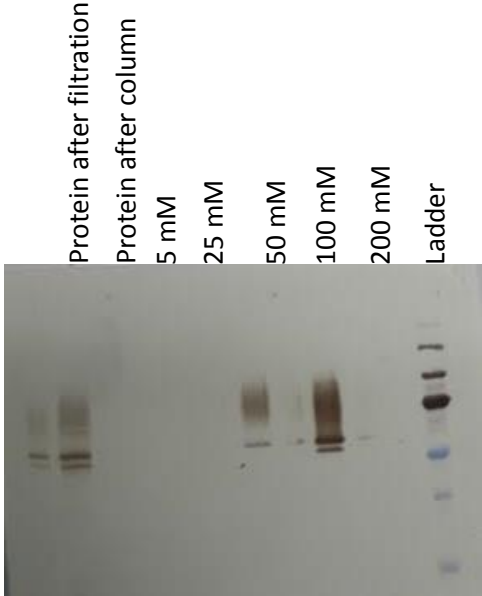
An antibody attaches to the histag tail of the protein and shows up as brown splotches seen above.

CHAPTER IV

CONCLUSION

The goal of this research, to express rAAS39 was accomplished. The remaining experiments are to optimize large scale expression and affinity purification. Once sufficient amount of rAAS39 is purified subsequent experiment will be performed. Subsequent experiments will be to: (1) determine if rAAS is a functional serine or cysteine protease inhibitor, and (2) to investigate the role(s) of AAS39 in the tick and/or at the tick-feeding site. The other take way from the thesis research is that I have been introduced to basic molecular biology applications, particularly recombinant DNA technology and basic protein analysis application.

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